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Antibiotics of the Virginiamycin Family, Inhibitors Which Contain Synergistic Components

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INTRODUCTION

The study of synergistic effects among antibiotics is an interesting development of the branch of biological sciences which deals with inhibitors of cell growth and multiplication. Within this domain, the antibiotics of the virginiamycin family have captivated the interest of scientists and physicians because of their unique features. The crude product obtained by the producing organisms, a group of streptomycetes, contains several components which display a synergistic inhibitory effect in sensitive cells.

Numerous virginiamycin-like antibiotics have been obtained in laboratories of different countries (6, 7, 18, 20, 40, 45, 48, 85, 100, 102, 127, 135, 151, 187, 209, 235, 247-249, 270, 298, 301, 318, 323, 328), and, in fact, most of the leading pharmaceutical industries have patented products of this kind. In Table 1, the main commercial preparations and their sources are reported.

In spite of the large number of preparations available, however, virginiamycin-like antibiotics represent a very small and homogeneous group of drugs. Two basic chemical structures,

TABLE 1. *Commercial preparations of virginiamycin-like antibiotics*

Antibiotic name	Company	Producing organism
Doricin	Squibb	
Patricin	Squibb	
Vernamycin	Squibb	
Etamycin	Bristol	*Streptomyces lavendulae*
Geminimycin	Chas. Pfizer	*S. olivaceus*
Synergistin (PA114)	Chas. Pfizer	*S. olivaceus*
Mikamycin	Kanegafuchi	*S. mitakaensis*
Ostreogrycin (E129)	Glaxo	*S. ostreogriseus*
Plauracin (A2315A)	Eli Lilly	*S. diastaticus*
Streptogramin	Eli Lilly	*S. diastaticus*
Pristinamycin (RP7293)	Rhône-Poulenc	*S. pristinaespiralis*
Pyostacin	Rhône-Poulenc	*S. pristinaespiralis*
Streptogramin	Merck	*S. graminofaciens*
Vernamycin	Olin Mathieson	*S. loidensis*
Virginiamycin (Staphylomycin)	R.I.T. (Recherche & Ind. Thérapeutiques)	*S. virginiae*
Viridogrisein	Parke Davis	*S. griseus* NRRL 2426
Griseoviridin	Parke Davis	*S. griseoviridus*

A and B, are shared by the components of all known inhibitors of this family; various products differ only in minor functional groups.

The aim of the present review article is to report basic data and recent findings concerning the structure and the mechanisms of action of virginiamycin-like antibiotics. Main results in the field will be summarized, and theories explaining the synergistic effect of the components will be discussed. Earlier reviews on these antibiotics were made by Tanaka (282) and Vazquez (312, 315). Reference can be made also to more general articles on protein synthesis inhibitors (27, 33, 125, 161, 162, 177, 239, 241, 244, 314, 316, 331), where data on virginiamycin-like antibiotics are reported and discussed.

CHEMISTRY AND PHYSICS OF VIRGINIAMYCIN-LIKE ANTIBIOTICS

Chemical Structure and Synthesis

All of the antibiotics of the virginiamycin family can be assigned to either one of the two basic primary structures, A and B (Fig. 1 and 2; Table 2). Although the two formulas are completely different, yet a similarity exists in the overall architecture of the two molecules: both of them are macrocyclic lactone peptolides (10, 20, 30, 74, 127, 179, 187, 235, 288, 295, 321, 322).

Compounds of the A group are polyunsaturated cyclic peptolides, which can be considered as highly modified depsipeptides (30). The basic

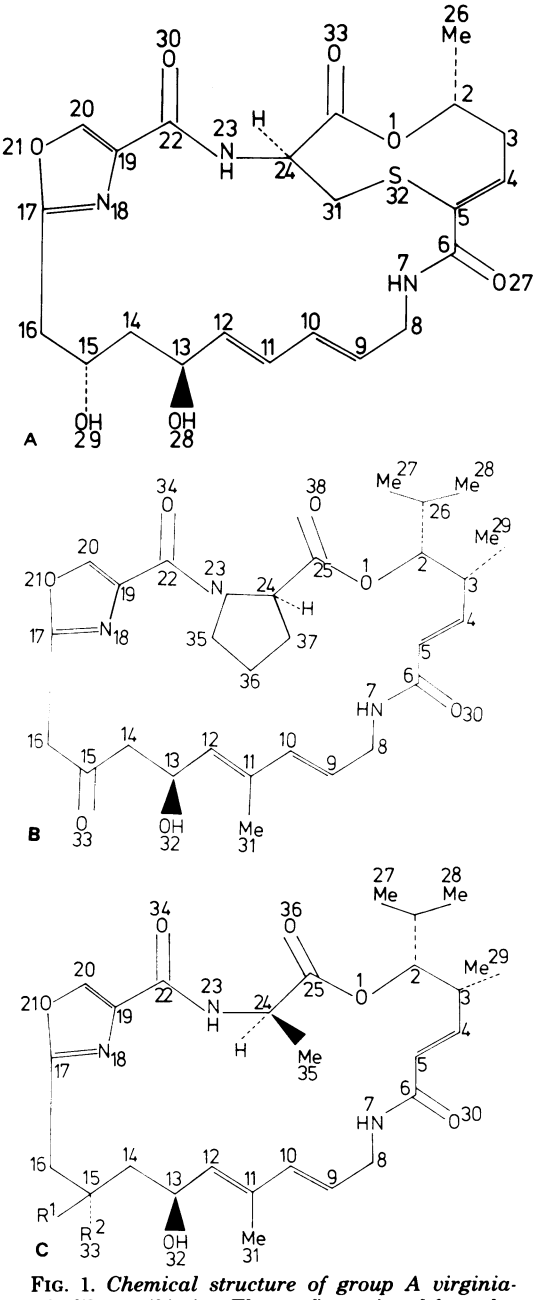
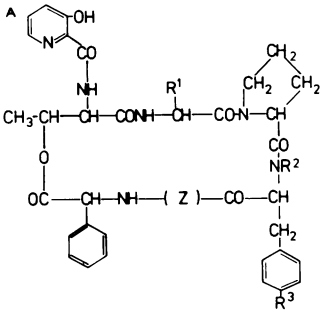


FIG. 1. *Chemical structure of group A virginiamycin-like antibiotics. The configurational formulas of three compounds of this group, which have been chemically characterized to date, are as follows: (A) griseoviridin; (B) ostreogrycin G; (C) madumycin II. Related to the former structures are those of two other well characterized antibiotics: ostreogrycin A ($\Delta^{24,37}$ in formula B) and madumycin I ($R^1R^2 > 0$ in formula C). Mikamycin A, PA114A, pristinamycin IIA, streptogramin A, vernamycin A, and virginiamycin M1 share the formula of ostreogrycin A. Pristinamycin IIB and virginiamycin M2 have the structure of ostreogrycin G. According to references 30 and 75.*

structure of these compounds, which has a molecular weight of about 500, was mainly established by X-ray crystallography and mass spectrometry (26, 45, 82, 83, 169) and also by identification of the hydrolysis products (220–222). Four antibiotics of type A have been chemically characterized already: griseoviridin (Fig. 1A), ostreogrycins A and G (Fig. 1B), and madumycin II (Fig. 1C). Virginiamycin M1, ostreogrycin A, pristinamycin IIA, streptogramin A, PA114A1, vernamycin A, and mikamycin A share the same formula ($C_{28}H_{35}N_3O_7$, molecular weight 525). The double bond A-2,3 is saturated in virginiamycin M2, ostreogrycin G, and pristinamycin IIB. All components of the A group contain a substituted aminodecanoic acid and an unusual oxazole system, presumably derived from a cyclized didehydroserine residue (30).



Name(s)	R^1	R^2	R^3	Z
Patricin B	C_2H_5	CH_3	H	Pipecolic acid
Virginiamycin S1	C_2H_5	CH_3	H	4-Oxopipecolic acid
Virginiamycin S4	CH_3	CH_3	H	4-Oxopipecolic acid
Virginiamycin S2	C_2H_5	H	H	4-Hydroxypipecolic acid
Virginiamycin S3	C_2H_5	CH_3	H	3-Hydroxy-4-oxopipecolic acid
Streptogramin B Mikamycin IA PA114B1 Pristinamycin IA Vernamycin Bα Ostreogrycin B	C_2H_5	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin 1C Vernamycin Bγ Ostreogrycin B1	CH_3	CH_3	$N(CH_3)_2$	4-Oxopipecolic acid
Pristinamycin IB Vernamycin Bβ Ostreogrycin B2	C_2H_5	CH_3	$NHCH_3$	4-Oxopipecolic acid
Vernamycin Bδ	CH_3	CH_3	$NHCH_3$	4-Oxopipecolic acid
Ostreogrycin B3	C_2H_5	CH_3	$N(CH_3)_2$	3-Hydroxy-4-oxopipecolic acid
Vernamycin C Doricin	C_2H_5	CH_3	$N(CH_3)_2$	Aspartic acid
Patricin A	C_2H_5	CH_3	H	Proline

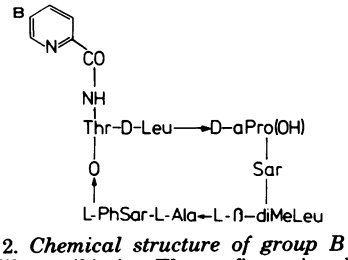


FIG. 2. *Chemical structure of group B virginiamycin-like antibiotics. The configurational formulas of the two compounds of this group, which have been characterized chemically to date, are as follows: (A) streptogramin B; (B) etamycin (viridogrisein). Most synergimycins B share the basic structure in (A), as detailed in the annexed schema, which is taken from reference 75.*

TABLE 2. *Components of antibiotics of the virginiamycin family*

Complex antibiotic	Type A components	Type B components
Madumycin (A2315A)	Madumycin II	Madumycin I
Mikamycin	Mikamycin A	Mikamycin B
Ostreogrycin (E129)	Ostreogrycins A, C, D, G, Q	Ostreogrycins B (B1, B2, B3)
Patricin		Patricins A and B
Plauracin	Plauracin II	Plauracin I
Pristinamycin (Pyostacin)	Pristinamycins II (A and B)	Pristinamycins I (A, B and C)
Streptogramin	Streptogramin A	Streptogramin B
Synergistins (PA114)	Synergistin A	Synergistins B (1 and 3)
Vernamycin	Vernamycin A	Vernamycins B (α, β, γ, δ)
	Griseoviridin	Viridogrisein (Etamycin) (Doricin) (C)
Virginiamycin (Staphylomycin)	Virginiamycins M (1 and 2)	Virginiamycins S (1, 2, 3, and 4)

Compounds of the B group are cyclic hexadepsipeptides of molecular weight of about 800. The primary structure, which was largely established by chemical identification of the hydrolysis products (9, 13, 19, 21, 26, 47, 72, 94, 134–136, 145, 146, 155, 165, 166, 168, 170, 226, 258, 267, 268, 294–297, 324–330), is reported in Fig. 2B. Most antibiotic preparations contain several components possessing similar structures. Thus, patricin A, PA114B1, doricin, mikamycin IA, ostreogrycins B (B1, B2, B3), pristinamycins I (IA, IB, IC), streptogramin B, vernamycins B (Bα, Bβ, Bγ, Bδ), and virginiamycins S (S1, S2, S3, S4) present minor modifications (hydrogens replaced by alkyl and methylamino groups) of the same basic structure (Fig. 2A) (30, 74, 288). Moreover, although most members of this group contain one molecule of pipecolic acid or derivative, this compound is replaced by either aspartic acid or prolin in doricin, patricin A, and vernamycin C (Fig. 2). Some of the amino acids in the basic structure of Fig. 2A are replaced by other amino acids in etamycin (Fig. 2B) and plauracin (209). The possibility of amino acid

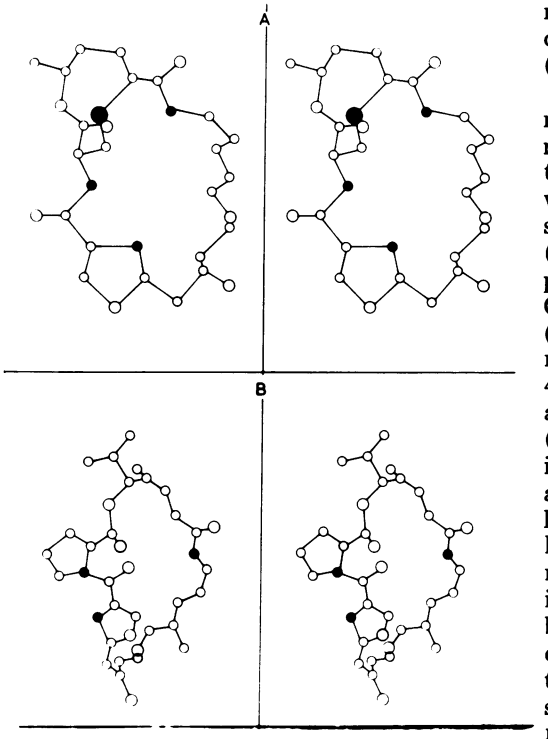


FIG. 3. *Crystal conformation of griseoviridin and ostreogrycin A. Perspective drawing by program pluto of X-ray analysis data of griseoviridin (A) and ostreogrycin A (B). According to reference 30.*

replacement with conservation of the biological activity suggests that formation of virginiamycin-like substances might be a phenomenon widely spread in nature, and that numerous antibiotics of this type are likely to be discovered in the future.

Very recent studies of nuclear magnetic resonance, mass spectrometry, and X-ray diffraction have allowed the spatial configuration of type A and B compounds to be unraveled.

The crystal structure analysis of several group A antibiotics has just been accomplished (30, 93). The crystal conformation of griseoviridin and ostreogrycin A is shown in Fig. 3. Low-temperature diffractometry of single crystals of virginiamycin M1 with one molecule of dioxane has allowed a tridimensional model of the antibiotic to be built up (Fig. 4). A planarity of atoms $C_{30}C_{29}C_{28}C_{26}O_{27}N_{25}C_{24}$ on the one hand, and of $C_{24}C_{23}C_{22}C_{20}C_{19}C_{17}$ on the other hand, has suggested the possibility of a resonance through the two planes at 80°C. More important, this study has pointed to the presence of one hydrogen bridge O_{18}—H_{18} ... O_{27} (2.79Å [0.279 nm]); the possibility of a second bridge $N_{25}H_{25}$ ... O_7 (3.42Å [0.342 nm]), which would stabilize the molecule by linking the two halves of it, is decreased by the greater distance of the partners (93).

From the ^1H (at 300 MHz) and ^{13}C nuclear magnetic resonance studies of several components of the B group, the Pauling-Corey-Koltrum space-filling models of these antibiotics were constructed (Fig. 5) (5, 31, 32). In this study, the following conclusions were reached. (i) Three H bonds are present: proline (C=O)/phenylglycine (NH), lactone (either C=O ... or O ...)/D-aminobutyric acid (NH), and picolinic (OH)/threonine (NH). (ii) Peptide bond conformation is *trans* for 1-1 and 2-3, and *cis* for the 4-5 couple. (iii) The 4-oxopipecolic residue has a "non-chair" "twist-boat" distorted structure. (iv) The benzyl side chain of N-Me-Phe, which is totally folded underneath the 4-oxopipecolic acid side chain in virginiamycin S, gradually leaves this favored position when changing the keto function, to an axial hydroxyl function, to no functional group at all. This indicates an important dipole-induced dipole interaction stabilizing the side chain conformation of the parent virginiamycin S. (v) Patricin A differs from the virginiamycin S in that it has a more open structure, since the phenylglycine side chain is now rotated away from the depsicycle backbone. Accordingly, virginiamycin S molecule was depicted as the one possessing a polar hydrophilic side (Fig. 5A) and a lipophilic side (Fig. 5B and C), the polar function of D-aminobutyric acid carbonyl being screened by hydrophobic structures. The expanded model of the molecule (Fig. 5D) shows an extreme conformation around the depside bond 6 (CO), which acquires an "inwards" or an "outwards" orientation according to the solvent (5, 31, 32).

The crystalline structure of virginiamycin S (an antibiotic of group B) has been unraveled through the joint effort of two groups of scientists belonging to the Crystallography Unit of the University of Louvain and the Physics Department of the University of York (79, 80). X-ray analysis of several crystalline preparations of the antibiotic has yielded a primary structure that is consistent with that previously established by chemical methods (297). The stereoscopic view of the tertiary structure of virginiamycin S (Fig. 6A) shows a macrocyclic ring constrained by a transannular hydrogen bond. A few details of such structure are worthy of mention. (i) Only one peptide bond out of seven has the *cis* configuration, the one which involves the N(26) deprived of H. (ii) The penta- and hexacycles are parallel to one another. (iii) O atoms have a ring-like disposition around the globular protein, with the exception of O(13), which is engaged in an intramolecular hydrogen bonding.

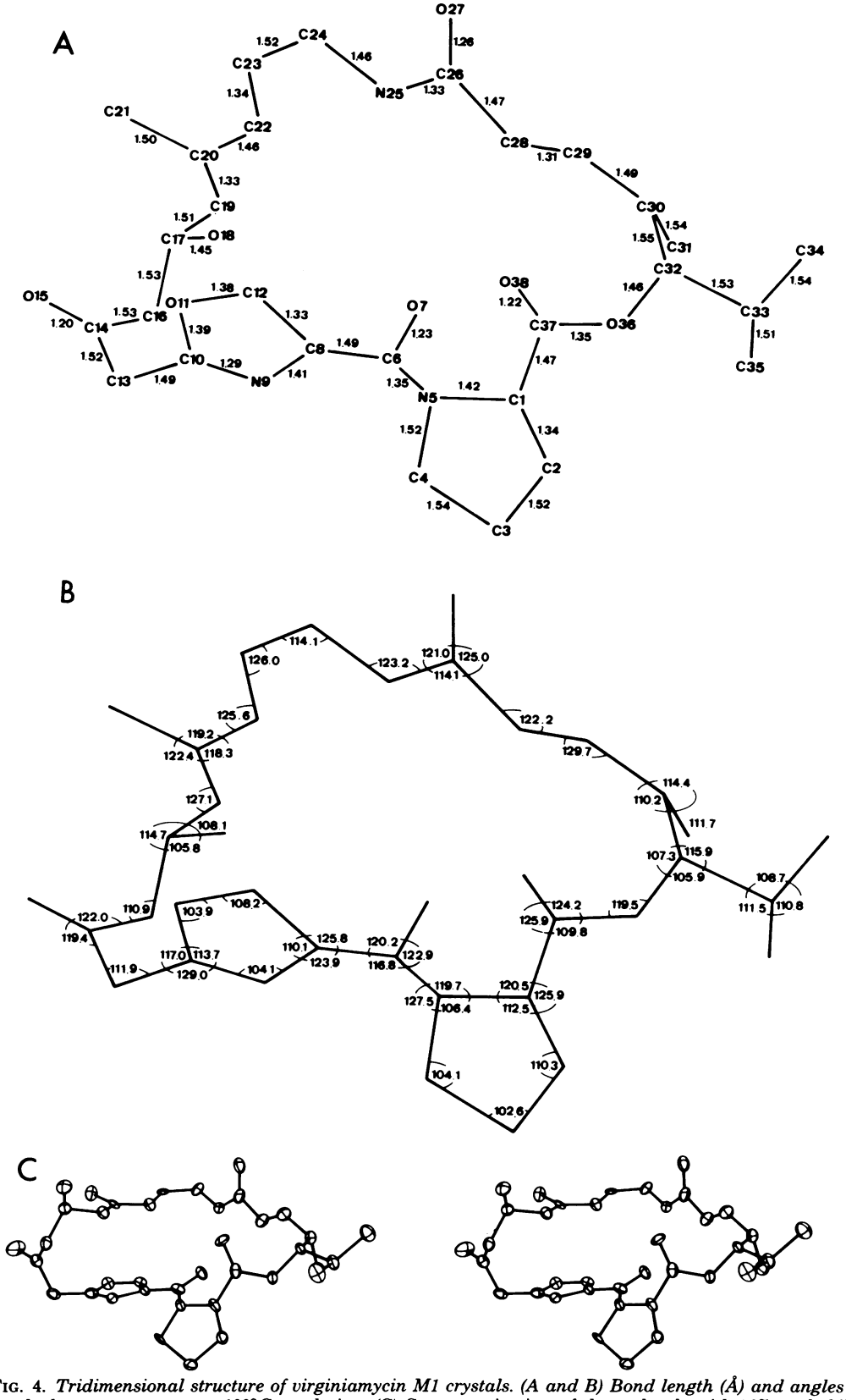


FIG. 4. *Tridimensional structure of virginiamycin M1 crystals. (A and B) Bond length (Å) and angles (°) of nonhydrogen atoms at a −100°C resolution. (C) Stereoscopic view of the molecule with 50% probability thermal ellipsoids at −100°C. From reference 93 (structure at 20°C) and unpublished data (G. Evrard, F. Durant, C. Dorval, and M. Melebeck: refinement of the structure at −100°C).*

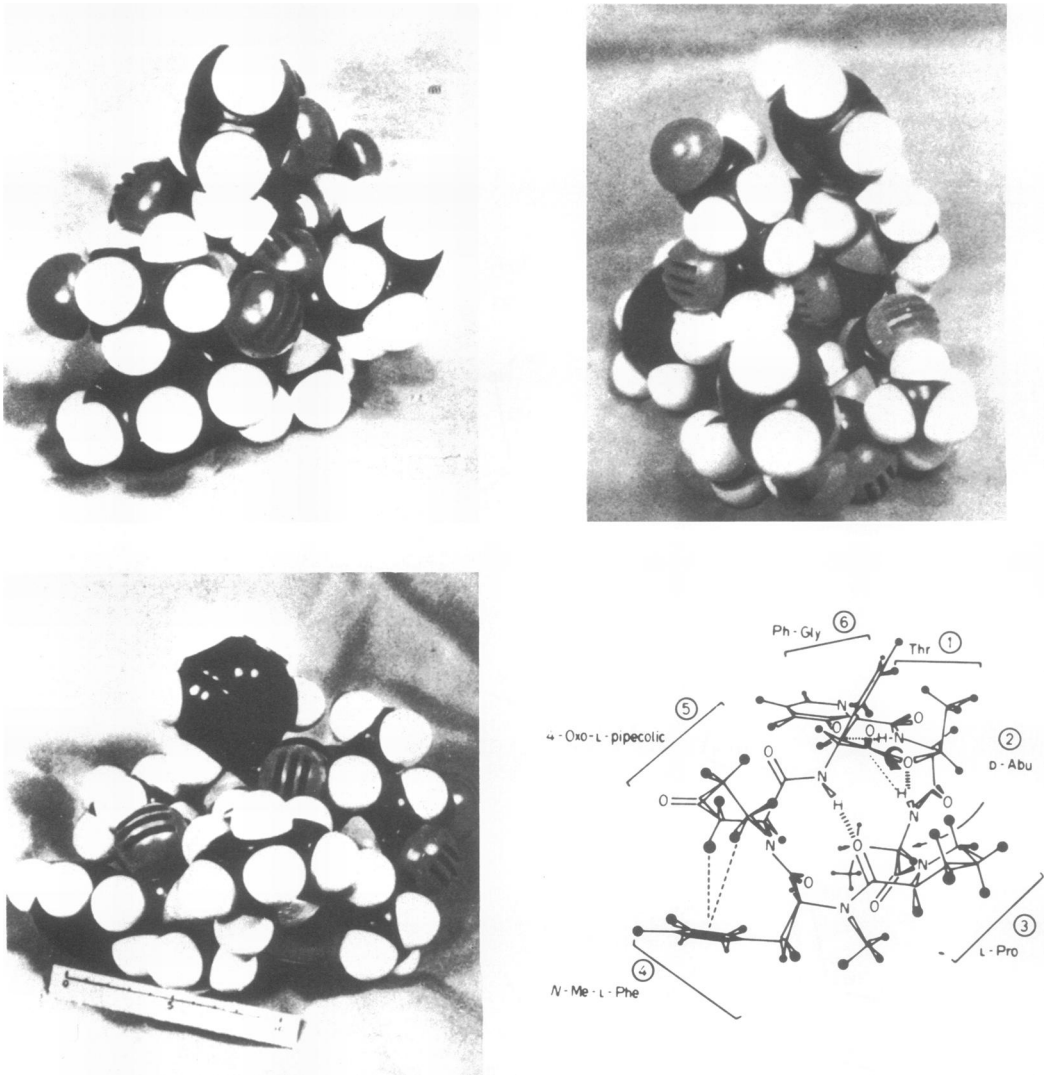


FIG. 5. *Tridimensional structure of VS. (A, B, and C) Pauling-Corey-Koltum space-filling models. (A) Top view of the molecule (6-CO upwards) showing a hydrophilic regions. (B) Side view of the molecule (6-CO inwards) showing a lipophilic region. (C) Back view of the molecule, with the lipophilic region at the right side. (D) Expanded model of virginiamycin, showing an extreme conformation around the depside bond (CO outwards). These models are based on ^{1}H and ^{13}C nuclear magnetic resonance spectra of virginiamycins S1 and S4 and vernamycin B. According to reference 5.*

The disposition of the molecules within the crystal mesh is shown in Fig. 6B.

Virginiamycin-like antibiotics are sensitive molecules; most reagents entail a loss of biological activity. Particularly important is, therefore, the restricted reductive reaction which has been discovered for virginiamycin S, for it allows the specific labeling of the molecule without decrease of its inhibitory power. Reduction of the carbonyl group under the experimental conditions described yields the two epimers of the dihydroderivative of the 4-oxopipecolic acid: the normal form bearing an OH group in *trans* with respect to the adjacent peptide bond, and the allo form possessing the OH in *cis* (Fig. 7). Both of these compounds inhibit the growth of sensitive microorganisms and are present in small concentrations in the usual preparation of virginiamycin (the allo form corresponds to virginiamycin S2) (154, 295).

Finally, the complete synthesis of patricin A (226) and etamycin (266) and the assembly and

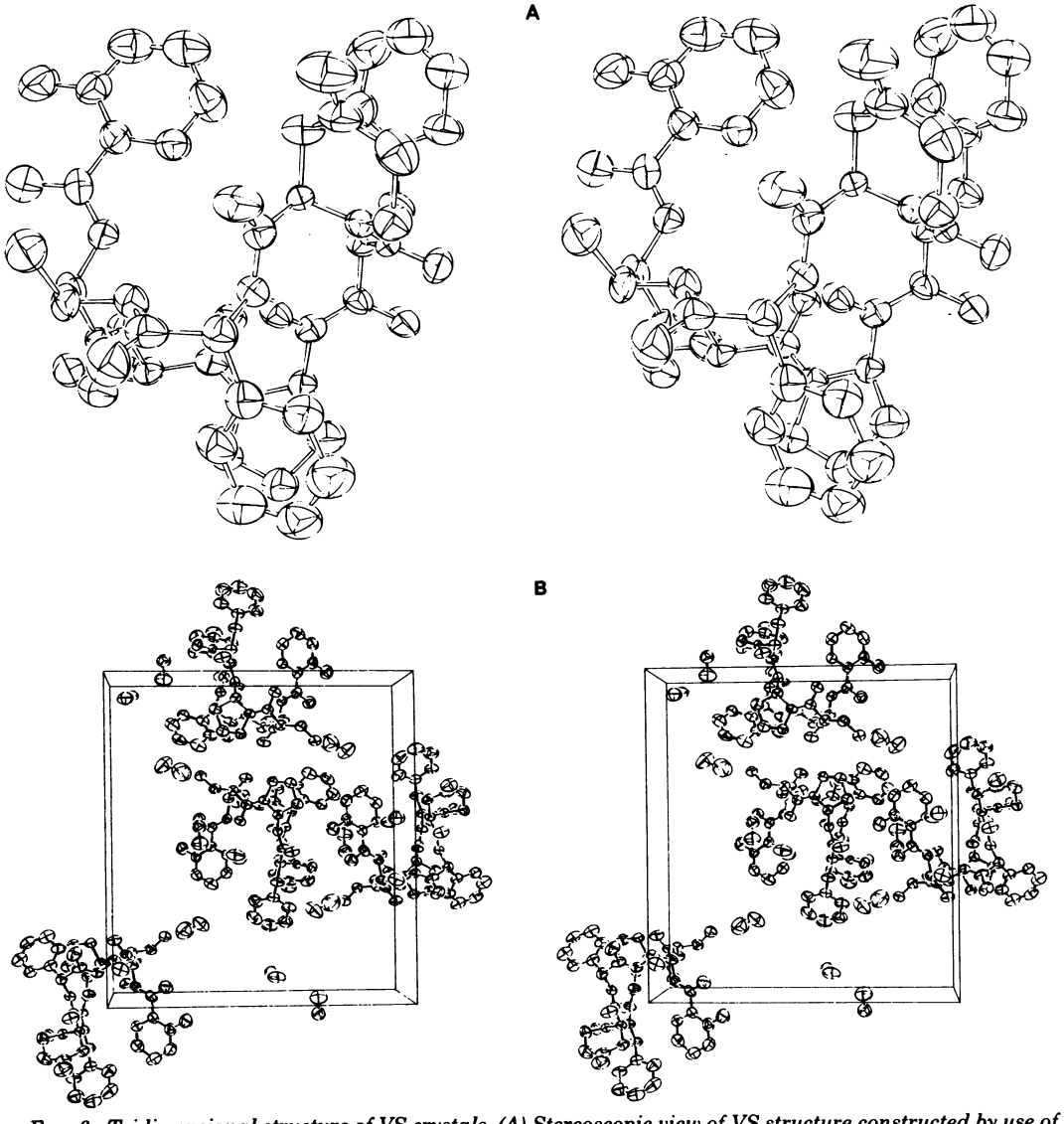


FIG. 6. *Tridimensional structure of VS crystals. (A) Stereoscopic view of VS structure constructed by use of a modified program Multan analysis of diffractometric measurements of the solvate* $C_{43}H_{49}N_7O_{10} \cdot 3\ CH_3OH$. *(B) Disposition of the molecules in the crystal mesh (orthorhombic crystals). According to reference 79.*

characterization of oligopeptides related to virginiamycin (142, 143, 155, 163, 195, 226) ought to be mentioned.

Biophysical Properties of Virginiamycin-Like Antibiotics

The components of both A and B groups have a very low solubility in aqueous solvents, whereas they are highly soluble in organic solvents (Table 3). This explains the partition coefficient of these drugs among subcellular fractions of both eucaryotic and procaryotic cells, and also the limitations in their therapeutic use and the technical difficulties for titration. The procedures for extraction, purification, and crystallization of these antibiotics are also based on their solubility properties. The separation patterns, by thin-layer chromatography, of virginiamycins of type A and B, the epimers of dihydrovirginiamycin S, and the amino acid of type B virginiamycins are reported in Fig. 7 (cf. also references 92, 129, 154, 295, 297). Virginiamycins A and B are unstable at low and high pH (253–257).

The absorption spectra of antibiotics of the A group in two different solvents are shown in Fig. 8A. In chloroform, there is a plateau at 270 nm, which undergoes little modification in water. Spectra of compounds of the B group in chlo-

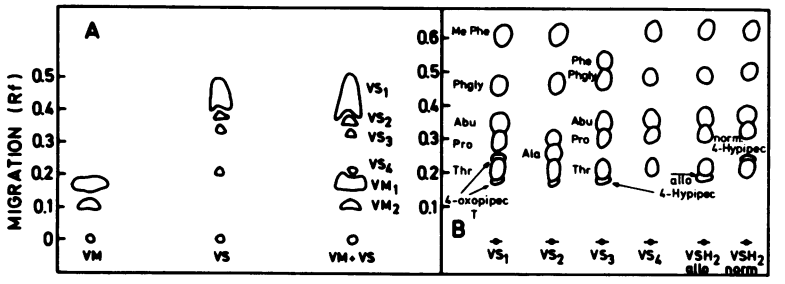


FIG. 7. *Chromatographic separation of virginiamycin components and hydrolysis products. (A) Thin-layer chromatography of purified virginiamycin, showing the separation of VM derivatives (M1 and M2) (type A compounds) and of VS derivatives (S1 to S4) (type B compounds) (M. Di Giambattista and C. Cocito, unpublished data). (B) Separation by paper chromatography of the hydrolysis (6 N HCl) products of the four natural components of VS (VS1 to VS4), and of the epimers obtained by catalytic hydrogenation of VS (allo-4-hydroxypipecolic acid has an OH/COOH cis configuration, whereas this is trans in the reduced form). According to reference 295.*

TABLE 3. *Solubility of virginiamycin-like antibiotics in different solvents*

Type A components (mg/ml)	Solvents[a]	Type B components (mg/ml)
	I	
100	Dimethyl formamide	100
100	Dimethyl sulfoxide	
50	Chloroform	100
50	Dioxane	80
	II	
20	Ethanol	50
20	Methanol	5
20	Acetone	170
10	Isopropanol	
10	Butanol	
10	Methylethylketone	25
10	Butyl acetate	25
	III	
5	Ethyl acetate	250
4	Amylacetate	
3	Benzene	
2	Toluene	80
1	Ether	10
	IV	
10^{-1}	Hexane	10^{-1}
10^{-2}	Carbontetrachloride	10^{-2}
10^{-2}	Petroleum ether	10^{-2}
10^{-3}	Ethanol:water (1:99)	10^{-3}
10^{-4}	Water	10^{-4}

[a] Solvent groups: I, very high solubility; II, good solubility; III, low solubility; IV, very low solubility.

roform (Fig. 8B) show a major peak at 305 nm, which is shifted to 350 nm in water. Moreover, the spectrum produced by mixing equimolar solutions of antibiotics of the two groups corresponds to the superposition of the spectra of single substances, and no shift of the absorption peaks is apparent when small amounts of one component are added to a concentrated solution of its partner. In other words, no evidence for a physical interaction of the A and B types of antibiotics, either in water or in organic solvents, has been gathered thus far.

Unlike the members of the A group, type B compounds yield fluorescent solutions. The emission and excitation spectra of virginiamycin S show a broad peak with half-band width of 40 to 50 nm. Maxima are at 342 nm for excitation and 406 nm for emission spectra (Fig. 8C).

The infrared spectra of type A and B virginiamycins indicating the main functional groups of the molecules are shown in Fig. 9A and B.

Chemical and Physical Determinations of Virginiamycin-Like Antibiotics

Virginiamycin solutions can be titrated spectrophotometrically (cf., for example, 22, 247–249, and 253–256). Linear relationships of absorbance and concentration are obtained with aqueous solutions of group A antibiotics at 270 nm, and of type B compounds at 350 nm (Fig. 10C). In addition, a colorimetric titration of type A compounds is possible, upon incubation with the Ehrlich reagent (Fig. 10A), whereas for type B components a very sensitive spectrofluorimetric procedure of titration is available (Fig. 10B).

Virginiamycin solutions strongly absorb ultraviolet light. Chromatographic spots and bands containing these antibiotics can, thus, be visualized by use of 254-nm-peaked ultraviolet lamps. Indeed, procedures for quantitative determination of these substances by the fluorescence quenching procedure have been developed. Attention has been recently drawn to the fact that irradiation with ultraviolet light produces a degradation of these antibiotics, and its use in preparative procedures must be avoided as much as possible (69).

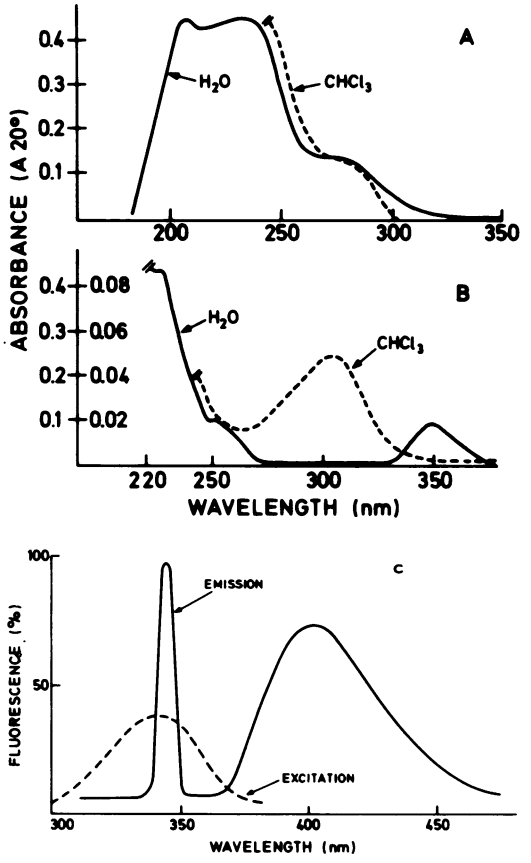


FIG. 8. *Ultraviolet-visible spectra of virginiamycin-like antibiotics of group A and B. (A) Spectra of VM in aqueous and nonaqueous solvents; (B) same as (A) for VS (solutions containing 10 μg/ml, either in 100% chloroform or in water containing 0.3% ethanol, were measured in a Beckman double-beam spectrophotometer with 1-cm quartz cuvettes). (C) Fluorescence excitation and emission spectra of VS (solutions containing 5 μg/ml in a water-ethanol [94: 6] mixture were scanned in a Perkin Elmer 204 spectrofluorometer). According to reference 230.*

ACTION OF VIRGINIAMYCIN-LIKE ANTIBIOTICS ON BACTERIA

Alterations of Growth and Viability in Bacilli

Virginiamycin-like antibiotics do not alter the growth of most eucaryotic protists (Protophyta, Protozoa, and Hysterophyta), but strongly inhibit the multiplication of many procaryotic protists (schizophyces and schizomyces) (125, 282, 312). Gram-positive bacteria are more sensitive (minimum inhibitory concentration of 0.1 to 5 μg/ml) than gram-negative bacteria (minimum inhibitory concentration, 5 to 200 μg/ml). Exceptions to this rule are: mycobacteria, some of which are relatively resistant, and haemophilus and neisseria, which proved quite sensitive to these drugs (Table 4) (312). Divergence in the sensitivity of different bacteria to virginiamycins is due in most cases to permeability, since ribosomes (which are the target of these antibiotics, as described in Inhibition of Cell Division in *Bdellovibrio*) from gram-negative organisms are as sensitive as those from gram-positive strains.

A mixture of type A and B antibiotics causes a more pronounced inhibition of bacterial growth than do single components separately; 10-fold to 100-fold increase of growth inhibition was observed in different microorganisms (Table 5) (2, 8, 11, 16, 18, 50, 53, 64, 103, 198, 231, 286, 288, 312, 324). In Fig. 11 the action of virginiamycin on the growth of *Bacillus subtilis* is depicted. Quite high levels (~50 μg/ml) of either component, virginiamycin M or S (VM or VS), are required to block completely the increase in turbidity of a growing culture, and a similar effect is produced by a far lower concentration of a VM + VS mixture (~0.5 μg/ml). This means a 100-fold potentiation of the antibiotic activity (53).

The synergistic action on cell multiplication varies according to the relative proportions of type A and B components in the mixture. The growth-inhibiting power of different combinations of components A and B on three gram-positive microorganisms is reported (see Fig. 33). The bell shape of the curves indicates a sharp lowering of biological activity when either substance is withdrawn, whereas their asymmetry suggests that type A components are indeed the limiting factor, since a lowering of their concentration below a 15% level produces a rapid drop of the antibiotic activity. Ratios of A/B ranging from 2:1 to 1:1 are the most active and are those which are found in nature.

Single virginiamycins do not reduce the viability of most bacteria, unless the incubation with the drugs is exceedingly long. Nevertheless, the growth of cells previously incubated with virginiamycin type A is subsequently retarded upon transfer to antibiotic-free medium. This phenomenon, which was previously described as "bacteriostasis" or "bacteriopause" (42, 44, 210, 319), is conceivably due to the difficulty in removing by dilution cell-bound type A compounds, and perhaps to the adaptative formation of inactivating enzymes (see below). Consequently, agar plates containing these drugs must be incubated for at least 2 days before being discarded to avoid an erroneous conclusion of viability loss.

On the other hand, mixtures of components A and B produce a sharp decrease of colony-forming capacity, not necessarily associated with the lysis of bacteria. In the most favorable cases,

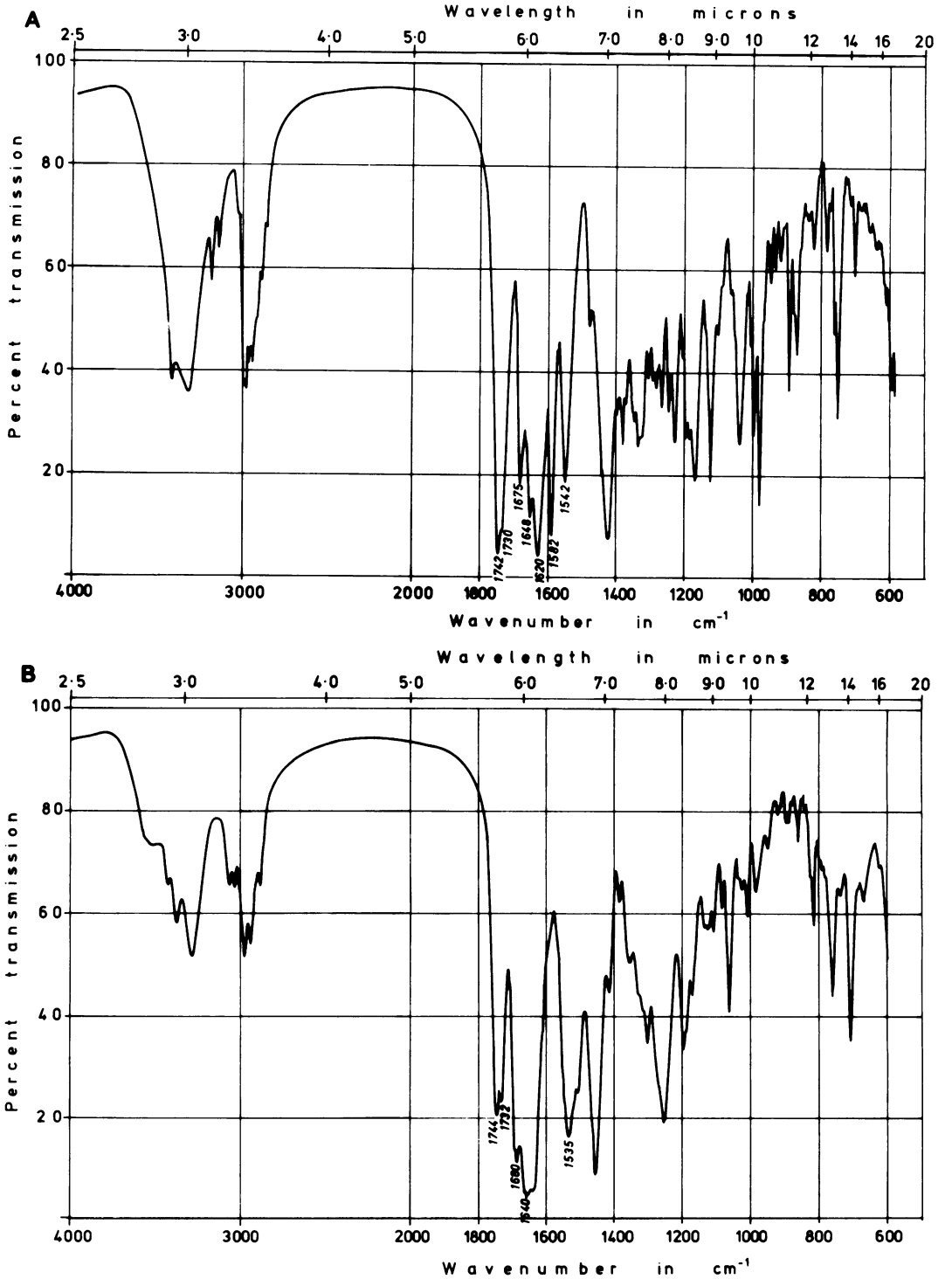


FIG. 9. *Infrared spectra of group A and B virginiamycins. (A) Spectrum of virginiamycin M1 (1% solution in KBr). Bands in 3,500 cm^{-1} region—NH and OH stretching vibrations; bands in 3,000 cm^{-1} region—CH stretching vibrations; 1,742 cm^{-1}—lactone; 1,730 cm^{-1}—ketone; 1,675 cm^{-1}—secondary amide band I; 1,542 cm^{-1}—secondary amide band II; 1,648, 1,620, and 1,582 cm^{-1}—tertiary amide, C=C stretching vibration of ethylenic bonds, and C=C and C=N of oxazole ring. (B) Spectrum of virginiamycin S1 (0.5% solution in KBr). Bands in 3,500 cm^{-1} region—NH and OH stretching vibrations; bands in 3,000 cm^{-1} region—CH stretching vibrations; 1,744 cm^{-1}—lactone; 1,732 cm^{-1}—ketone; 1,680 cm^{-1}—secondary amide band I; 1,535 cm^{-1}—secondary amide band II; 1,640 cm^{-1}—tertiary amide and C=C stretching vibrations of aromatic nucleus (H. Vanderhaeghe et al., unpublished data, and reference 70).*

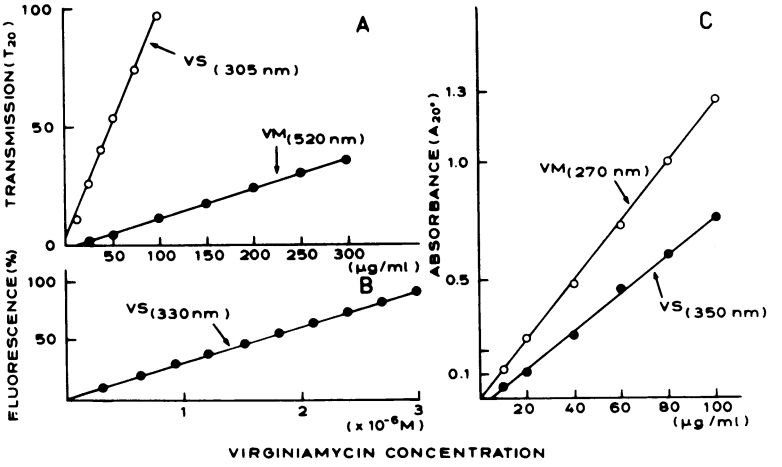


FIG. 10. *Biophysical determination of virginiamycin-like antibiotics. (A) Colorimetric determination of VM (group A component) and of VS (group B). To VM solutions of 0.1 to 1.0 mg/ml in isopropanol, equal volumes of Ehrlich reagent (2.5 N HCl-2% solution of 4-dimethylaminobenzaldehyde in isopropanol [4:6] mixture) were added, samples were incubated for 15 min at 75°C and 30 min at 20°C, and transmission was measured at 520 nm. VS solutions in 100% chloroform were measured at 305 nm. (B) Spectrofluorimetric titration (λExc = 330) of VS in water. (C) Spectrophotometric titration (20°C) of VM and VS in a water-ethanol-chloroform (89:10:1) mixture (cf. reference 230).*

TABLE 4. *Minimum concentration of single components inhibiting the growth of different bacteria*[a]

Organisms	Inhibitory concn (µg/ml)							
	Viridogrisein	Streptogramin		Synergistin		Virginiamycin		Mikamycin
		A	B	A-1	B-1	M	S	A	B
Gram-positive bacteria									
Bacillus megaterium		40	3					800	20
Bacillus subtilis	2.50			100	3.12	50	13	800	10
Staphylococcus aureus	0.31	6	10	0.78	6.25	5	125	20	100
Sarcina lutea						2.5	14		
Streptococcus pyogenes	0.63			0.19	50			40	40
Gram-negative bacteria									
Escherichia coli	200	40	100					800	800
Haemophilus pertussis	5							4	100

[a] According to reference 312.

99% of the cells become nonviable within one generation time (Fig. 12D). Although a similar effect was observed upon alternated incubation of microorganisms with single virginiamycin components (62), the difficulty of completely and rapidly removing type A compounds by washing renders this type of experiment questionable.

Antibiotics of the A and B groups act synergistically in double-sensitive organisms. The situation has been more clearly investigated in *B. subtilis*, where two types of resistance to type A compounds and one type of resistance to type B virginiamycins were found. As discussed later, a most intriguing observation is that the viability loss produced by a mixture of A and B components occurs in mutants resistant to type A, but not in those resistant to type B virginiamycins. The conclusion is that, in *B. subtilis*, the gene controlling the sensitivity to type B components is directly concerned with the lethal effect of A + B mixtures (64).

The two synergistic effects on bacterial growth and viability are quite specific: virginiamycins of one group increase and render irreversible the inhibitory action of their partners, but not that of other translational inhibitors. Thus, for example, virginiamycin M and S do not potentiate, at least in *B. subtilis*, the reduction of cell growth and viability caused by chloramphenicol,

TABLE 5. *Growth inhibition of different microorganisms by mixture of A and B components*[a]

Organisms	Inhibitory concn (μg/ml)				
	Streptogramin	Synergistin	Virginiamycin	Mikamycin	Pristinamycin
Gram-positive bacteria					
Bacillus megaterium	2			64	
Bacillus subtilis		0.78	1	32	0.70
Staphylococcus aureus	0.60	0.19	0.20	4	0.20
Sarcina lutea			0.10	1	
Streptococcus pyogenes	0.05	0.08	0.07		0.10
Streptococcus faecalis	1.49	0.39	0.50		0.20
Diplococcus pneumoniae	0.25	3.12	0.07	6	0.15
Corynebacterium diphtheriae	0.04	0.39		1	0.02
Mycobacterium sp. 607	11	6.25		280	
Mycobacterium tuberculosis	5		20	200	
Gram-negative bacteria					
Salmonella typhosa	11.80	100		1,600	
Escherichia coli	40	100		1,600	50
Aerobacter aerogenes		100	100		250
Haemophilus pertussis	0.04	3.12			
Neisseria gonorrheae		3.12			0.20
Pseudomonas aeruginosa	50	100		1,600	250
Yeast					
Saccharomyces cerevisiae	85			1,600	
Candida albicans		100	100	1,600	
Fungi					
Aspergillus niger	85				
Aspergillus oryzae				1,600	
Protozoa					
Trichomonas vaginalis	490				
Trichophyton sulfureum		100			

[a] According to reference 312.

erythromycin, fusidic acid, and oleandomycin (Fig. 12). All of these antibiotics are known to act on the 50S ribosomal subunits.

Not only are virginiamycins unable to increase the inhibitory power of other compounds interfering with protein synthesis, but in several instances they proved capable of blocking the action of other antibiotics. Thus, for example, mikamycin A was reported to prevent the killing effect of streptomycin and kanamycin (345). Since erythromycin and chloramphenicol have similar capacity, the conclusion has emerged that there is an antagonistic effect among protein synthesis inhibitors which act on the 50S (mostly bacteriostatic drugs) and on the 30S (mostly bactericidal drugs) ribosomal subunits, respectively. Unfortunately, this antagonism among protein synthesis inhibitors has not been analyzed further, and its molecular basis is unknown.

Also, the observation that mikamycins prevent the bacteriolytic action of penicillins is merely an example of the well-recognized antagonism between bacteriostatic drugs in general and cell wall synthesis inhibitors. The latter type of antibiotics acts exclusively on exponentially multiplying bacteria and is, thus, ineffective on resting cells (333).

Macromolecule Formation in the Presence of Virginiamycins

During the last two decades, conflicting reports on macromolecule metabolism in cells treated with virginiamycins have been published. In fact, different results can be obtained according to (i) the length of incubation of bacteria with the drug, and (ii) the technique used for the evaluation of the synthesis of a given polymer. This conclusion stems from the data that follow.

If the kinetics of incorporation of labeled precursors, pyrimidine bases, and amino acids into deoxy- and ribo-polynucleotides and polypeptides, respectively, are traced in *B. subtilis* in the presence of virginiamycins, it can be seen that the last type of synthesis is blocked without delay, whereas the former types are inhibited after a considerable lag (Fig. 13). The conclusion is, therefore, that polypeptide formation is directly affected by compounds of both A and B groups, and that this entails the reduction of nucleic acid synthesis (53, 103, 123, 286, 315). The first observations along these lines were reported by Yamaguchi and Tanaka (338–341).

Kinetics of amino acid incorporation into polypeptides in antibiotic-resistant mutants con-

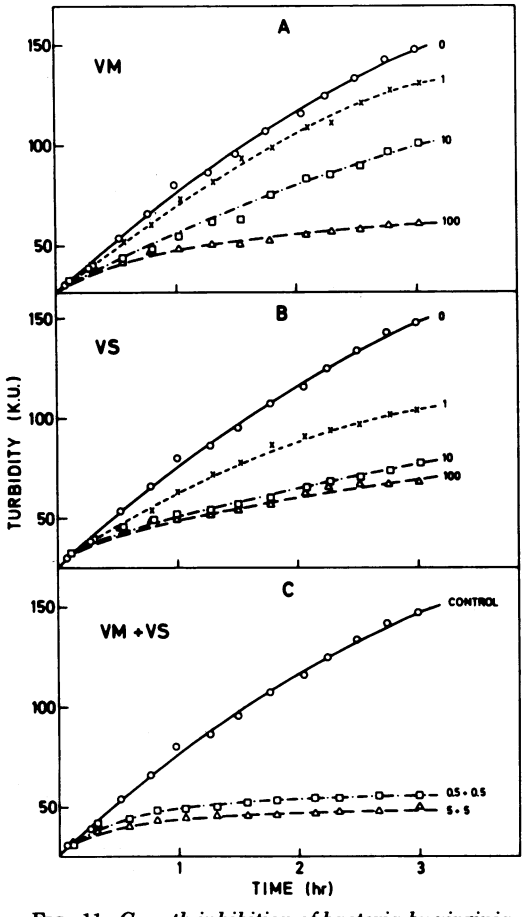


FIG. 11. *Growth inhibition of bacteria by virginiamycins. B. subtilis cells were incubated with increasing concentrations (1, 10, and 100 μg/ml) of single virginiamycin components, VM (A) and VS (B), or their mixture (either 0.5 (□) or 5 (△) μg of VM + VS per ml) (C), and growth was measured spectrophotometrically. According to reference 53.*

firm that the inhibition of protein synthesis is the molecular basis of both the bacteriostatic action of single components and the bactericidal effect of their combination (Fig. 14). This was conclusively proved by showing (i) that VM and VS produce a transient inhibition of polypeptide formation separately, and a permanent effect jointly, and (ii) that the mixture of VM + VS blocks irreversibly protein synthesis in VM-resistant, but not in VS-resistant, mutants (64).

Kinetics of uracil incorporation into polyribonucleotides are increased during the first period of incubation of bacteria with these antibiotics and level off slowly (Fig. 13B). Similar observations were previously made with cells treated with chloramphenicol and erythromycin (144). This is due to two simultaneous alterations of ribonucleic acid (RNA) metabolism that shall be discussed later: (i) ribosomal RNA (rRNA) is still formed without being wrapped into neosomes, and (ii) untranslated messenger RNA (mRNA) accumulates (53).

In spite of this initial increase in polyribonucleotide level, a very early inhibition of 16S and 23S rRNA formation in virginiamycin-treated *B. subtilis* has been observed (53). Moreover, rRNA which accumulates under those conditions is undermethylated. This structural alteration accounts for its metabolic instability. The conclusion is that stability of rRNA's, nucleic acids which are highly conserved and transferred to progeny cells during bacterial multiplication, relies on (i) their methylation by an apparently unstable methylase activity, and (ii) their binding to ribosomal proteins. Similar inference was drawn for chloramphenicol (90, 128, 211, 262, 263). Thus, rRNA formation and stability are tightly coupled to protein synthesis, and this equilibrium is broken by virginiamycin-like antibiotics.

Also, an increase in the half-life of mRNA was observed shortly after the addition of virginiamycin to bacterial cultures (53). This finding can be explained by the work of Fan et al. (116) describing two contrasting situations in bacteria submitted to growth inhibitors. The half-life of mRNA is decreased in the presence of antibiotics which, like puromycin, produce the dissociation of translational complexes, and is increased by inhibitors which mimic chloramphenicol in freezing the complexes. Indeed, evidence for an increased stability of polysomes in bacteria treated for short lapses of time with virginiamycins was gathered (55) (as mentioned later, it is only after prolonged incubation with these inhibitors that a dissociation of translational complexes occurs).

The influence of virginiamycins on cellular deoxyribonucleic acid (DNA) metabolism has not been exhaustively analyzed. Apparently, the incorporation of labeled precursors into trichloroacetic acid-insoluble material is affected only after prolonged incubation with these inhibitors (Fig. 13A) (53). This agrees with the finding that: (i) vernamycin does not inhibit DNA polymerase in vitro, and (ii) inhibition of protein synthesis prevents the initiation of cell chromosome formation, leaving the elongation steps unaffected.

Metabolism of Polysomes and Ribosomes in *Bacillus subtilis*

Formation of ribosomal subunits is blocked in bacteria treated with virginiamycin (55, 56). This effect is shared by the other inhibitors of protein synthesis, because the formation of ribosomal proteins is particularly sensitive to antibiotics.

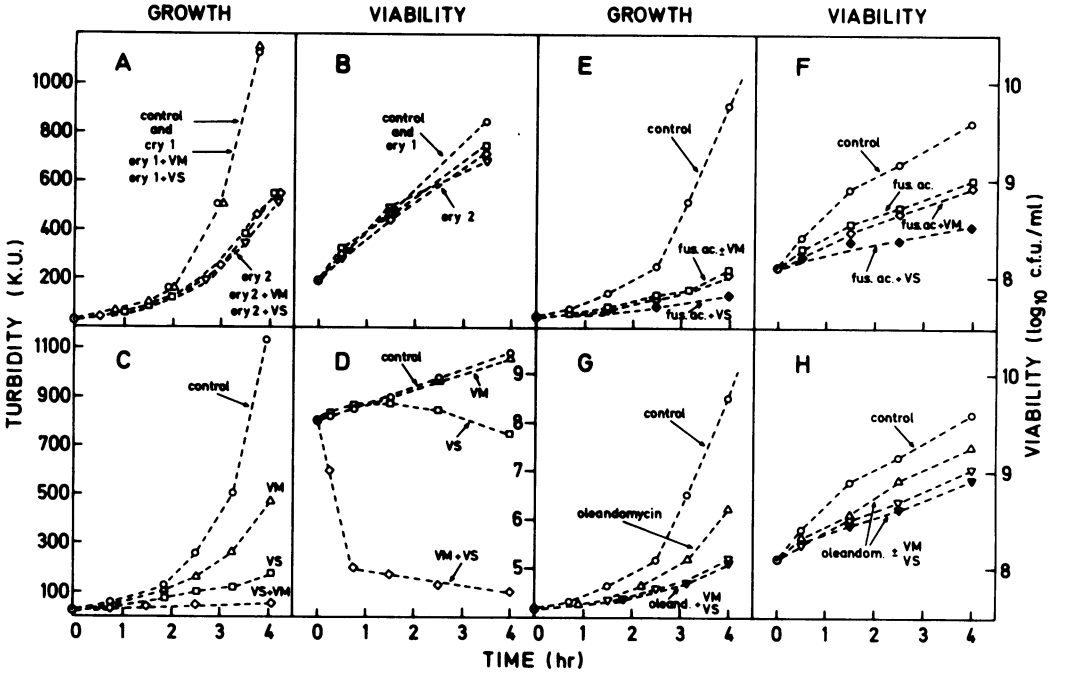


FIG. 12. *Synergistic inhibitory activity of virginiamycin components (VM and VS) and other 50S inhibitors. B. subtilis cells were incubated with different antibiotics and their combinations; cell multiplication (A, C, E, G) and cell viability (B, D, F, H) were evaluated. Inhibitors: (A and B) None (control) (○); erythromycin, 0.1 μg/ml (△, ery₁) and 0.05 μg/ml (▽, ery₂); VM + erythromycin, 0.01 μg of VM and either 0.01 or 0.05 (□) μg of erythromycin per ml; VS + erythromycin, same as for VM + erythromycin (◇). (C and D) None (○); VM, 0.01 μg/ml (△); VS, 1 μg/ml (□); VM + VS, 0.1 μg of VM + 1 μg of VS per ml (◇). (E and F) None (○); fusidic acid (fus. ac.), 10 μg/ml (□); VM, 0.1 μg/ml, + fus. ac., 10 μg/ml (◇); VS, 0.5 μg/ml, + fus. ac., 10 μg/ml (◆). (G and H) None (○); oleandomycin, 1 μg/ml (△); VM, 0.1 μg/ml, + oleandomycin, 1 μg/ml (▽); VS, 0.5 μg/ml, + oleandomycin, 1 μg/ml (▼).*

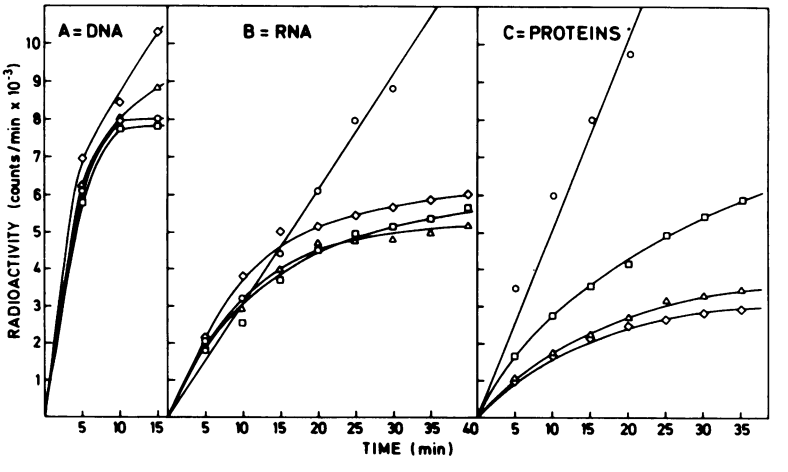


FIG. 13. *Macromolecule formation in virginiamycin-treated bacteria. B. subtilis cells were incubated with the virginiamycin components VM and VS and labeled with [^{3}H]thymidine (A) (15-min pulses given at 15-min intervals to virginiamycin-treated cells), [6-^{3}H]uracil (B), and ^{14}C-amino acid mixture (C). Radioactivity incorporated into DNA (A), RNA (B), and proteins (C) was measured. Virginiamycin: none (control) (○); VM, 50 μg/ml (△); VS, 50 μg/ml (□); VM + VS, 2.5 μg/ml (◇). According to reference 53.*

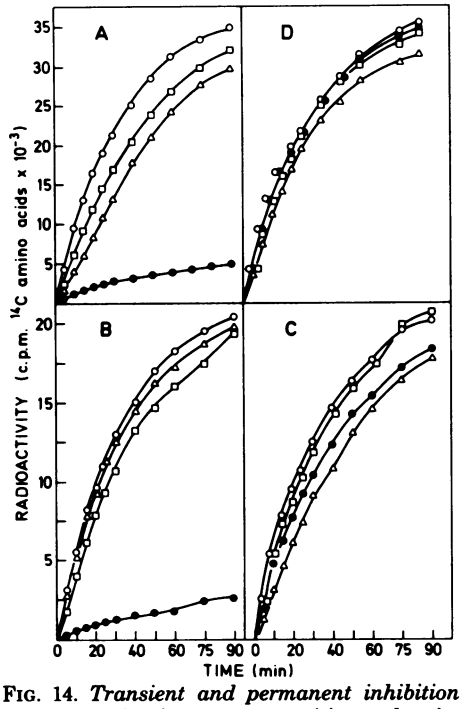


FIG. 14. *Transient and permanent inhibition of protein formation in mutants sensitive and resistant to virginiamycins. B. subtilis mutants, after 30-min incubation with the virginiamycin components VM and VS, were transferred to antibiotic-free medium and labeled with ^{14}C-amino acids. Radioactivity incorporated into proteins was measured. Mutants: M^IS^I, sensitive to both VM and VS (A); $M_E^RS^I$, resistant to VM (B); M^IS^R, resistant to VS (C); and M^RS^R, resistant to both VM and VS (D). Virginiamycin: none (control)* (○); *VM, 1 μg/ml* (△); *VS, 1 μg/ml* (□); *VM + VS, 0.05 μg/ml* (●). *According to reference 64.*

The rRNA synthesized under these conditions binds to cytoplasmic proteins made before the administration of the drug, since a negligible pool of free ribosomal proteins exists in exponentially growing bacteria (262, 263). The ribonucleoprotein complexes which accumulate are heterogeneous in size (sedimentation coefficients of 18S to 25.8S have been recorded) and composition. They mimic the "relaxed particles" which appear in relaxed mutants starved for an essential amino acid. Extensive studies of the composition and fate of such particles in chloramphenicol-treated bacteria brought about a revised interpretation of their origin and function (they were formerly considered as physiological precursors of nascent ribosomes or neosomes) (178, 348).

Upon removal of single virginiamycins, formation of ribosomal subunits resumes without delay (56). Apparently, rRNA molecules within relaxed-like particles dissociate from their protein partners and rapidly associate to newly formed ribosomal proteins. This recovery process is rather puzzling, for rRNA which accumulates under the condition of halted protein synthesis is undermethylated and has lower molecular weight (little or no 23S rRNA is formed under these conditions) (53). When sensitive cells are incubated with a mixture of virginiamycins A and B, no such recovery occurs upon removal of the drug (56); this is due to the permanent halt of protein synthesis occurring under these conditions (64).

Short incubation of growing cells with virginiamycin "freezes" polysomes. Indeed, an increase of the half-life of the translational complexes has been recorded by labeling-chasing experiments (55). A similar observation was made with chloramphenicol, hence, the routine use of this drug for the preparation of bacterial polyribosomes, which are known to "run off" even at low temperature.

If the incubation of bacteria with virginiamycin is prolonged, most polysomes disappear and monosomes and ribosomal subunits accumulate (59, 75, 107, 110, 241). The picture is different in cells treated with compounds of the A and B groups. In the former case, a large amount of ribosomal subunit has been found, a situation mimicking that produced by transcriptional inhibitors. In the latter case, ribosomes accumulate, as they do in chloramphenicol-treated cells and in auxotrophs starved for an essential amino acid (59).

When cells incubated with virginiamycins of the A group are lysed, and lysates are submitted to high-speed centrifugation (100,000 × *g* in sucrose gradients), an unusual ribosomal peak, the "60S component," appears (Fig. 15). Its formation is prevented either by particle fixation with glutaraldehyde (Fig. 15E) or by particle fractionation at low speed (50,000 × *g*) (57, 59). Recent studies on pressure sensitivity of bacterial ribosomes have contributed to explain these findings. Ribosomes, which are produced under certain instances of halted protein synthesis, dissociate when they reach a certain distance from the axis of rotation while traveling in a dense medium at a critical speed (the steepness of the density and pressure gradients contribute to the sharpness of the peak) (59, 132, 148, 212-215, 273, 299, 300). Although the molecular basis of 60S component formation in the presence of virginiamycin M is unknown, it is surprising that no 60S component is formed in cells grown in the presence of type B compounds (Fig. 15C).

Mutation to Virginiamycin Resistance and Mapping of Resistance Genes

Bacterial resistance to antibiotics in general,

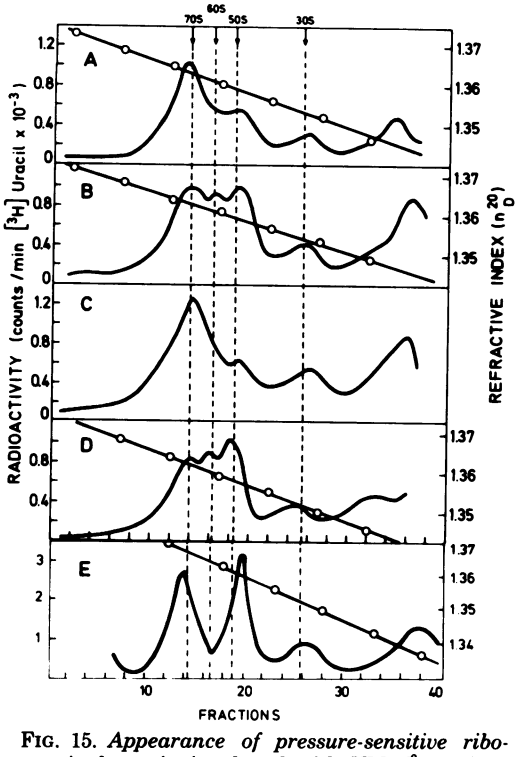


FIG. 15. *Appearance of pressure-sensitive ribosomes in bacteria incubated with VM. [^{3}H]Uracil-labeled B. subtilis cells were incubated with the virginiamycin components VM and VS for three generations, disrupted, and fractionated by ultracentrifugation in density gradients. Virginiamycin: none (A); VM, 1 μg/ml (B); VS, 1 μg/ml (C); VM + VS, 0.1 μg/ml (D). Sample (E) corresponds to sample (B) submitted to glutaraldehyde fixation. The three peaks in the control (A) are those of 70S, 50S, and 30S particles. The 60S peak (B and D) is located between those of monosomes and subunits, and its appearance is prevented by glutaraldehyde fixation.*

and to virginiamycin in particular, is due to one of three mechanisms: (i) alteration of permeability; (ii) modification of the target (the 50S ribosomal subunit, in the case of virginiamycins); and (iii) inactivation of the drug. Antibiotics can be inactivated either by hydrolysis (sensitivity of the virginiamycin lactone ring is comparable to that of the β-lactam ring of penicillins and cephalosporins) or by coupling (acylation, adenylation, and phosphorylation are the most common mechanisms) (17). Moreover, genetic determinants for antibiotic resistance can be either chromosomal or episomal in nature: in most cases, drug inactivation is due to plasmids, target modification is a chromosomal type of resistance, and permeability loss is caused by either mechanism. Although no systematic study of different mutations to virginiamycin resistance in different microorganisms has been made, all of the three mentioned mechanisms were reported for antibiotics of the A and B groups.

Ennis compared the behavior of *B. subtilis* (wild type sensitive, and mutants resistant to vernamycin) to that of *Escherichia coli* (wild type resistant, and mutants sensitive to vernamycin) (106, 109). The conclusion was that, in the three mutants which were analyzed, resistance to this antibiotic was due to permeability loss, since ethylenediaminetetraacetic acid treatment and protoplast conversion rendered the protein synthesizing machinery of resistant cells sensitive to the antibiotics.

The situation in strain 168 of *B. subtilis* is as follows. Growth of the wild type is blocked without restriction in the presence of VS (type B component), but is inhibited for a limited lapse of time (5 to 10 generations) by VM (type A component). This situation, indicated as "late" resistance to VM or M_L^R, differs from that of "early" resistance (M_E^R), i.e., unrestricted inhibition by the antibiotic. From the wild type, mutants sensitive to both virginiamycins (M^IS^I) were produced by mutagenization and penicillin selection. They were the starting point for the preparation, by mutagen treatment, of VM-resistant ($M_E^RS^I$ and $M_L^RS^I$), VS-resistant (M^IS^R), and double-resistant (M^RS^R) mutants. The phenotype of these five strains is depicted in Fig. 16. The colony-forming ability of sensitive and "early" resistant mutants, which were incubated with single virginiamycins and their mixture, is shown in Fig. 17. It can be seen that incubation of double-sensitive strains with a mixture of both A and B components produces a sharp drop in viability (Fig. 17A); this lethal effect still operates in VM-resistant (Fig. 17B) but not in VS-resistant (Fig. 17C) mutants. Likewise, a mixture of virginiamycins A and B produces an irreversible inhibition of protein synthesis in the double-sensitive as well as in the VM-resistant mutants, but not in the VS-resistant bacteria (Fig. 14). The overall conclusions are: (i) lethality of virginiamycins relies on the gene for VS sensitivity and disappears when this mutates to resistance; and (ii) VS acts synergistically with VM in VS-sensitive cells, and antagonistically in VS-resistant mutants (cf. Fig. 14C, 16C, and 17C) (cf. 64 and G. Fraselle, Ph.D. thesis, University of Louvain, Brussels, Belgium, 1972).

Although unproven, it is probable that resistance to virginiamycin, which was dealt with in the cited work on bacilli, was chromosomal resistance. Instead, it is in staphylococci that plasmid-mediated resistance was mainly studied. Thus, for example, from a strain of *Staphylococcus aureus* able to inactivate several antibiotics

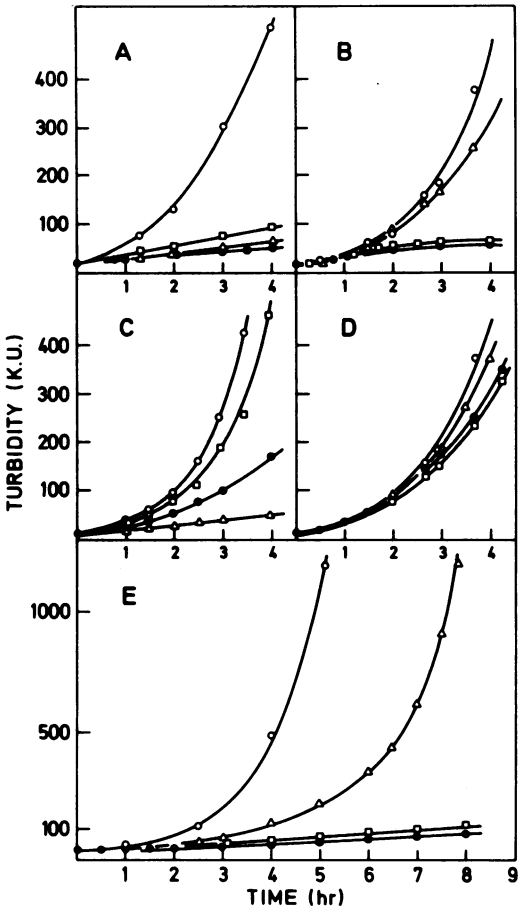


FIG. 16. *Multiplication of resistant mutants in the presence of virginiamycins. The growth of the following resistant mutants of B. subtilis, in the presence of the virginiamycin components VM and VS, was measured turbidimetrically: M^IS^I, sensitive to VM and VS (A); M^IS^R, resistant to VS (C); $M_E^RS^R$, resistant to VM and VS (D). Mutants $M_E^RS^I$ (B) and $M_L^RS^I$ (E) carry two types of mutations (E = early and L = late resistance) against VM. Virginiamycin concentrations: none (control) (○); VM, 1 μg/ml (△); VS, 1 μg/ml (□); VM + VS, 0.1 μg/ml (●). According to reference 64.*

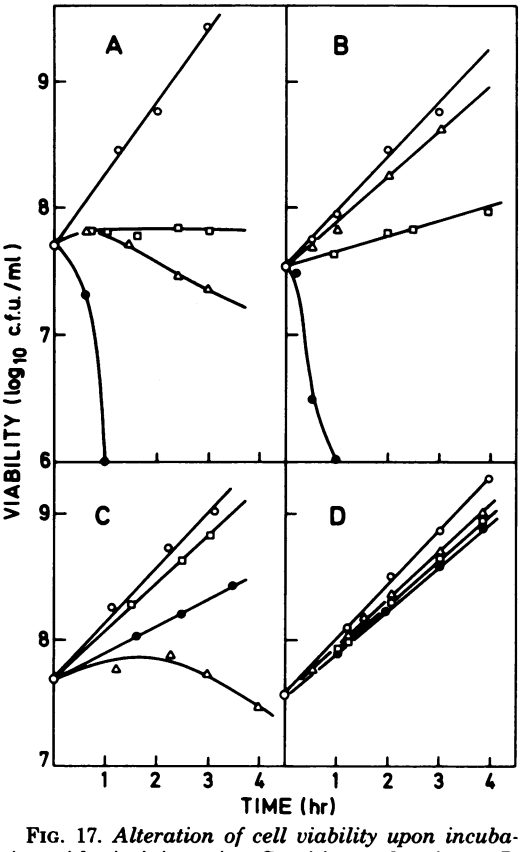


FIG. 17. *Alteration of cell viability upon incubation with virginiamycins. Sensitive and resistant B. subtilis mutants were incubated with the virginiamycin components VM and VS, and colony-forming ability was measured. Mutants M^IS^I (A), $M_E^RS^I$ (B), M^IS^R (C), M^RS^R (D) (cf. legend to Fig. 16). Virginiamycin: none (control) (○); VM, 1 μg/ml (△); VS, 1 μg/ml (□); VM + VS, 0.1 μg/ml (●). According to reference 64.*

including pristinamycin IIA, a plasmid (PAC-IIA) was isolated, which directed the synthesis of an acetyltransferase capable of *O*-acetylating the drug. The inactivated product was characterized by nuclear magnetic resonance and mass spectrometry (184, 185). Another strain of *S. aureus* isolated from humans and resistant to type A components (100 μg of virginiamycin M per ml) was found capable of inactivating the drug by an inducible and strain-specific acetyltransferase, presumably coded for by a plasmid (84).

A plasmid of this sort, pAM-77, has been isolated from *Streptococcus sanguis* and characterized. This episome, carrying the genes for resistance to erythromycin, lincomycin, and vernamycin Bα, was obtained as a covalently closed thymine-labeled satellite DNA band by CsCl centrifugation. pAM-77 had a sedimentation coefficient of 25S, a molecular weight of 4.8×10^6, and a contour length of 2.3 μm. Restriction endonuclease segments of this plasmid were used to transfect the transformable Challis strain of *Streptococcus* to erythromycin resistance. Unstable transformants were obtained, however, and their resistance towards vernamycin was not further assessed (337). pAM-77 and several cases of episomal resistance to vernamycins which were studied to date seem to share the following properties: (i) the capacity

to withstand not only virginiamycins, but also macrolides and lincosamides (the so-called "MLS pattern"); and (ii) the "induction type" of resistance to macrolides, by which exposure of bacteria to subinhibitory concentrations of erythromycin was followed by the appearance of a refractory capacity towards high doses of antibiotics of the MLS group (cf. also 24 and 280). It was recently reported that this phenomenon, the molecular basis of which is unknown, requires a threshold level of ribosome modification to be attained in order to produce a resistance dominant to sensitivity. Moreover, the possibility of induction by different macrolides and their modified products indicates that the inducer and inhibitory inactivities can be dissociated and have different targets (3, 4).

Inactivation of virginiamycins does not occur merely by coupling; indeed, the cleavage of the lactone ring by lactonases has been related. The attention of scientists has been mainly drawn to the lactonases of streptomycetes, particularly of those strains used for the production of commercial preparations. Such enzymes would, in fact, reduce the product yield in fermentation processes. Thus, from *Actinoplanes missouriensis*, a virginiamycin β-lactonase has been purified. The enzyme had a molecular weight of about 3.5×10^4, a K_m value of 3.73×10^{-4} M, and a pH optimum of 7.8 (145, 146). Likewise, *Streptomyces mitakaensis* was found to produce a lactonase able to inactivate mikamycin B and to yield the corresponding acid (166). The purified enzyme had a molecular weight of 2.9×10^4 and a K_m value of 1.43×10^{-5} M (165). These enzymes might play the role of physiological regulators of the antibiotic formation during the growth cycle of the producing organisms.

Cross-resistance between virginiamycins and macrolides in gram-positive microorganisms has been reported by several authors (42–44, 52, 122, 203, 283). Chabbert et al. have analyzed systematically the pattern of cross-resistance between pristinamycin, macrolides, and lincomycin group antibiotics in gram-positive pathogenic organisms isolated from humans. They found three types of resistance called: (i) "heterogeneous dissociated" (resistance to erythromycin only, sensitivity to the other antibiotics); (ii) "homogeneous dissociated" (resistance to all macrolides, sensitivity to lincomycin and pristinamycin); and (iii) "undissociated" (resistance to macrolides and lincomycin, sensitivity to pristinamycin). The overall conclusion is that, although a cross-resistant pattern to macrolides and virginiamycin-like antibiotics is frequent, a dissociation of the two types of resistance can be obtained (cf. 42–44, and 91, 157, 280).

Thus far only virginiamycin B-resistant genes have been mapped in *B. subtilis*, but work is in progress to map more precisely the genes for resistance to both VM and VS in *E. coli* and in *B. subtilis*.

As shown in Table 6, an 18% cotransduction of the virginiamycin S resistance gene with purine A16 and a 49% cotransduction with cysteine A14 was recorded (M. P. de Béthune, unpublished data). Efficient cotransformation (40 to 100%) of VS resistance (VS^R) with the genes for streptomycin resistance (SM^R) ($VS^R - SM^R$ = 59%), elongation factor G (VS^R – EFG = 98.2%), and cysteine ($VS^R - Cys^+$ = 40.5%) was also observed (E. Ron, unpublished results). From these data, the following position has been tentatively assigned to a VS resistance gene in *B. subtilis*: . . . Cys-Sm-EFG-VS. . . . Note that all the genes linked to the VS resistance locus are close to the origin of the *B. subtilis* chromosome, as well as to the resistance loci for ribosome-binding antibiotics. It can thus be concluded that genes of resistance to type B components map in the ribosomal protein region.

Inhibition of Cell Division in *Bdellovibrio*

The case of *Bdellovibrio* is discussed separately, because this system proved particularly interesting for a study of virginiamycin action. *Bdellovibrio bacteriovorus* is a microorganism which penetrates and grows in the periplasmic space of other gram-negative bacteria, utilizing the host components as sources of energy and of precursors. Intracellular growth of *Bdellovibrio* results in the loss of flagella and in the formation of an elongated body which divides—daughter cells acquire flagella and become free through the lysis of the host. In addition to this host-

TABLE 6. *Mapping of virginiamycin S-resistance genes in B. subtilis chromosome*

Gene couples[a]	Cotransformation (%)[b]	Cotransduction (%)[c, d]
VS^R EFG	98.2	
VS^R Sm^R	59.0	
VS^R CysA14	40.5	49(cys) 46(VS^R) 47.5 (avg)
VS^R PurA16		20(ade) 15(VS^R) 17.75 (avg)
Sm^R CysA14	61.3	
EFG CysA14	38.7	

[a] Symbols: VS^R, virginiamycin S resistance; EFG, elongation factor G; Sm^R, streptomycin resistance; CysA14, cysteine A14; PurA16, purine A16.

[b] Data from E. Rone et al. (unpublished).

[c] Data from M. P. de Béthune, Ph.D. thesis, University of Louvain, Brussels, Belgium, 1975.

[d] Selected marker in parentheses.

dependent strain which is an obligate parasite, two other types of *Bdellovibrio*, namely the saprophytic (nonparasitic, also called host-independent), and the facultatively parasitic strains, which can grow in axenic culture non-supplemented with bacterial extracts, have been described (cf. Shilo [269], Starr [275], Starr and Huang [276], Starr and Seidler [277], and Varon [304] for review). The life cycle of *Bdellovibrio* is, thus, unique among bacteria (which multiply by binary fission), particularly in the fragmentation step involving the cleavage of the filament into 20 to 100 mobile vibrios.

Although the growth of *Bdellovibrio* in axenic culture is highly resistant to single antibiotics of the A and B group, sharp inhibition of *Bdellovibrio* multiplication and host cell lysis occurs upon incubation with a mixture of both components, and similar observation was made for the symbiotic multiplication of this microorganism (305). This finding is still another example of the synergistic growth-inhibitory action of antibiotics of the virginiamycin family.

Moreover, unlike their partners of the A group, type B components proved able to dissociate the formation of the multicellular filament from its fragmentation. In fact, in axenic cultures treated with VS, the accumulation of elongated bodies was observed. This effect was reversible, since transfer to antibiotic-free medium allowed cleavage to occur, and relied on protein synthesis. Moreover, VS seemed to block specifically an early step of the *Bdellovibrio* life cycle, since its addition during the second half of the cycle did not prevent division (305).

More recent work has shown that elongation of *Bdellovibrio* is a polar and unidirectional process, and that division into daughter cells requires an unimpaired protein synthesis, as well as the accumulation of a low-molecular-weight division protein (101). The latter is apparently released by dividing cells and is similar to the division proteins found by several investigators in different bacterial species (149, 150, 271, 351). Virginiamycins of the B group either prevent the synthesis, or specifically inactivate, such division protein. The importance of this finding is obvious, in view of the fact that the molecular mechanism of these antibiotics is still unknown.

TRANSIENT AND PERMANENT BLEACHING OF ALGAE BY ANTIBIOTICS OF THE VIRGINIAMYCIN FAMILY

Cell Growth and Chlorophyll Synthesis in *Euglena*

Addition of single virginiamycin components did not alter the growth curve of *Euglena gracilis* in heterotrophic media (62). Although an inhibition of the photoautotrophic multiplication by type A antibiotics was expected (see below), such inhibition could not be observed for technical reasons.

In spite of the lack of growth alterations, type A virginiamycins were found to produce a reversible block of chlorophyll formation (Fig. 18). Type B antibiotics per se had no apparent action on the synthesis of photosynthetic pigments, but increased and rendered irreversible the inhibitory effect caused by their partners. Hence, a transient bleaching was produced by type A

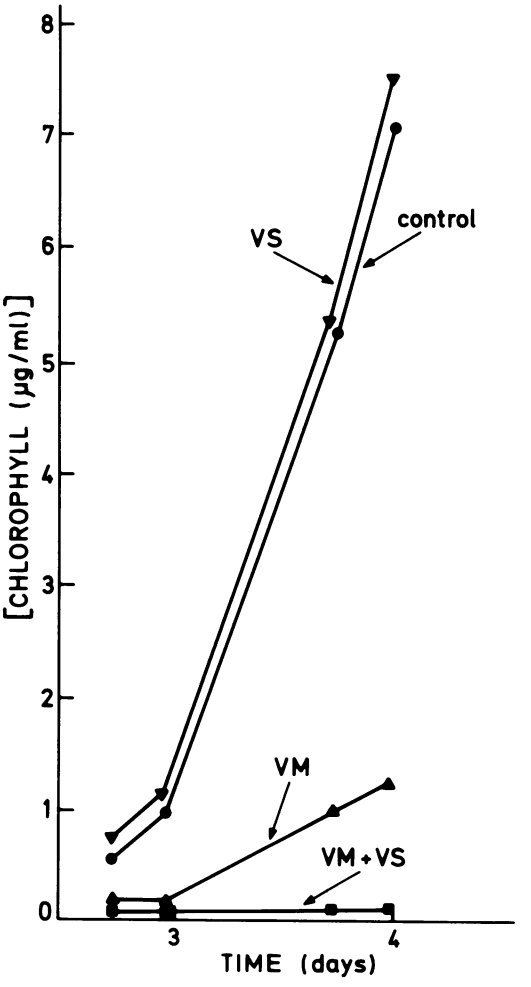


FIG. 18. *Inhibition of chlorophyll synthesis in algae by virginiamycins. Euglena gracilis was grown in light in the presence of single virginiamycin components, VM and VS, and their mixture. Chlorophyll was extracted and measured. Inhibitor concentration: none (●); 100 µg of VM per ml (▲); 100 µg of VS per ml (▼); 50 µg of VM + VS per ml (■). From reference 302.*

inhibitors, and a permanent bleaching was observed in the presence of a mixture of type A and B antibiotics (Table 7) (62). Whereas colonies of *E. gracilis* on control plates were green, those on agar containing 100 μg of virginiamycin M per ml were reddish (due to the accumulation of carotenes), and those formed in the presence of VM and VS were white. When such colonies were replica plated in agar without antibiotics, reddish cells yielded green colonies, and white cells produced white colonies (302). The morphological alterations accounting for these observations were described. Although chloroplasts from algae grown in the presence of type B components were indistinguishable from the controls, the organelles from cells treated with type A virginiamycins presented a loss of pyrenoid and spindle structures, and a dissociation of thylakoids; yet such altered organelles were still capable of recovery, upon removal of the antibiotic. By contrast, in cells incubated with a mixture of A and B components, chloroplasts were replaced by reticulated bodies (302) mimicking the proplastids which were observed in certain etiolated cultures found in nature. These altered organelles, in which the lamellar structures were replaced by tubular bodies, had lost the ability to yield chloroplasts upon exposure to light in the absence of antibiotics (Fig. 19).

TABLE 7. *Efficiency of bleaching of Euglena gracilis by virginiamycin*[a]

Virginiamycin[b]			Colonies[c]		
Component	Concn (μg/ml)	Contact (days)	Total (no.)	Bleached (no.)	Efficiency of bleaching (%)
VS	200	1	69	0	0
		2	77	0	0
		3	167	0	0
		4	162	0	0
VM	100	1	85	0	0
		2	55	0	0
		3	134	4	3
		4	198	16	8
	200	1	91	0	0
		2	118	0	0
		3	325	41	18
		4	238	27	11
VM + VS	25 + 25	9	41	6	14
	50 + 50	1	162	0	0
		2	99	18	18
		3	197	125	63
		4	90	82	91
	100 + 100	1	84	0	0
		2	72	31	43
		3	144	134	93
		4	334	313	93

[a] According to references 62 and 302.

[b] *Euglena gracilis* was incubated for different periods with single virginiamycin components (VM and VS) or their combination.

[c] Cells were washed and plated on agar without antibiotics in the light, and colonies were counted.

Chloroplast Morphogenesis and Ribosome Formation in Algae

Since virginiamycin inhibits protein synthesis by interacting with 70S ribosomes, a kind of particle which is present in chloroplasts, it is at this level that the molecular basis of the bleaching effect was sought for. Indeed, it was found that 70S ribosome formation was blocked in *E. gracilis* grown in the presence of VM. The inhibition was reversible in cells treated with type A components and irreversible when type A and B inhibitors were both present (303). Quite unexpectedly, however, formation of chloroplast rRNA was also found to be blocked by virginiamycin. In fact, the light-dependent synthesis of 16S rRNA did not take place in dark-adapted cells previously incubated with a mixture of A and B components (303).

The unitary hypothesis, which was proposed to explain all the above findings, postulates that the biosynthetic pathways for chlorophyll, RNA, and protein formation are integrated within the chloroplasts. Consequently, the morphogenesis of these organelles is strictly coupled with an active synthesis of proteins, which is the target of virginiamycins. This view is supported by a large body of evidence.

Ebringer (95, 96) has systematically screened in *Euglena* the bleaching property of most of the commercially available antibiotics; his survey included several hundred products (cf. also 41, 234). The conclusion was that a few drugs preventing DNA and protein formation, but none of the RNA inhibitors, were endowed with permanent bleaching capacity. Among the inhibitors of protein synthesis, antibiotics acting on both the 30S subunit (aminoglycosides) and the 50S subunit (lincomycin, oleandomycin, erythromycin, sparsomycin, carbomycin, and streptogramin) were included (Table 8). By culturing *E. gracilis* in the presence of type A and B virginiamycins, the author of this review obtained leucophytes, i.e., permanently bleached algae, with a frequency close to 100% (Table 7) (62). This finding was explained by postulating that the genetic continuity of chloroplasts relies on their structural integrity as a whole. An irreversible inactivation of chloroplast ribosomes would, thus, have an effect comparable to that produced by a damage of the organelle chromosome. Likewise, an alteration of viral coat proteins prevents host infection and virion pro-

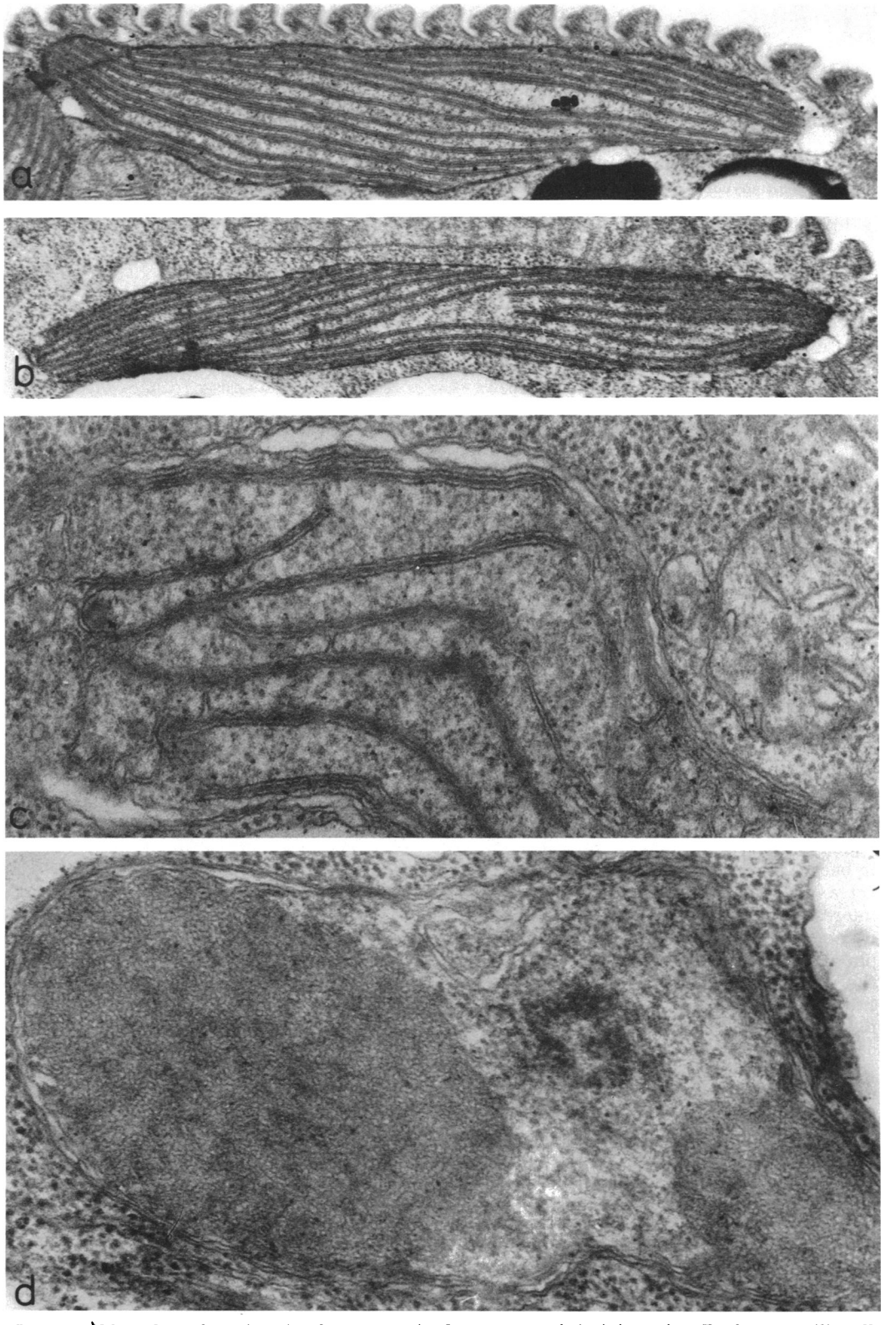


FIG. 19. *Chloroplast alterations in algae grown in the presence of virginiamycins. Euglena gracilis cells were incubated for 4 days in the presence of single virginiamycin components, VM and VS, and their mixture. Electron micrographs were made on ultrathin sections. Virginiamycin: none (a; ×25,000); VS, 100 μg/ml (b; ×20,000); VM, 100 μg/ml (c; ×50,000); VM + VS, 100 μg/ml (d; ×50,000). According to references 62 and 302.*

TABLE 8. *Bleaching capacity of antibiotics which inhibit protein synthesis*[a]

Antibiotic	Killing concn (A) (μg/ml)	Bleaching concn (B) (μg/ml)	Bleaching index (C) [(A:B)/(A − B)]	Relative bleaching power (D) (1/100 C)
30S inhibitors				
Streptomycin	2,000	10	0.100	1
Dihydrostreptomycin	2,000	100	0.011	9.1
Bluensomycin	1,000	100	0.011	0.1
Kanamycin	1,000	200	0.00625	16.0
Spectinomycin	1,000	50	0.0211	14.7
Gentamicin	15	10	0.300	0.3
Kasugamycin	1			
Neomycin	400			
50S inhibitors				
Streptogramin	500	200	0.00833	12.0
Pactamycin	200	150	0.0260	3.8
Angolamycin	2,000	500	0.00267	37.5
Carbomycin	700	100	0.00117	85.5
Erythromycin	5,000	800	0.00149	67.1
Kitasamycin	2,000	700	0.00236	42.4
Oleandomycin	5,000	4,000	0.00125	80.0
Spiramycin	1,200	600	0.00333	30.0
Tylosin	2,000	500	0.00267	37.5
Lincomycin	4,000	1,500	0.00104	96.1
Clindamycin	3,500	300	0.00363	27.5
Chloramphenicol	1,000	—		
Tetracycline	500	—		
Sparsomycin	100	—		
Puromycin	1,000	—		

[a] According to references 95 and 96.

duction just as the inactivating mutation of an essential viral gene does.

Inhibition of Growth and Macromolecule Synthesis in Cyanophyces

Since types A and B virginiamycins are equally effective in inhibiting protein synthesis in bacteria, whereas only type A antibiotics are active on algal chloroplasts, it was of obvious interest to find which situation would apply to cyanophyces. In fact, these protists combine the photosynthetic pathway of eucaryotic algae with the procaryotic organization of bacteria (336). Indeed, it was found that the inhibition pattern displayed by cyanophyces mimicked that of eucaryotic algae rather than that of bacteria. Type B components did not show an inhibitory action per se, but enhanced the effect of type A compounds and rendered it permanent (67). The evolutionary and taxonomic implications of these findings are obvious: they may reveal biochemical events linked to the development of the photosynthetic apparatus in the course of evolution, and also point to possible differences in the structure of ribosomes from procaryotic organisms. Indeed, virginiamycin-like antibiotics apparently are able to discriminate between 70S particles from bacteria and blue-green algae.

Another important difference is that the enhancement by type B components of the inhibition produced by type A antibiotics was far higher in bacteria (100-fold to 1,000-fold increase) than in cyanophyces (2-fold to 10-fold enhancement). The two types of metabolic organization prevailing in bacteria and in blue-green algae were alleged to account for such a difference. In fact, the growth of heterotrophic bacteria relies on a complex network of anaplerotic pathways. The inhibition of such a system is proportional to the antibiotic concentration within wide ranges. By contrast, the photoautotrophic growth of cyanophyces is likely to yield an all-or-none type of response to the inhibitors.

Type A virginiamycins were found to block chlorophyll formation in *Plectonema boryanum*. This effect, which paralleled the inhibition of CO_2 photoassimilation and took place after an appreciable lag, was considered as a consequence of protein inhibition (67). On the other hand, DNA synthesis was found halted without delay, and this effect was claimed responsible for the lethal effect of VM in this filamentous cyanophyces. To account for this finding, it was proposed that the genomes of blue-green algae and bacteria have different structures: the DNA of the former microorganisms is possibly coupled to basic proteins capable of binding inhibitors

which do not have affinity for the naked DNA of bacteria (97, 99, 191, 193, 349, 350). Finally, inhibition of protein synthesis by single virginiamycin components proved reversible, and that produced by a combination of A and B compounds proved irreversible, an effect comparable to that taking place in bacteria.

Action of Virginiamycin on Isolated Plant Chloroplasts

In the previous sections, the evidence was given for a permanent block of proplastid development in protists by combinations of virginiamycin components. Single components caused a transient inhibition of protein synthesis in algal chloroplasts, whereas a permanent effect was produced by their combination. If a similar cooperative effect were obtained with isolated chloroplasts, the latter type of organelle would be an ideal cell-free system for investigating the synergistic action of this group of antibiotics in vitro. Unfortunately, this did not turn out to be the case.

The work was done with isolated spinach chloroplasts incorporating labeled amino acids into proteins through a light-dependent reaction. This system proved insensitive to type B virginiamycins, but was strongly inhibited by type A compounds (Table 9). However, no increase of this inhibitory effect by a combination of A and B type antibiotics was observed. The reason for such a failure is unknown. It is possible that the isolation process entails structural changes of the translational machinery of the organelles. Such alterations are revealed by the loss of the virginiamycin cooperative effect. Two indirect proofs for such inference were found: (i) protein synthesis in isolated organelles is a short-lived process, and (ii) inhibition of peptide bond formation by type A components does not increase beyond a certain limit, even when inhibitor concentration is increased.

TABLE 9. *Inhibition by virginiamycin of the light-dependent protein synthesis in isolated plant chloroplasts*[a]

Experimental conditions	Inhibitors[b]	Radioactivity in protein (cpm/sample)
Dark		5,042
Light		44,112
Light	VM (1 μg/ml)	18,666
Light	VS (10 μg/ml)	35,623
Light	VM + VS (1 μg/ml)	17,489

[a] According to C. Cocito, O. Tiboni, and O. Ciferri (unpublished results).

[b] Inhibitors: virginiamycin components, VM and VS.

In a further section, the inhibition of protein synthesis in organelle-free systems from plant chloroplasts will be mentioned. In agreement with the above conclusions, it will be shown that type A virginiamycins block peptide bond formation. No enhancement by group B compounds has been observed.

Virginiamycin-like antibiotics are expected to have comparable inhibitory action on chloroplasts and mitochondria, since both organelles harbor 70S ribosomes which are the target of these inhibitors. Unexpected was, therefore, the report that mitochondria of *E. gracilis* are unaffected by virginiamycin, presumably because of a permeability barrier at the mitochondrial membrane. The latter situation cannot be extrapolated to other eucaryotic cells of both protist and nonprotist origin, however, as shown by the fact that some strains of yeasts (131, 147) and mammalian cells (88, 89) were claimed to be sensitive to antibiotics of the virginiamycin family. Obviously, further biochemical studies on whole cells and isolated mitochondria are needed to clarify this problem.

MACROMOLECULE METABOLISM IN VIRUS-INFECTED PROCARYOTES IN THE PRESENCE OF VIRGINIAMYCIN-LIKE ANTIBIOTICS

Interference with the Multiplication of Virulent Bacteriophages

No information is available concerning the action of these antibiotics on the replication of single-stranded DNA and RNA phages. The best-known phages of these groups replicate in gram-negative bacteria, which are refractive to these drugs. Conversely, the action of virginiamycin has been studied in *B. subtilis* infected with phage 2C, a virus containing a double-stranded DNA genome, in which thymine is replaced by the unusual base hydroxymethyluracil (196, 232). The DNA of phage 2C is replicated discontinuously and semiconservatively (141), presumably by a virus-specific polymerase which is fully active in permeabilized cell systems (137). Okazaki fragments are then joined by a virus-specific ligase (138), and progeny DNA molecules within the vegetative pool undergo extensive genetic recombination (139, 140). All of these steps of viral DNA replication rely on the synthesis of enzymes coded for by the phage genome (cf. e.g., 232 and 233 for review); hence, the entire process is highly sensitive to protein synthesis inhibitors.

Virginiamycins were found to interfere with

the lysis of the host cell and the release of newly formed particles. The overall effect was different, depending on the dose of the antibiotic and the time of addition. When cells were infected with phage 2C in the presence of high concentrations of single components of either type, the growth of the host cells was progressively reduced, but no lysis occurred. Lower doses of these antibiotics, though unable to prevent the lysis of infected cells, reduced the yield of viable particles. Very small amounts of a mixture of type A and B components were required, however, to block irreversibly the formation of virions and the lysis of the host cell (Fig. 20). Addition of single virginiamycin components at the end of the eclipse phase produced an acceleration of the replication cycle and an anticipation of the lysis. Yet, the combination of A and B components still prevented the lysis of the host when added during the maturation phase (54).

A study of the effect of virginiamycin on the yield of phage 2C virions offers still another example of the synergistic action of the components of this antibiotic. Indeed, a quite limited reduction of the number of particles was obtained after short incubation with single components, when the lytic cycle was allowed to go to completion in the absence of inhibitors. No virion crop was produced upon exposure of infected cells to a mixture of type A and B compounds for a 10-min period during the eclipse and the beginning of the maturation phase. The dose producing such irreversible effect was 1,000-fold smaller than that required for blocking the viral cycle with single components (54, 138).

Formation of virus 2C DNA starts at mid-eclipse phase and continues linearly until lysis occurs. This synthesis was blocked completely when single virginiamycins and their combination were added to the culture at the moment of infection. However, when addition was made during the second part of the eclipse phase and the maturation period, formation of 2C DNA was merely reduced. The extent of inhibition was inversely related to the length of the interval elapsed between the infection and the antibiotic treatment (138). These observations are similar to those reported for phage T2 DNA synthesis in *E. coli* B and can be explained in a similar way. Viral genome is made by a phage-specific polymerase, the formation of which takes place during the early eclipse phase and is blocked by a precocious addition of inhibitors. When the enzyme is allowed to accumulate, the incubation of the host with protein synthesis inhibitors is ineffective (290). Moreover, virginiamycin inhibits recombination of phage 2C DNA (139), just as chloramphenicol does in the T2-*E. coli* system (35, 173).

Virginiamycins not only interfere with viral DNA formation, but also alter RNA metabolism in phage-infected bacteria. It is worth remembering that formation of cellular RNA in *E. coli* is halted soon after the attachment of T-even phages and ghosts. rRNA, tRNA, and mRNA are equally affected in this system, which is

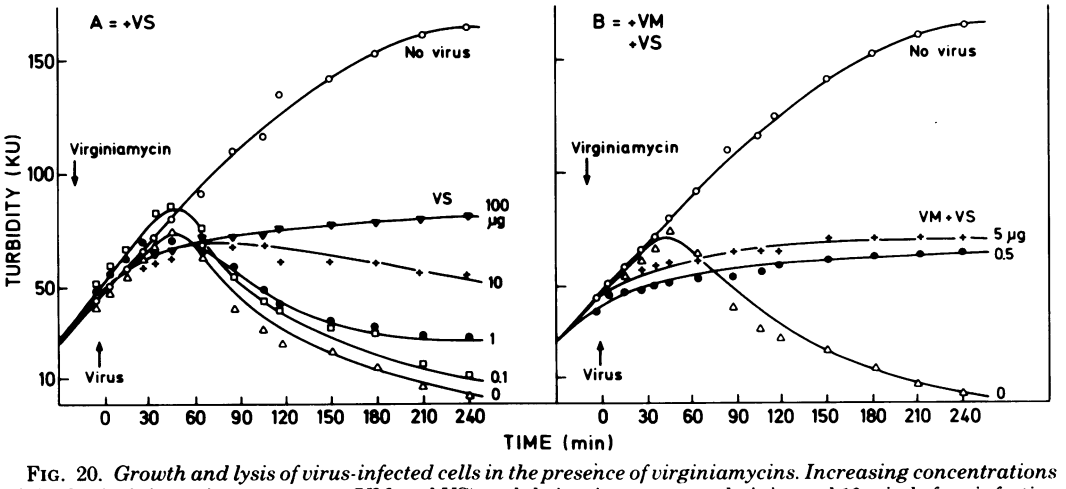


FIG. 20. *Growth and lysis of virus-infected cells in the presence of virginiamycins. Increasing concentrations of single virginiamycin components (VM and VS) and their mixture were administered 10 min before infection (10 plaque-forming units of phage 2C per cell). Growth and lysis of the host (B. subtilis) were followed turbidimetrically. (A) 0.1 to 100 µg of VS per ml (results with VM were similar to those with VS); (B) 0.5 µg of VM + VS per ml. According to reference 54.*

taken as the model of virion-host interaction (217, 218). In reality, such a metabolic pattern is quite unusual, since in most virus-host systems the synthesis of cellular and viral nucleic acids continues side by side, at least during the eclipse phase. This is the case, for example, of 2C-infected *B. subtilis*, where rRNA formation and ribosome assembly were found to take place at a reduced rate during the entire latent period. The extent of rRNA inhibition was a function of the multiplicity of infection, i.e., the number of copies of viral genome competing with the cell genome for transcription (58, 60). Since rRNA is methylated, and virginiamycins are known to interfere with the process of RNA methylation (53), it was of obvious interest to explore the action of these antibiotics in 2C-infected cells. This study showed that viral infection did not reduce, and in many instances increased, the rRNA methylation process. On the other hand, virginiamycin sharply inhibited this process during the entire replication cycle. The degree of repression of RNA methylation was inversely related to the multiplicity of infection, thus indicating a pronounced antagonistic effect of the antibiotic and the virus. To account for these findings, it has been proposed that rRNA methylation requires an active protein synthesis, a process which is not affected by virus development, but that is blocked by virginiamycin-like antibiotics.

Virginiamycins also interfere with the metabolism of viral mRNA. As a matter of fact, the genome of virus 2C undergoes an asymmetrical transcription, whereby the two DNA strands are copied with unequal efficiencies during the viral cycle. This means that the RNA polymerase transcribes more genes from the H strand in one direction than it does from the L strand in the opposite direction. Virginiamycin alters the regulatory mechanism underlying such asymmetrical transcription, as shown by a more even distribution of pulsed RNA hybridizing with the two strands of viral DNA (58). It is proposed that the programmed sequential expression of viral genes is the result of a cascade-type of regulation, whereby each cistron is repressed by its own product. The latter derepresses, instead, the next gene of the metabolic pathway. Conceivably, virginiamycins block the translation of the viral message and, thus, interfere with the entire sequence of biochemical reactions leading to virion production.

Alteration of the Lytic Cycle of a Temperate Cyanovirus

Cyanophages, a recently discovered group of viruses which multiply in cyanophyces, include virulent and temperature species. Cyanophages so far isolated have double-stranded linear DNA genomes. Their replication cycle has unique features, due to the photoautotrophic nature of their hosts, blue-green algae. In fact, the production of virulent cyanophages drastically impairs the photosynthetic function of the host, as shown by the invagination of the photosynthetic lamellae and the complete halt of CO_2 photoassimilation during the eclipse phase. Conversely, a very limited number of viral particles are produced under conditions preventing photosynthesis (particle yield is reduced to one-tenth in the dark, and to a few hundredths after block of photosystem II) (cf. 28 and 228 for review).

Interference of virginiamycins with cyanophage replication is best known in the case of the LPP group of viruses, which have as common hosts the filamentous cyanophyces *Lyngbya*, *Phormidium*, and *Plectonema*. The LPP group includes, among others, the virulent LPP1 and the temperate LPP2 phages (260, 261). The latter lysogenizes *P. boryanum* and produces a lysogenic immunity similar to that by phage λ in *E. coli* (34, 98, 229). The action of protein synthesis inhibitors was mainly explored on the induction process of LPP2 lysogens. For this purpose, *Plectonema* carrying a temperature-sensitive mutation of the LPP2 prophage repressor was used. Such mutants were lytically induced by exposure to light and heating to nonpermissive temperature; the two events were simultaneously needed for successful induction (251). An additional requirement, protein synthesis, proved essential for the onset of a lytic cycle. In fact, when the translation of the induction message was prevented, no virion was produced (61, 65). No plausible explanation has thus far been found for such a multiple requirement for illumination, repressor inactivation, and peptide-bond formation.

Virginiamycins were found to interfere in the following way with the LPP2 induction process. Type B inhibitors did not prevent the induction nor subsequent replication. Type A compounds prevented the lytic induction, but produced a reversible halt of lytic cycle progression (Fig. 21). A combination of type A and B factors produced an irreversible block of both processes. Note that cyanophyces, in which induction was blocked by type A components, multiplied normally in the absence of the drug and retained the capacity of being subsequently induced. On the other hand, irreversible halt of the lytic cycle by a mixture of type A and B inhibitors was accompanied by viability loss of lysogens (65).

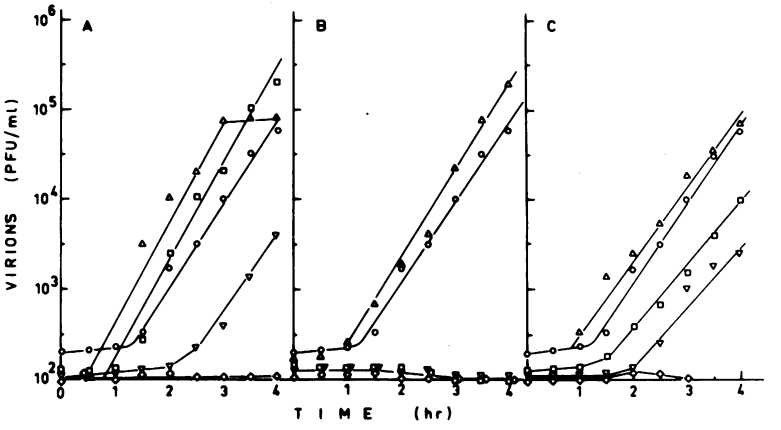


FIG. 21. *Inhibition of lytic induction in lysogenic cyanophyces by virginiamycins. Cultures of lysogenic Plectonema boryanum PI(CtsI) carrying a temperature-sensitive mutation of the prophage repressor were induced (40°C, light, 30 min) in the absence and in the presence of the virginiamycin components VM and VS. Virions produced in antibiotic-free medium were counted. Virginiamycin: none (○); VS, 100 μg/ml (△); VM, 100 μg/ml (▽); VM + VS, 50 μg/ml (◇). Chloramphenicol, 100 μg/ml (□), was used for comparison. A 30-min contact with the inhibitors was allowed: before (A), during (B), and after (C) induction. According to references 65 and 61.*

ACTION OF VIRGINIAMYCIN-LIKE ANTIBIOTICS IN CELL-FREE SYSTEMS FROM BACTERIA AND IN SUBORGANELLAR SYSTEMS FROM EUCARYOTES

Inhibition of Protein Synthesis in Cell-Free Systems from Bacteria and in Lysates of Cytoplasm Organelles

Group A virginiamycins proved very powerful inhibitors of polyphenylalanine formation directed by polyuridylic acid in cell-free systems from *E. coli* (66, 104, 181, 241, 309, 314, 342–344). This reaction was completely blocked by drug concentrations as small as 0.1 μg/ml, one of the most striking inhibitions observed with this system. Type A components have comparable activities with all synthetic messengers (282, 343), but apparently produce lesser inhibition in bacterial systems for protein synthesis directed by natural messenger (240). The latter report agrees with the observations that these antibiotics do not inhibit peptide bond formation by native polysomes in vitro (239, 240) and do not bind to isolated bacterial polysomes (71, 75).

On the other hand, the inhibitory action of group B components on cell-free systems for protein synthesis is still controversial. Thus, for example, vernamycin B (105, 181), ostreogrycin B (309), and mikamycin B (343) were found capable of inhibiting the polyadenylic acid-directed polylysine formation in *E. coli* cytoplasm. However, several authors have failed to show a block of polyuridylic acid-directed polyphenylalanine formation (66, 239), and others have occasionally observed a reduced effect with this system (M. P. de Béthune and K. H. Nierhaus, unpublished experiments). Differences in the preparation and functional tests of ribosomes may account for several conflicting results. Also, discrepancies in literature data can partly be explained by the work of Yamaguchi and Tanaka (343), in which mikamycin B is shown to be ineffective in the polyuridylic acid system but fully inhibitory in the polyadenylic acid system of *E. coli* (Fig. 22).

VM (group A compound) also proved capable of preventing polyuridylic acid-directed polyphenylalanine formation in a suborganellar system from spinach chloroplasts (Table 10). In this system, group B compounds did not display any evident inhibitory activity.

A synergistic inhibitory effect of A and B components on protein synthesis in cell-free systems has been claimed by some authors (104, 111, 282). However, no such synergism was found by others, using the Nirenberg systems for peptide-bond formation (66, 314, 315). Similar failure was met with chloroplast homogenates (Table 10). Indeed, the absence of synergistic effects in all these cases is not surprising, the systems used being insensitive to group B components.

Interference with Initiation and Elongation of Peptide Chains In Vitro

Virginiamycin-like antibiotics block protein synthesis by interacting with the 50S ribosomal subunits. The list of other antibiotics known to interact with bacterial ribosomes is reported in Table 11. Reference will be made to this list, as

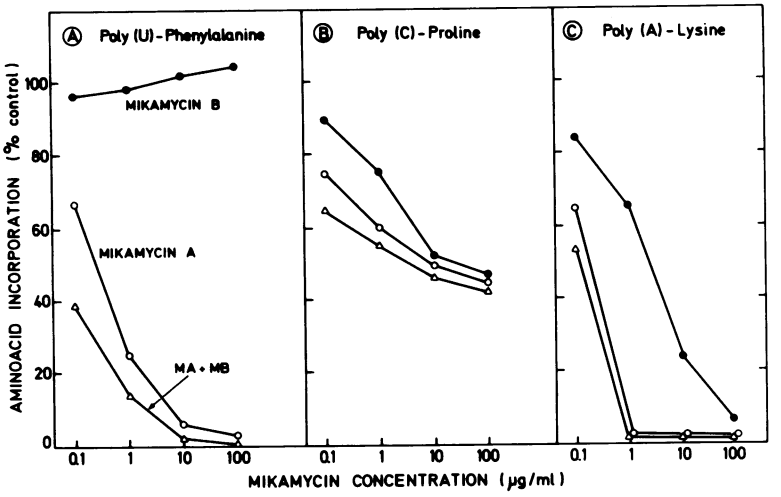


FIG. 22. *Inhibitory action of mikamycins on peptide bond formation directed by different polynucleotides in cell-free systems. Incorporation of [^{14}C]phenylalanine (A), [^{14}C]proline (B), and [^{14}C]lysine (C) directed by polyuridylic acid (A), polycytidylic acid (B), and polyadenylic acid (C), respectively, in a cell-free E. coli system was measured in the presence of increasing concentrations of mikamycin A (○), mikamycin B (●), and their mixture (△). According to reference 344.*

TABLE 10. *Inhibition by virginiamycin of protein formation in extracts of plant chloroplasts*[a]

Samples	Inhibitors[b]	Radioactivity in protein (cpm/sample)
Blank		334
Complete system		6,270
Complete system	VM (0.1 μg/ml)	1,229
Complete system	VS (1 μg/ml)	5,979
Complete system	VM + VS (0.1 μg/ml)	844

[a] From C. Cocito, O. Tiboni, and O. Ciferri (unpublished results).

[b] Virginiamycin components VM and VS.

well as to some review articles on the subject (27, 161, 162, 183, 186, 188, 190, 219, 239, 243, 246, 274, 314, 316, and 331), in the discussion that follows. Also, the initiation and elongation reactions which are the possible targets of the inhibitory activities of these antibiotics are reported in the schematic drawing of the protein biosynthetic pathway (see Fig. 30).

Initiation includes the reactions preceding the formation of the first peptide bond (see Fig. 30, steps II, III, and IV), namely: (i) fixation of initiation factors and mRNA to the 30S subunit, (ii) binding of formylmethionyl (fMet)-transfer RNA_{fMet} (fMet-$tRNA_{fMet}$), and (iii) association of the 50S subunit and recycling of the initiation factors. All of these steps were analyzed by myself and found unaffected by VM, whether free subunits or ribosomes were used (68). Figure 23 and Table 12 show, for example, that formation of the 40S initiation complex containing 30S subunits, MS2-RNA, and fMet-tRNA is not affected by VM. Likewise, addition of the 50S subunit to the previous complex and assembly of the 75S initiation particles is not prevented by the antibiotic (Fig. 24). Conversely, the nonenzymatic binding of fMet-tRNA to ribosomes and 50S subunits was found to be inhibited by vernamycin A (112), whereas still another report claimed the formation of the 40S complex (mRNA-30S subunit-fMet-tRNA) to be insensitive and that of the 75S initiation complex (mRNA-70S ribosomes-fMet-tRNA) to be sensitive to mikamycin A (282, 343). Such discrepancies may be accounted for by differences in the experimental systems used.

Elongation of peptide chains includes the following: (i) the guanosine 5'-triphosphate (GTP)-dependent elongation factor Tu (EFTu)-directed binding of aminoacyl-tRNA to the A site of the mRNA-70S complex; (ii) the peptidyltransferase-dependent peptide-bond formation between fMet on the P site and the next amino acid on the A site; and (iii) the GTP-dependent elongation factor G-directed translocation of peptidyl-tRNA from the A to the P site (reactions leading to stages V, VI, and VIII in Fig. 30). The first two steps were found to be inhibited by group A virginiamycins (Tables 14 and 15), whereas the third step apparently was unaffected (68, 182, 236-242, 245, 313, 317). In more detail, the enzymatic binding of phenylal-

TABLE 11. *Ribosomal subunits—specificity of some inhibitors of protein synthesis*[a]

Supernatant	30S subunit	50S subunit
A. Kirromycin (folic acid antagonists)	Aminoglycosides[b]	Althiomycin
	Colicins	Bottromycin
	Edeins	Chloramphenicol group[c]
	Negamycin	Lincosamides[d]
		Macrolides[e]
		Micrococcin
		Pleuromutilin
		Virginiamycins A
		Virginiamycins B
		Thiostrepton group[f]
B. Fusidic acid (GTP analogs)	Aurintricarboxylic acid	Amicetin
	Pactamycin	Blasticidin
		Bamicetin
		Gougerotin group[g]
		Plicacetin
		Puromycin
		Sparsomycin
		Tetracyclines[h]

[a] According to references 241, 243, 244, 314, and 316. A, Inhibitors of procaryotes; B, inhibitors of both procaryotes and eucaryotes.

[b] Bluensomycin, dihydrostreptomycin, gentamicin, hygromycin, kanamycin, kasugamycin, neomycin, paramomycin, spectinomycin, and streptomycin.

[c] Chloramycetin, D-adenosine 5'-monophosphate-3,*d*-thiomycetin, and D-Win 5094.

[d] Celesticetin, clindamycin, and lincomycin.

[e] Angolamycin, carbomycin, chalcomycin, erythromycin, forocidin, lancamycin, leucomycin, methymycin, neospiramycin, niddamycin, oleandomycin, spiramycin and tylosin.

[f] Althiomycin, multhiomycin, scomycin, sporangiomycin, thiopeptin, and thiostrepton (bryamycin).

[g] Bamicetin, gougerotin, and plicamicetin.

[h] Chlortetracycline, doxycycline, oxytetracycline, and tetracycline.

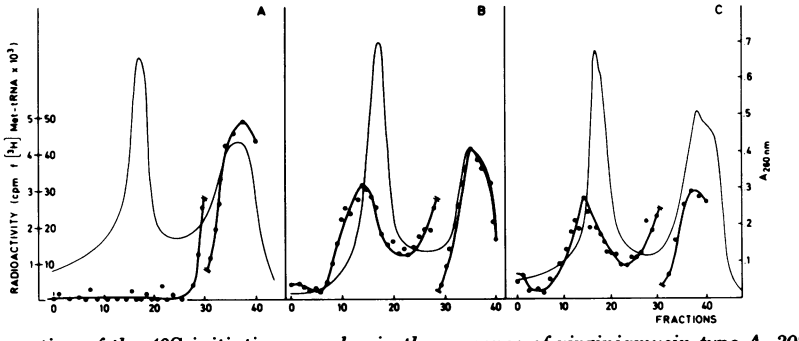


FIG. 23. *Formation of the 40S initiation complex in the presence of virginiamycin type A. 30S ribosomal subunits of E. coli (one absorbance unit at 260 nm [1 A_{260} unit]) and mRNA (0.4 A_{260} unit MS2-RNA) were incubated with f[^{3}H]Met-tRNA (0.4 A_{260} unit) in the presence and in the absence of VM (100 μg of VM/ml), and of optimum concentration of IF1, IF2, IF3, GTP, and inorganic ions. After fixation, ribosomes were fractionated by density gradient centrifugation, and the A_{260} (solid line) and radioactivity (●) of the fractions were recorded. (A) Control without mRNA; (B) control with mRNA; (C) sample with VM. According to reference 68.*

anyl-tRNA at 6 mM Mg^{2+} was completely blocked by VM, whereas the nonenzymatic binding at 12 mM Mg^{2+} was 50% inhibited (cf. Tables 13 and 14). Accordingly, site A of the elongation complex (the one involved at low Mg concentration) was indicated as the target of the antibiotic. Indeed, the EFTu-directed binding of alanyl-tRNA to a 75S initiation complex was prevented by VM (68).

VM proved capable not only of preventing the binding of alanyl-tRNA to the elongation complexes, but also of promoting its detachment (68). No such ejection takes place, however, in the case of fMet-tRNA, a phenomenon which

TABLE 12. *Action of type A virginiamycin on the binding of fMet-tRNA to ribosomes and subunits*[a, b]

Ribosomes and subunits	VM (100 μg/ml)	f[^{3}H]Met-tRNA (pmol of ribosome-bound tRNA)
30S	−	1.39
	+	1.29
30S + 50S	−	4.57
	+	3.43
70S	−	3.89
	+	3.44

[a] Initiation complexes were formed upon incubation of mRNA, f[^{3}H]Met-tRNA, either 70S ribosomes or comparable amounts of 30S and 50S subunits, and optimum concentrations of IF1, IF2, IF3, GTP, and inorganic ions, in the presence and in the absence of virginiamycin M. Radioactivity of complexes was measured.

[b] According to reference 68.

shall be discussed in the last section of this chapter.

Interference of group A virginiamycins with peptide bond formation was shown in different ways. First of all, fMet and phenylalanine, which were positioned at the P site of ribosome in the presence of GTP, did not react with puromycin in the presence of VM (Fig. 24) (66, 68). Likewise, peptide bond formation between either acetylphenylalanyl-tRNA or polylysyl-tRNA positioned at the P site of ribosomes and puromycin was blocked by vernamycin A (38, 39, 117, 118, 133, 237, 243, 272). Moreover, formation of the dipeptide fMet-alanine directed by MS2-RNA on 70S ribosomes was blocked by VM (Table 15). Finally, the "fragment reaction," in which amino acids attached to the terminal pentanucleotide of tRNA were made to react with puromycin in the presence of 50S ribosomal subunits, was found to be inhibited by streptogramin A (Fig. 25) (204–208). The latter finding furnishes additional evidence for a reaction of group A virginiamycin-like antibiotics with the large subunit and rules out possible involvement of the small subunit.

An interference of group A components with both the EFTu and the peptidyl-transferase reactions does not necessarily imply a binding of these inhibitors to different sites of the 50S subunit. The attachment of certain inhibitors and analogs (for example, acetyl-aminoacyl-tRNA) to the A site not only produces a steric hindrance effect on P site functions, but also peptidyl-tRNA is prevented from reacting with the aminoacyl-tRNA on the A site by the presence of EFTu within the translation complexes. A well documented case of this sort is that of kirromycin, an antibiotic which binds to EFTu. The EFTu-kirromycin complex is still able to catalyze the GTP-dependent attachment of aminoacyl-tRNA to ribosomes, but is unable to leave the 50S subunit as EFTu-guanosine 5′-diphosphate. Under those conditions, the interaction between the peptidyl radical on the P site and the aminoacyl-tRNA on the A site does not take place; hence, the inhibition of peptide bond formation results without direct interference with the peptidyl-transferase center (51, 334, 335).

On the other hand, no firm evidence was given for an interference of group B components with the peptidyl-transferase reaction. Accordingly, Munro and Vazquez (208) found no inhibitory activity on the fragment reaction catalyzed by 50S subunits. In conclusion, it can be said that all of the steps involved in the initiation and the elongation of peptide chains directed by natural messengers in *E. coli* cell-free systems have been explored; none of them was found to be clearly blocked by antibiotics of group B. It can be inferred, therefore, that no in vitro functional test for these inhibitors is available at the present time.

Binding of Type A Virginiamycins to Bacterial Ribosomes In Vitro

A study of antibiotic fixation to ribosomes has provided essential information concerning the targets of different inhibitors, the mechanisms of antibiotic resistance, the enzymatic functions of ribosomes, and the topography of catalytic centers on subunit surface (46, 124, 174–177, 264, 265, 279, 281).

Binding of group A components to ribosomes was shown by different techniques: retention on micropore filters (108), gel chromatography (66), and sedimentation from buffers with high ethanol content (72). Very recently, a new method for evaluation of ribosome-bound VM was described. This is based on the preferential adsorption of the free drug to Norite A, which can be sedimented by low-speed centrifugation, leaving in the supernatant the ribosome-bound fraction. The binding of the drug to both 50S subunits and 70S monosomes was confirmed (Fig. 26); the small subunits fixed negligible amounts of this antibiotic. The environmental factors involved in the antibiotic binding have been explored. The reaction was found to be strictly dependent on inorganic ions below a limiting concentration (1 mM Mg^{2+} and 100 mM K^+ or NH_4^+), but above this level showed little modification within quite broad limits (Fig. 27) (63, 108). The reaction was relatively slow, as compared to that of group B compounds, and temperature dependent (63). From the Scatchard plot, an association constant of 3.2×10^5 M^{-1} and a $\bar{\nu}$ value of 0.85 were computed for VM (Fig. 28A). The former value, which is different

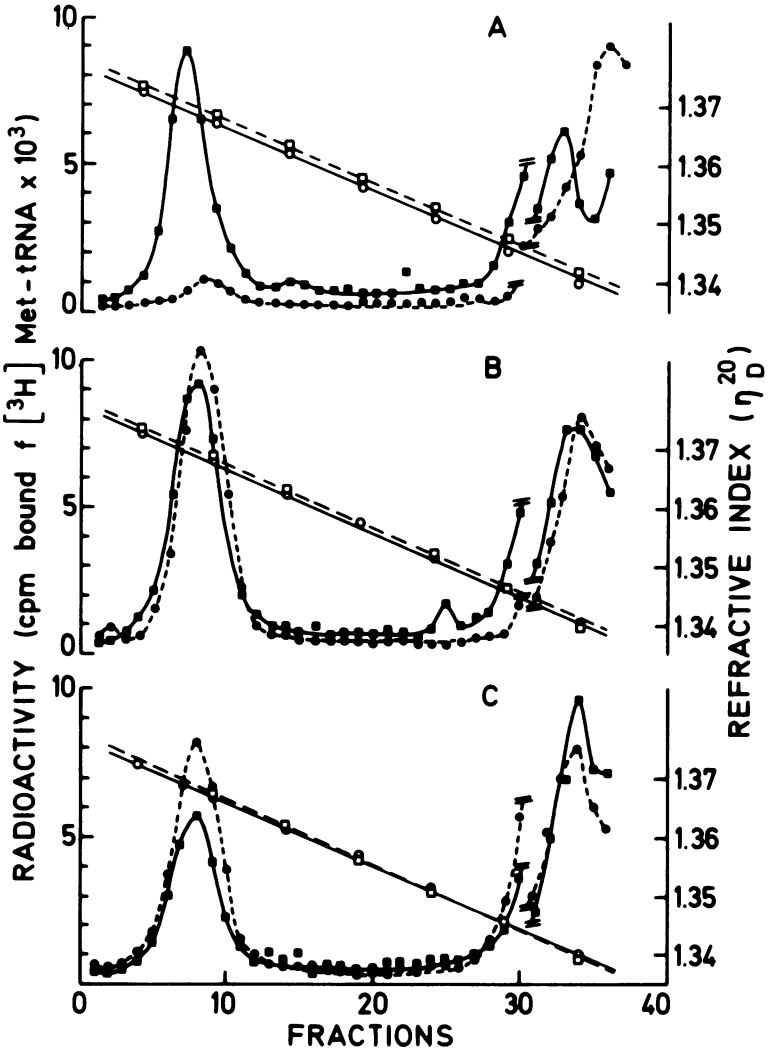


FIG. 24. *Inhibition of peptidyl-puromycin formation by type A virginiamycin. The following reactions were carried out sequentially: (i) 40S initiation complex formation (from 30S subunits, MS2-RNA, f[³H]Met-tRNA, IF1, IF2, IF3, and GTP); (ii) assembly of 75S complex from 30S and 50S subunits, in the presence and in the absence of VM (200 μg of VM/ml); (iii) reaction with puromycin of one aliquot of each sample; (iv) density gradient fractionation of samples ± puromycin, and radioactivity measurement of fractions. (A) − VM ± puromycin; (B) + VM ± puromycin. In section C, step (i) was carried out with the nonhydrolyzable analog Gua-5'-P-P-CH₂-P replacing GTP. Symbols: − puromycin (■); + puromycin (●). According to reference 68.*

from that obtained with the filter retention technique (108), accounts for the relative lability of their complex with ribosomes. The latter value points to a monomolecular association of these drugs with ribonucleoprotein particles. An important finding, the implication of which shall be discussed later, is that the association constant remained unchanged in the presence of group B virginiamycins (63).

Competition for binding to ribosomes is taken as an indication that two antibiotics have partly overlapping fixation sites. When the ability of different protein synthesis inhibitors to prevent the binding of [³H]vernamycin A was analyzed by different methods, however, discrepancies were sometimes reported. Thus, according to the filter retention methods, erythromycin, spiramycin, leucomycin, macrocin, tylosin, and carbomycin were good competitors, whereas no inhibition of binding was afforded by antibiotics of the chloramphenicol, macrolide (oleandomycin), lincomycin, and type B virginiamycins

TABLE 13. *Action of type A virginiamycin on the nonenzymatic binding of aminoacyl-tRNA to ribosomes at different Mg^{2+} concentrations*[a, b]

VM (μg/ml)	Mg^{2+} concentration (mM)	Sp act of complex (cpm per 100 μg of ribosome)
1.	15	1,671
0.5	15	1,031
2.	5	669
0.5	5	693

[a] Ternary complex was formed by incubating at 24°C for 20 min polyuridylic acid, ribosomes, and [^{14}C]phenylalanyl-tRNA, in the presence and in the absence of VM. Ribosome-bound aminoacyl-tRNA was measured.

[b] According to reference 66.

TABLE 14. *Action of type A virginiamycins on the EFTu-dependent binding of alanyl-tRNA to initiation complexes*[a] *containing fMet-tRNA*[b]

EFTu	VM (μg/ml)	Ribosome-bound alanyl-tRNA (pmol of bound tRNA per sample)
+		2.528
−		1.823
+	200	0.395
+	20	0.439

[a] Initiation complexes containing mRNA, fMet-tRNA, and 70S ribosomes were made as in the legends to Table 14 and Fig. 24, and incubated with EFTu, [^{3}H]alanyl-tRNA, and GTP, in the presence and in the absence of VM. Ribosome-bound radioactivity was measured.

[b] According to reference 68.

(108). By use of the Norite technique, some competition for the binding of VM (group A component) was observed with chloramphenicol, oleandomycin, and erythromycin, but not with VS (group B components) (63). In the latter work it was also pointed out that rough competition data are of little value, if the association constants of competing antibiotics are not taken into account. The correspondent K_a values are, in fact, 7.2×10^7, 2.5×10^6, 0.32×10^6, and 0.21×10^6 M^{-1} for erythromycin, VS, VM, and chloramphenicol, respectively. This means that, although crude data suggested a higher competition effect by erythromycin than by chloramphenicol, the reverse conclusion stems from the K_a values. Such inference agrees with previous reports that fixation of [^{14}C]chloramphenicol to 50S subunits is prevented by streptogramin A (237, 242, 306–308, 310, 311).

Type A components were found to bind either to free ribosomal subunits or to run-off ribosomes. By contrast, reduced amounts of these drugs are fixed by polypeptide-free polyribosomes, and still less are fixed by native polysomes (71). Accordingly, these inhibitors do not prevent peptide bond formation by polysomes carrying endogeneous mRNA strands (240, 242, 244).

TABLE 15. *Puromycin reactivity of f[^{3}H]Met-tRNA within initiation complexes made in the presence of type A virginiamycins*[a]

MS2-RNA[b]	VM	f[^{3}H]Met-tRNA (pmol/sample)		
		Ribosome fraction[c]		Ethyl acetate fraction[d] + puromycin
		− Puromycin	+ Puromycin	
−	−	1.520	1.001	3.505
+	−	5.149	1.573	6.750
+	+	3.831	5.170	0.426

[a] According to reference 68.

[b] Complexes were formed upon incubation (37°C, 10 min) of mRNA, f[^{3}H]Met-tRNA, 70S ribosomes, and optimum concentrations of IF1, IF2, IF3, GTP, and inorganic ions, in the presence and in the absence of VM (200 μg/ml).

[c] Aliquots of complexes were allowed to react with puromycin.

[d] Ribosome-bound and unbound radioactivity was measured.

Fixation of Type B Virginiamycins to Ribosomal Subunits and Components

The binding of type B components to ribosomes has been demonstrated by different methods, i.e., isolation of the complex by exclusion chromatography (66), retention of the complex on membrane filters (Millipore Corp.) (111), centrifugal sedimentation of particles (55), and equilibrium dialysis (78). Since these procedures, except the last, are susceptible to alter the association $\rightleftarrows$ dissociation equilibrium, a technique for direct measurement of the antibiotic-ribosome complex has been recently developed (230). VS is fluorescent in solution, and the fluorescence intensity increases upon addition of 50S ribosomal subunits. Such variation (ΔI_{416nm}) is proportional to the particle concentration. This technique has been used to confirm the binding of VS to both 50S subunits and 70S monosomes (negligible amounts of the drug are fixed by the small subunits). In addition, it has allowed the association constant of VS and the 1:1 stoichiometry of the antibiotic-ribosome reaction to be assessed (Fig. 28B).

The K_a value determined by spectrofluorometry (2.5×10^6 M^{-1}, according to Fig. 28) agrees with the values previously established with other methods, i.e., equilibrium dialysis (78) and sedimentation of the complex (71). Note that

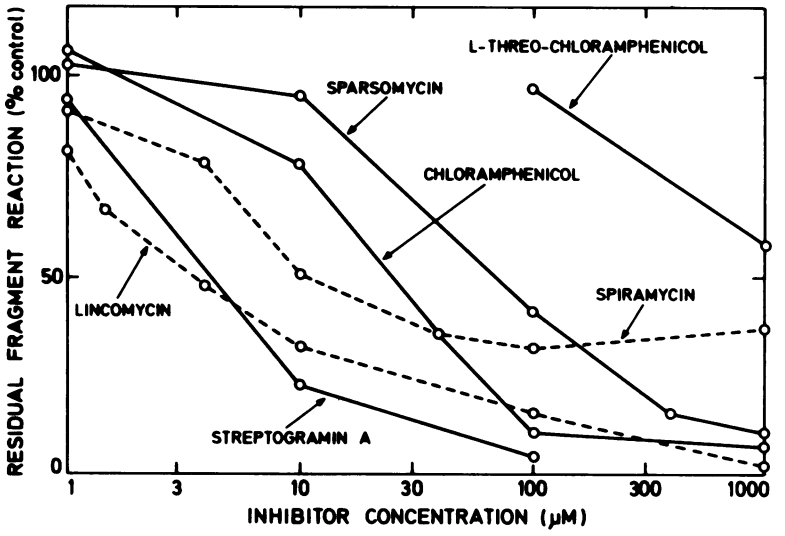


FIG. 25. *Inhibition of the "fragment reaction" by group A streptogramin and other 50S inhibitors. E. coli ribosomes (1 mg) were incubated with [^{35}S]FM-T1 fragment (3.7 × 10^4 dpm/8 nmol of formylmethionyl-CAACCA oligonucleotide) and puromycin (1 mM), in the presence of different antibiotics, as indicated. The amount of labeled fMet-puromycin formed was measured. According to reference 208.*

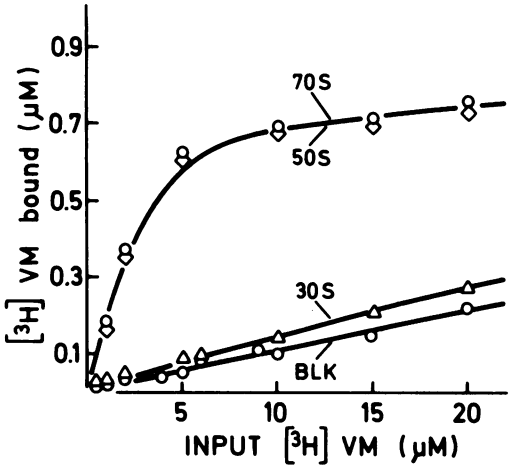


FIG. 26. *Binding of VM to ribosomes and subunits. Samples of 70S (○), 50S (◇), and 30S (△) particles (160 pmol in 200 μl of buffer) were incubated at 37°C for 20 min with increasing concentrations of [^{3}H]VM, and bound radioactivity was determined. Blank (○), no ribosomal particles. According to reference 63.*

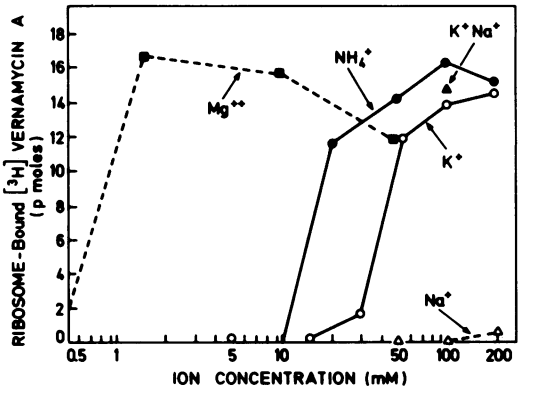


FIG. 27. *Influence of ion concentration on the binding of type A vernamycin to ribosomes. E. coli ribosomes (15 pmol) and [^{3}H]vernamycin A (2 × 10^{-7} M) were incubated at 37°C in tris(hydroxymethyl)-aminomethane buffers containing different concentrations of Mg acetate, KCl, NaCl, and NH$_4$Cl. Ribosome-bound radioactivity was measured by the filter retention technique. Sample K$^+$ Na$^+$ had 100 mM K$^+$ in addition to the Na$^+$ ions. According to reference 108.*

the K_a value of group A compounds is about one-eighth that of group B, and that, although the former type of antibiotics increases the affinity of ribosomes for the latter type, the reverse is untrue. Very interesting is the observation that the ΔI of the VS-ribosome complex increases upon addition of VM (group A component) to the reaction mixture. The corresponding K_a values are 2.5 × 10^6 M^{-1} in the absence of VM and 15 × 10^6 M^{-1} in its presence (Fig. 29). Thus, the attachment of VM to ribosomes increases their affinity for VS sixfold. This accounts for the synergistic effect of group A and B components in vivo. Related to this phenomenon is the "ethanol effect" described by Con-

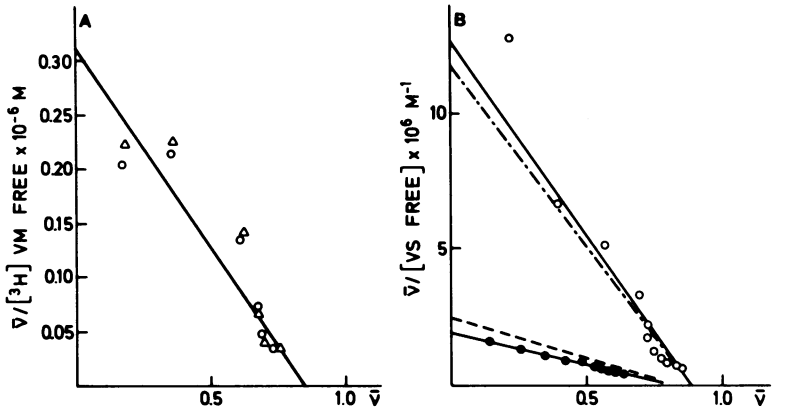


FIG. 28. *Scatchard plots of the binding reactions of virginiamycin components to ribosomes. (A) VM (reference 63); and (B) VS (reference 230). The two groups of plots in (B) correspond to measurements of VS binding in the presence of different amounts of VM—none (●), 0.2 μm (---), 0.8 μM (-·-), and 1.6 μM (○). $\bar{\nu}$ = moles of bound antibiotics per mole of 50S ribosomal subunits present.*

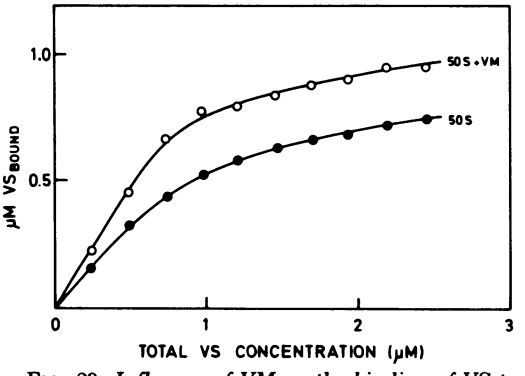


FIG. 29. *Influence of VM on the binding of VS to ribosome. 50S ribosomal subunits of E. coli (1.1 μM) were incubated for 20 min at 37°C with (○) or without (●) VM (1.6 μM), and then for 1 min with different amounts of VS. ΔI was measured and converted into micromoles of VM bound. According to reference 230.*

treras and Vazquez (71), whereby the binding of vernamycin B to 50S subunits is progressively inhibited by increasing concentrations of ethanol, and the adverse effect of this solvent is counteracted by vernamycin A. Although the molecular mechanisms of the fluorescence enhancing and ethanol reversal effects are unknown, both of them point to a conformational change induced by type A components, a change which facilitates the fixation of type B compounds.

By using the equilibrium dialysis technique, strong inhibitions of VS (^{3}H-labeled VS) binding to ribosomes by erythromycin and of the [^{14}C]-erythromycin binding by VS were shown (78), whereas chloramphenicol, puromycin, and tetracycline did not interfere with VS attachment (78, 111).

An attempt to identify the VS-binding protein was made by use of the dissociation-reconstitution technique (Nomura and Held [216]). Groups of ribosomal proteins were detached from 50S subunits, upon incubation with salt solutions of increasing concentrations, and the cores were tested for their ability to bind the antibiotic and to catalyze peptide bond formation. The 0.8 M LiCl cores lost these capacities upon treatment with 1.3 M LiCl, and regained it when 1.3 M LiCl-split proteins were added back to the system. The latter fraction contained proteins L_1, L_5, L_8, L_9, L_{16}, and L_{25}, and the L_{16} component proved essential for fixations of the antibiotic (Table 16). Although this result does not prove that L_{16} is the VS-binding protein, it demonstrates its stringent requirement for the drug attachment (78).

Molecular Mechanism of Virginiamycin-Like Antibiotics

Although an inhibitory activity of group A components on initiation was claimed, most reports point to an interference of these inhibitors with elongation. More precisely, the metabolic block caused by these inhibitors seems clearly situated between stages IV and VI of the protein synthesis schema in Fig. 30. As a matter of fact, two effects of type A virginiamycins have been well demonstrated in cell-free systems: inhibition of aminoacyl-tRNA binding and of peptide bond formation. Since the binding of these antibiotics to ribosomes is definitely a monomolecular reaction (63, 71, 230), it is unlikely that more than one target site is present in the 50S subunit (Fig. 28). A possible interference with the A site is likely to affect P site functions more

TABLE 16. *Binding of type B virginiamycins to reconstituted ribosomes*[a]

Ribosomal particle or subparticle (composition)[b]	Polyuridylic acid-directed polyphenylalanine formation (% of control)[c]	^{3}H-labeled vs binding (% of input)[d]
1. 50S subunit	100	100
2. 0.8 core		31
3. 1.3 core	2	2
4. 1.3 core + 1.3 split proteins	39	24
5. 1.3 core + proteins L_1, L_5, $L_{8/9}$; L_{16}, L_{25}		40
6. 1.3 core + proteins L_1, L_5, $L_{8/9}$, L_{25}		1
7. 1.3 core + protein L_{16}		35

[a] According to reference 78.

[b] Cores and split protein samples were obtained by incubation of 50S subunits with 0.8 M and 1.3 M LiCl.

[c] Polyuridylic acid-directed polyphenylalanine synthesis was measured in the presence of 30S subunits.

[d] Eight absorbance units at 260 nm of ribosomal particles or subparticles and 1,250 pmol of [^{3}H]dihydrovirginiamycin S were incubated in dialysis cells.

easily than would a block of the P site inhibit the aminoacyl-tRNA binding in an in vitro assay.

VM (type A component) proved able to induce the detachment of previously bound alanyl-tRNA, but not that of fMet-tRNA, from the mRNA-ribosome complex (68). The simplest explanation is that the initiator aminoacyl-tRNA has two attachment sites on the 30S and 50S subunits, whereas the elongation aminoacyl-tRNA's have only one fixation point on the large subunit. Under these circumstances, the competitive binding of VM to the elongation complex would cause the detachment from the 50S subunit of all aminoacyl-tRNA species but fMet-tRNA, which remains hooked by the small subunit. Such an interpretation is supported by the finding that, within an elongation complex built up in the presence of VM, fMet-tRNA is present in a puromycin-unreactive form (cf. Fig. 24 and Table 15).

A tentative schema of type A virginiamycin action is depicted in Fig. 31, which accounts for most inhibitory effects described to date. If present during initiation, VM allows the formation of a complete initiation complex holding fMet-tRNA under an unreactive form. The binding of other aminoacyl-tRNA's is prevented by VM. In addition, this antibiotic causes a rejection of bound aminoacyl-tRNA, and presumably of peptidyl-tRNA in a pre-translocational stage at the A site. Thus, in the presence of VM, unreactive ribosomes accumulate, which are good candidates for the 60S particles found in bacteria treated with type A, but not with type B, components (Fig. 15).

No biochemical reaction of the protein biosynthetic pathway was shown conclusively to be blocked by group B components. There is, thus, an evident discrepancy between in vivo and in vitro data with bacterial systems, which to date has received no obvious explanation. The simplest hypothesis is that in vitro experiments entail conformational changes of the ribosomes, which become insensitive to the inhibitory action of these antibiotics. In this connection, it must be recalled that, although all procaryotes share 70S ribosomes undistinguishable by the usual functional tests, group B compounds (which inhibit protein synthesis in schizomyces but not in schizophyces) apparently are able to recognize the structural dissimilarity of ribosomal particles from the two groups of cells. Conversely, it might be postulated that protein inhibition (which applies exclusively to bacteria) and synergistic viability loss (which applies to both bacteria and blue-green algae) are distinct inhibitory activities of type B virginiamycins, possibly occurring on different targets.

In conclusion, several lines of evidence favor an interaction of virginiamycins with the A site of the 50S subunits, to which proteins L_6, L_{15}, L_{16}, and L_{18} have been assigned. Indeed, protein L_{16} proved essential for the binding of group B components (78). The proximity of the P site, on which proteins L_2, L_{11}, L_{18}, L_{20}, and L_{27} are held, can explain the interference of vernamycin with puromycin in vitro (208). The peptidyltransferase center (Fig. 32), which contains proteins L_2, L_6, L_{11}, L_{15}, L_{16}, L_{18}, L_{20}, $L_{26/27}$, and $L_{32/33}$, in addition to 23S rRNA, is likely to have its catalytic function altered by antibiotics holding affinities for the A and P sites (27, 243, 246). In fact, the catalytic center shares protein components with the A and P sites (proteins L_{15}, L_{16}, L_{18}, and L_{20}, for example) and also with the nascent peptide groove, to which proteins L_2, L_{20}, L_{24}, L_{27}, and $L_{32/33}$ have been assigned (244). Further investigation along these lines is expected to provide more complete information on the relationships between virginiamycin-binding proteins and structural components of the catalytic center for peptide bond formation.

Since the distinguishing trait of virginiamycins is the synergistic inhibitory action of their components in vivo, the demonstration of such cooperative effect in vitro is the obvious goal of recent molecular and conformational studies. An interpretation of these findings is herewith attempted, and a theory explaining the molecular

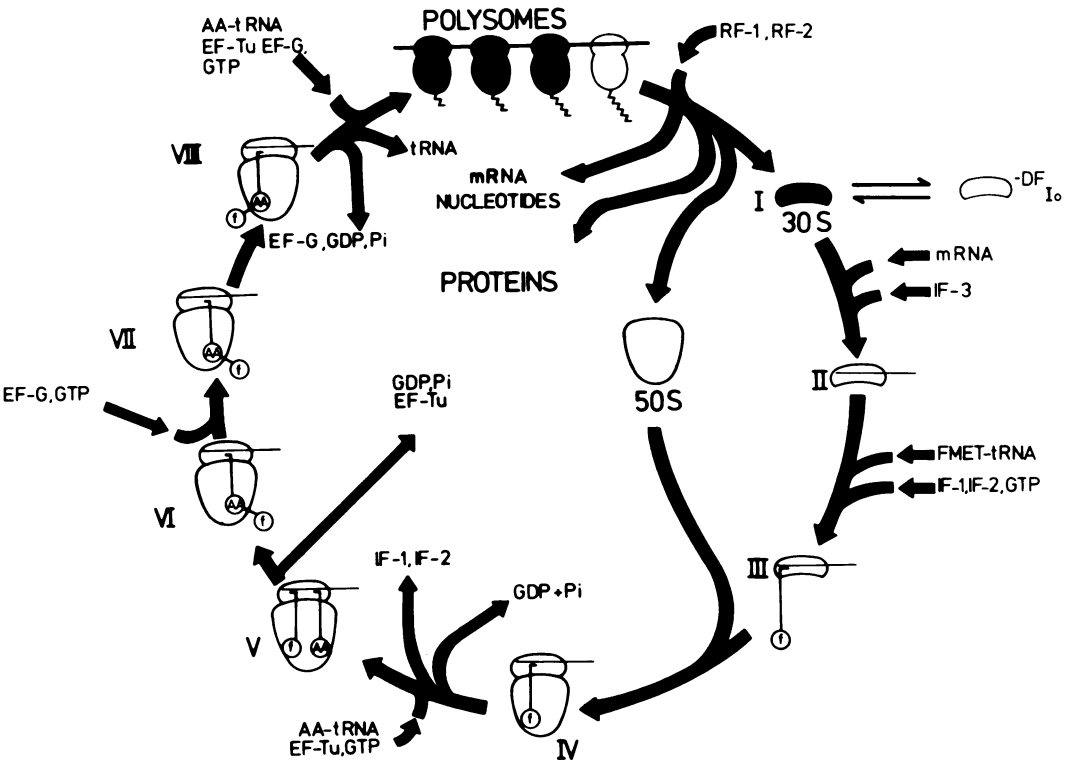


FIG. 30. *Ribosomal cycle and synthesis of proteins in procaryotes. According to references 239 and 243.*

mechanism of these antibiotics is proposed. Results gathered with different techniques are consistent in demonstrating that group A components facilitate the binding of their partners to ribosomes. The contrary, i.e., an increase of A component fixation by group B compounds, could not be shown (cf. 63 and 230). This suggests that binding of components A to ribosomes is the primum movens of the inhibitory action. This event entails a conformational change of the 50S subunit, for which several lines of evidence were provided: (i) reversal of the ethanol effect (71), (ii) increase of fluorescence enhancement (230), and (iii) production of pressure-sensitive particles (57, 59) (Fig. 19). Since the association constant of type B compounds with ribosomes undergoes a sharp increase upon the attachment of the A components (Fig. 29), a synergistic effect in one direction seems well established. A possible cooperation in the opposite direction is largely conjectural, although a bidirectional synergism would account for the 100-fold potentiation of the antibiotic activity in vivo. The most plausible and simple hypothesis is that fixation of group B compounds "locks in" previously bound A components. Since the latter type of drugs forms quite unstable complexes with ribosomes, this instability is expected to be reduced by the proposed mechanism. However, no conclusive evidence for such an effect has been gathered to date.

USE OF VIRGINIAMYCIN-LIKE ANTIBIOTICS IN HUMANS AND IN ANIMALS

Pharmacological and Therapeutic Studies

The solubility of virginiamycins in different solvents (see Chemical and Physical Determinations of Virginiamycin-like Antibiotics) partially accounts for their absorption, distribution in circulating fluids and organs, and excretion. In turn, the partition of circulating virginiamycins in different tissues of the organisms determines the local concentration of the drugs, and hence their effectiveness as therapeutic agents. It must also be noted that pharmacological studies with these antibiotics were rendered difficult not only by reason of their peculiar solubility, but also because the fixation to cells and the catabolism of A and B compounds are different, and the bactericidal potency of the mixture relies on the relative proportion of the two components.

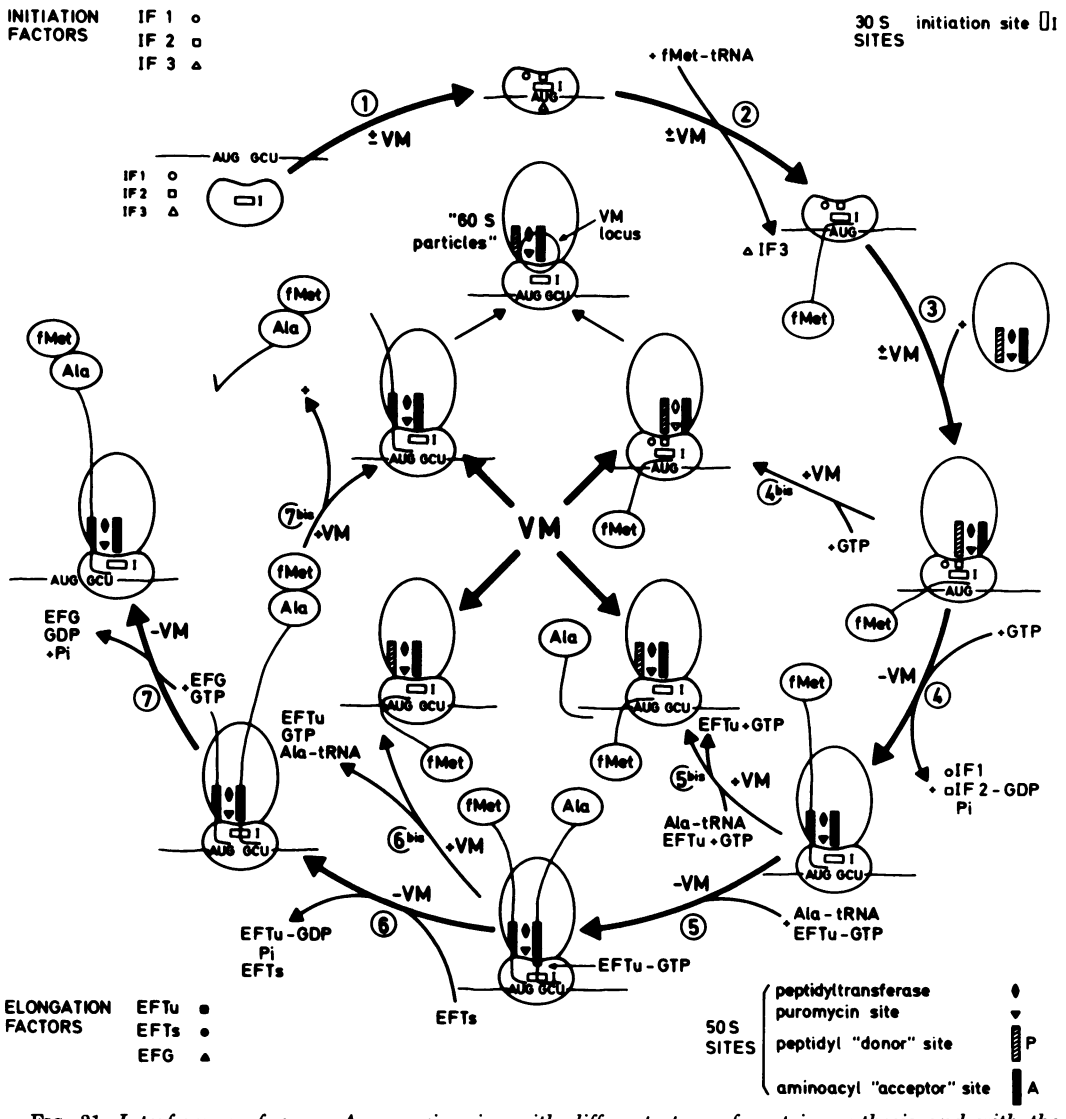


FIG. 31. *Interference of group A synergimycins with different steps of protein synthesis and with the ribosomal cycle. The drawing summarizes the main inhibitory effects of VM discussed in the text.*

A very small percentage of the administered antibiotics, whether introduced per os or by subcutaneous or intraperitoneal injection, appears in the circulation, as expected for substances endowed with low hydrosolubility. However, a higher proportion of the A component was found in the blood after subcutaneous administration, whereas more B component became solubilized after ingestion. Moreover, pristinamycin was claimed to be seven times more active when introduced parenterally than by the enteric route (14–16, 192).

In Table 17, the antibiotic activities in the blood and in three organs (liver, spleen, and kidney) of mice receiving a single dose of pristinamycin, either per os or subcutaneously, are reported. During the first 6 h after administration, the inhibitory activity was almost equally distributed among blood and organs, whereas after 1 day the drug could no longer be detected, except in the kidneys. It was also reported that pristinamycins do not pass the hematoencephalic barrier and do not accumulate in the bone tissue (14, 16). The obvious inference is that these drugs have no apparent affinity for mammalian cells, and are completely catabolized by mammals.

The relative concentrations of the two groups

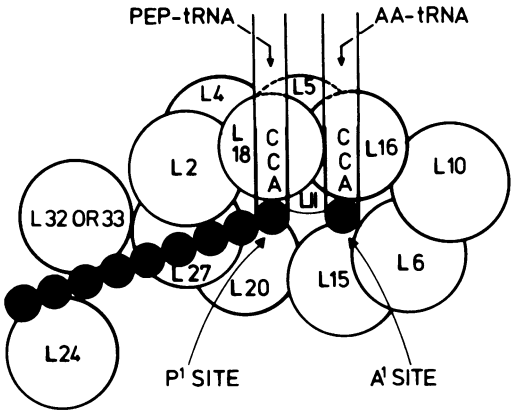


FIG. 32. *Topography of the aminoacyl (acceptor) and peptidyl (donor) sites, and of the peptidyltransferase locus on the surface of the 50S ribosomal subunits. According to reference 244.*

TABLE 17. *Distribution of pristinamycin in mice, after oral and subcutaneous administration*[a]

Pristinamycin[b] (route of administration)	Organs and tissues	Distribution of pristinamycin in the body (units/ml of blood or g of organ) after:		
		2 h	6 h	24 h
Per os	Blood	144	24	<1
	Liver	232	20	<1
	Spleen	136		<1
	Kidney	104	16	12
Subcutaneous	Blood	192	40	<1
	Liver	200	56	<1
	Spleen	96	24	<1
	Kidney	200	56	8

[a] According to reference 16.
[b] Single dose of 500 mg/kg.

of virginiamycins in the blood of mice receiving a single oral dose with equal concentrations of A and B were 5 to 25% for the A component, and 75 to 95% for the B component. Moreover, in the blood stream, about 80% of group A and 40% of group B components were apparently bound in a reversible fashion to serum proteins (92, 252, 320). Hence, type A compounds are the limiting therapeutic factors. Indeed, a specific adsorption of these substances into the erythrocytes and their rapid inactivation have been reported (16).

The excretion of virginiamycins is quite rapid, and takes place through the urine, the bile, and the feces. This explains the relatively high levels of drugs in the kidneys and liver soon after parenteral administration of labeled antibiotics and the persistence of radioactivity in the bladder and gallbladder (16, 23, 287).

The catabolism of virginiamycins in man is only partly known. It seems established that about 10% of the administered drug is excreted as such, whereas 90% is completely hydrolyzed (156, 327). It has been calculated that, in dogs receiving a single oral dose of 1 g/kg, about 12 and 20% of the etamycin is eliminated as an undegraded product through the feces and the urine, respectively (86). Only few degradation products of type B components have been chemically characterized, among which is a derivative of hydroxy-3-pipecolylglycine (15, 156).

In spite of their low hydrosolubility and the small percentage reaching the bloodstream, virginiamycins proved very active remedies for the prevention and treatment of experimental infections in laboratory animals. Thus, for example, pristinamycin administered either per os or subcutaneously (single daily dose for 3 days) protected mice from peritoneal infections by *S. aureus*, *Streptococcus pyogenes*, and *Diplococcus pneumoniae*, and were of evident therapeutic value in a staphylococcal septicemia with renal abscesses (the corresponding 50% therapeutic doses varied between 10^2 and 10^3 mg/kg per day) (15). Likewise, as shown in Table 18, intraperitoneal and subcutaneous injections of low doses of mikamycin had evident prophylactic and therapeutic effects on the evolution of an acute peritonitis by *Streptococcus hemolyticus* (285).

It is noteworthy that the synergistic effect of the A and B groups of components in the experimental animals is very similar to that observed with axenic bacterial cultures. The striking resemblance of the two graphs showing the biological activity of different A/B mixtures on the experimental staphylococcal infection of mice (Fig. 33B) and in axenic cultures of gram-positive microorganisms (Fig. 33A) (cf. also references 16 and 285) cannot be missed.

Toxicological Investigation

Toxicological studies in mice, rats, and dogs indicate that virginiamycin-like antibiotics, even if administered for prolonged periods at quite high doses, have extremely low toxicity. Thus, for example, mice were found to tolerate without appreciable alterations the intraperitoneal and subcutaneous injections of 300 mg of mikamycin per kg, as well as a per os administration of 600 mg/kg (284, 285). Also, a single oral dose of 800 mg/kg was found to be harmless for dogs (224). In addition, the intraperitoneal 50% lethal dose in mice was calculated to be 273 mg of etamycin per kg, and oral 50% lethal dose values of 1 g/kg for mice and 4 g/kg for rabbits were reported (86). Although some symptoms of acute toxicity were observed upon intravenous injection of very high doses of etamycin (50% lethal dose = 38 mg/kg) (1 ml of 0.08% suspension in water),

TABLE 18. *Therapeutic action of mikamycin in experimentally infected animals*[a]

Antibiotic	Treatment[b]		Dose (mg)	Survival[c]	
	Administration			Mean survival time (h)	Animals survived (no. after 200 h)
	Route	h			
1. None				22.5–33	0/8
2. Mikamycin	i.p.	1, 6, 12	0.8	159	6/8
			0.2	157	5/8
			0.05	110	2/8
3. Mikamycin	s.c.	1, 6, 12	0.8	200	8/8
			0.2	200	8/8
			0.05	200	8/8
4. Mikamycin	i.p.	6, 12, 18	0.8	200	8/8
			0.2	79	2/8
			0.05	76	1/8
5. Mikamycin	s.c.	6, 12, 18	0.8	200	8/8
			0.2	71	1/8
			0.05	28	0/8

[a] According to reference 285.

[b] Early (samples 2 and 3) and late (samples 4 and 5) administration of mikamycin (indicated doses) repeated three times by the intraperitoneal (i.p.) or the subcutaneous (s.c.) route.

[c] Mice infected by intraperitoneal injection of 500 (samples 1, 3, and 5) or 2,000 (samples 2 and 4) 50% lethal doses of *Streptococcus hemolyticus* (group A, type 19, strain J17D).

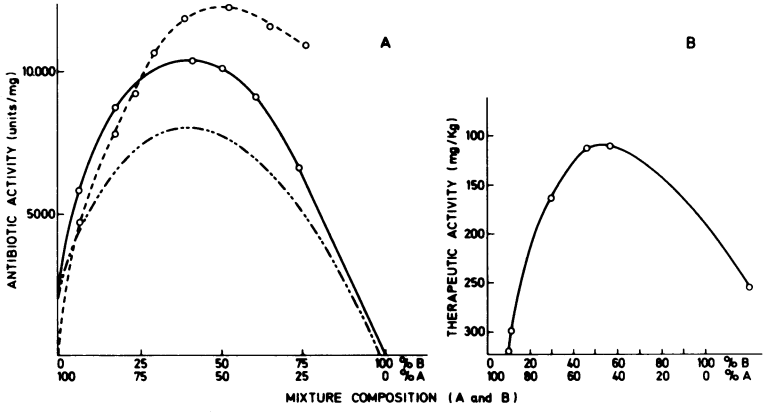


FIG. 33. *In vitro and in vivo synergistic effects of mixtures of type A and B pristinamycin components. (A) Inhibitory action of pristinamycin on axenic cultures of three gram-positive bacteria (Streptococcus pyogenes, —○—; Sarcina lutea, —·—; and Bacillus subtilis, - -○- -). (B) Therapeutic action of pristinamycin administered per os on staphylococcal peritonitis of mice (50% therapeutic dose expressed in milligrams of drug administered per os per kg of animal weight). According to reference 16.*

they are likely to be accounted for by the large amount of insoluble material introduced into the bloodstream (the administered dose exceeded by three orders of magnitude its hydrosolubility level). Upon intramuscular injection of high doses of antibiotic (1 ml of 20% suspension of etamycin in water), pockets of dried unadsorbed material became encapsulated in the muscle and surrounded by necrotic tissue (86).

When the treatment of mice per os was pursued for several months, dilatation and thinning of the cecum was observed (16, 160). These alterations were similar to those found in germ-free animals and were reversible. The size and thickness of the intestine became normal within 2 weeks after the suspension of the treatment and the reestablishment of a complex intestinal flora.

In cats and dogs receiving very high levels of etamycin for prolonged periods (250 to 700 mg/kg per day given orally for 1 to 3 weeks), a leukopenia was observed, in parallel with an increase of the body temperature and hemorrhages of the gastrointestinal tract. When the treatment was discontinued, there was a sharp increase of the leukocyte number and a decrease

of the rectal temperature. No such findings were reproduced in rabbits and mice; oral doses of 1 to 4 g/kg per day did not modify the blood cell counts in these animals (86).

Likewise, no significant abnormalities of the cardiovascular and nervous systems of laboratory animals receiving pristinamycin were detected, nor were teratogenic or cancerogenic alterations observed in rodents after prolonged treatment with these antibiotics (16, 160). These findings agree with tissue residue analyses of swine receiving high levels of virginiamycin for long periods (treatment with 170 ppm of 155 g/t for 18 weeks). Residues of the antibiotic greater than 0.1 ppm could not be detected in the muscle, liver, kidneys, fat, and skin of the animals (87).

Therapeutic Application of Virginiamycin-Like Antibiotics in Human Medicine

As the use of virginiamycin-like antibiotics in man is limited at the present time, the literature available on their therapeutic application is small, and recent data are missing. Moreover, because of the poor resorption, the clinical use of these antibiotics has been mainly focused on topical applications. In addition, the narrow spectrum of virginiamycins has limited their application to particular diseases produced by gram-positive bacteria in general, and by exceptional gram-negative microorganisms (121, 197).

One of the most successful applications is in the field of pediatrics. Since *Haemophilus pertussis* proved quite sensitive to virginiamycins, these antibiotics were used successfully in the treatment of whooping cough. About one-third of the treated patients showed reduced fits of coughing, gain in weight, and quick recovery, whereas the remaining two-thirds had moderate improvements; in all cases the pharmaceutical preparations were well tolerated by the infants (49, 119, 227). Virginiamycins found other applications in the therapy of several infectious illnesses of children. Thus, in one clinical investigation, cutaneous, respiratory, digestive, and bone disease (mostly acute staphylococcal infections) were treated—80% of the cases successfully, and 10% unsuccessfully (121, 152, 153).

Surgery is still another branch of medicine in which therapy with virginiamycins met with considerable success. Focal infections of bones (osteomyelitis) and articulations (acute arthritis), which proved resistant to other therapeutic agents, were found to respond favorably to virginiamycins (194). These antibiotics were also used in the therapy of open abscesses and peritonitis. In addition, a prophylactic application for the protection of the stumps of amputated limbs, and of surgical wounds from superinfection, has been reported (81).

In stomatology, these antibiotics were used to treat dental abscesses and to prevent septicemia after removal of infectious foci (apical granuloma) (1), and in otorhinolaryngology for the therapy of abscesses of sinuses, labyrinth, and tympanic cavity, abscesses which are particularly refractive to chemotherapy (25).

Virginiamycin-like antibiotics found numerous applications in dermatology. As a matter of fact, staphylococcal infections of the skin, impetigo and folliculitis in particular, proved very sensitive to these antibiotics (250). Also, virginiamycins were used with success to protect skin burns, to treat staphylococcal infections of burn sores, and to prevent the infection of skin transplants (126). In most cases, wounds were maintained aseptic, and rapid healing was obtained without production of allergic and irritative reactions. The use of these antibiotics for prevention and treatment of superinfected eczematous lesions has also been related.

Use of Virginiamycin-Like Antibiotics in Domestic Animals

During the last three decades, antibiotics have been largely used as food additives, to improve the growth of poultry, swine, and cattle. Although growth promotion by antibiotics is not conclusively explained, there is little doubt that this effect is due to an inhibition of the intestinal flora, particularly of gram-positive bacteria. As a matter of fact, antibiotics which are absorbed in very small amounts from the intestinal tract are very active growth promoters. Moreover, no growth enhancement is observed in germfree animals, unless they become infected with the fecal flora of conventional animals. Four main hypotheses have been proposed to explain these findings: (i) improved conservation of nutrients, particularly of amino acids (inhibition of the synthesis of decarboxylating and desaminating bacterial enzymes); (ii) vitamin-sparing effect (protection of hydrosoluble vitamins, particularly of those present in the diet at limiting concentrations); (iii) increased absorption of digested products (the intestinal wall is thinner in germfree and in antibiotic-treated animals, and has a higher absorption rate); and (iv) inhibition of the production of bacterial toxins (which lower the fattening of the livestock). It is possible that all these mechanisms, and others still unknown, play a role in the growth-promoting effect of antibiotics.

Use of virginiamycin-like antibiotics as growth promoters started soon after the discovery of

these antibiotics (12, 347). In one work, the improvement of feed utilization in chickens by virginiamycin was compared to that caused by oxytetracycline and bacitracin, antibiotics possessing "broad" (i.e., inhibition of gram-positive and gram-negative bacteria) and "narrow" (i.e., activity on gram-positive bacteria only) spectra, respectively, and currently used in commercial feeds. It was found that administration of virginiamycin within very wide ranges (4 to 100 g/t basal ration) significantly improved the feed efficiency over the controls. Growth promotion of chickens by virginiamycin was comparable to that by oxytetracycline and bacitracin, and similar results were obtained with turkey poults (347) and rabbits (167). Data shown in Table 19 indicate that addition of virginiamycin (5 to 30 g/t) to the diet of chickens and turkey poults produces a weight increase of 1 to 13% over the control, and an improvement of feed efficiency of 3 to 10%. Comparable results were obtained in several experimental centers (29, 67, 113–115). The recommended dosage for growing birds is 5 to 20 ppm for starter feeds and 5 to 10 ppm for finisher feeds.

The effect of virginiamycin on growth of suckling and fattening lambs (76, 159), swine (12, 36, 37, 72, 73, 130, 158, 199, 200, 201, 225, 259, 292, 293), and calves (164, 189) has been extensively investigated. It was concluded that these antibiotics promote nutrient adsorption and growth rate of several farm animals, as shown by the data reported in Table 20. The recommended dosages are as follows: 50, 50, 20, and 5 ppm for prestarter, starter, grower, and finisher pig feeds, respectively; 40 to 80 ppm for starter and 20 to 40 ppm for finisher vealcalf feeds.

At the present time, commercial preparations of virginiamycin are widely used as feed additives (352). The successful use of these compounds is due to their very favorable biological properties: (i) extremely low toxicity; (ii) lack of accumulation in animal tissues; (iii) practically undetectable production of resistant mutants in the intestinal flora; (iv) narrow spectrum, i.e., restricted inhibition of gram-positive microorganisms; (v) rare induction, if any, of episomal resistance carried by gram-negative plasmids; and (vi) biodegradability in cattle feces. Concerning the last point, it has been observed that a storage of feces of pigs fed upon a diet supplemented with virginiamycin brought about an inactivation of 80% of the antibiotic within few days.

In addition, virginiamycins have found successful application as therapeutic agents in veterinary medicine. Although these antibiotics were mainly used for the treatment of swine dysentery, during recent years, there is little doubt that the number of animal diseases controlled by these drugs will increase in the future (cf., for example, reference 76 concerning the use of virginiamycin in ovine dysentery).

Swine dysentery (bloody scours) is a mucohemorrhagic enteritis that affects pigs of all countries. The acute form is accompanied by diarrhea with mucus and blood in feces, dehydration, and frequent death (the autopsy shows

TABLE 19. *Growth promotion of birds by virginiamycin*[a]

Birds	Antibiotic[b] treatment (g/t)	Growth[c]			
		Wt increase		Feed conversion value	Increase in feed efficiency (%)
		Avg wt (g)	Increase over control (%)		
I. Chickens		332		2.70	
	10	376	+13.2	2.52	+6.7
II. Chickens		354		2.80	
	20	403	+13.8	2.58	+7.9
III. Chickens		1,192		2.207	
	7.5	1,227	+0.9	2.136	+3.3
	15	1,237	+4.5	2.071	+6.2
	30	1,230	+1.2	2.120	+4.0
IV. Turkey poults		589		1.97	
	10	660	+12.0	1.79	+9.2
V. Turkey poults		1,335		2.04	
	10	1,416	+3.1	1.83	+10.3

[a] According to reference 352.
[b] Virginiamycin added to the diet.
[c] Experimental condition: I, 50 males per group, 4 weeks; II, same as I, chickens of both sexes; III, 1-day-old chickens of both sexes, 6 weeks treatment, 30 birds per group; IV, groups of two strains of turkey poults kept for 4 weeks; V, same as IV, for 7 weeks.

TABLE 20. *Growth promotion of animals by virginiamycin*[a]

Animals	Antibiotic treatment (g/TON)	Growth[b]			
		Avg wt gain (g/day)	Wt increase over control (%)	Feed conversion value	Increase in feed efficiency (%)
I. Pigs		653		3.66	
	20	716	+9.6	3.43	+6.3
II. Pigs		503		4.69	
	10	540	+7.4	4.36	+7.1
	20	544	+8.2	4.37	+6.9
III. Pigs				3.48	
	40/20		+8.0	3.21	+7.8
IV. Calves		1,088			+5.0
	40		+12.4		+6.2
	80		+13.1		

[a] According to reference 352.

[b] Experimental conditions: I, 11 groups of 12 fattening pigs, 24-month treatment; II, groups of 7 fattening pigs, 2-month treatment; III, groups of 8 pigs treated for 6 months, from weaning to finish with 40 (6 weeks) and 20 (18 weeks) parts per million of antibiotic; IV, groups of 13 bull calves grown from 40 to 162 kg.

hemorrhagic and necrotic lesions of the colon). The evolution to chronic dysentery produces a lowering of growth and feed-conversion efficiency among survivors. The main etiological agent is *Treponema hyodysenteriae*. Indeed, the experimental production of the disease has been obtained by the oral administration of this spirochete. The growth of this microorganism is inhibited by virginiamycin, and hence its use in swine breeding. As a matter of fact, in the United States and Eastern Europe, this antibiotic proved very efficient in preventing swine dysentery, when administered at low doses as food additive (76, 199, 225, 332). In addition, at higher doses, this antibiotic was successfully used for the treatment of the acute disease and the prevention of the chronic state (180, 199, 332). Recommend doses were as follows: 10 to 25 g/t for a prophylactic use and 50 to 100 g/t for therapeutic purposes (long- and short-range control, respectively).

Comments on the Present Applications of Virginiamycin-Like Antibiotics

Since the publication of the "Swann Report" (281a) and the promulgation of its basic concept embodied in the "Medicines Act," several regulatory authorities have proscribed the use, as growth promoter in animals, of antibiotic having therapeutic value for humans. The aim of such a rule is to avoid the production in animals, and the transmission to men through the meat, of bacterial strains carrying plasmids with antibiotic-resistant factors (cf., for example, 171, 172). As the result of this policy, the therapeutic application of virginiamycins in human medicine has been neglected in favor of its utilization in animal husbandry. At the present time, the treatment of bacterial infections of men with these antibiotics is restricted to a few countries, whereas they are largely used around the world for stock-farming. The reason for the choice taken by most pharmaceutical industries is as follows: virginiamycin-like antibiotics meet all the requirements for growth promoters, whereas they are hardly competing with other therapeutically used antibiotics because of solubility and resorption problems.

Although the exceptional performance of virginiamycins in animal husbandry and veterinary medicine is unquestionable, the therapeutic capacity of these antibiotics in humans and their potential as remedies for selected diseases should not be underestimated. As a matter of fact, it is my opinion that virginiamycins are the ad hoc remedy for the treatment of focal gram-positive infections by in situ administration of concentration solutions of the antibiotic. Conversely, the infectious centers can be reached by antibiotics conveyed by the bloodstream. Concerning the experimental use of virginiamycins, it can be argued that most chemical trials and pharmacological studies in vivo were carried out by administering the antibiotics per os, whereas it is well established that a negligible portion of the ingested solid product is solubilized and absorbed in the digestive tract.

Finally, it must not be forgotten that the key problem for a generalized therapeutic use of virginiamycins is that of their solubilization in water. Although the attempts at obtaining commercial hydrosoluble preparations have been so far unsuccessful, this possibility cannot be excluded. This is particularly true for type A components, which are the limiting therapeutic factors because of their lower absorption, higher

affinity for serum proteins and erythrocytes, and faster inactivation. Since they play a predominant role in the synergistic effect of A and B components, the possible acquisition of water-soluble preparations of type A compounds is expected to considerably improve the therapeutic power of these antibiotics in vivo.

SUMMARY

A unique property of virginiamycin-like antibiotics is to contain several components endowed with a synergistic effect in microorganisms. One of the two basic structures, A and B, can be assigned to all the antibiotics of this family. The two formulas are completely unrelated, although both types of substances are macrocyclic lactone rings. The tridimensional structure of several compounds of the A and B groups has been elucidated.

The synergistic inhibitory effect is double: lowering of the minimum inhibitory concentration of each component by its partner, and irreversible action of the mixture of two components, which separately would induce a reversible inhibition. Hence, the bactericidal action of an association of bacteriostatic antibiotics.

Two patterns of biological activities have been recognized in nature. The first is that of bacteria, the growth of which is blocked reversibly by either the A or the B component and irreversibly by their mixture. The second pattern is that of algae, which are transiently bleached by group A compounds, whereas type B components, which are incapable of producing appreciable alterations per se, increase the bleaching effect of their partners and render it irreversible (Table 21). The presence of altered chloroplasts, and the absence of normal organelles which are replaced by reticulated bodies, have been shown, respectively, in algae treated with type A components and in those incubated with a mixture of A and B compounds. In the organelles exposed to these antibiotics, not only is the formation of chloroplast ribosomes prevented, but also the synthesis of rRNA is blocked; these two processes parallel the morphological plast development and chlorophyll synthesis. Cyanophyces, which share with bacteria a procaryotic organization and with eucaryotic algae photosynthetic functions associated with O_2 production, have the virginiamycin inhibition pattern characteristic of eucaryotic algae. The main difference is that group A antibiotics are lethal for cyanophyces, possibly due to a direct action on DNA formation.

In bacilli, the mixture of A and B virginiamycins is lethal not only for the double-sensitive strains, but also for mutants resistant to type A components. This effect is suppressed, however, when cells become resistant to the B components. The gene responsible for a chromosomal resistance to type B components maps in the ribosomal protein region, close to the origin and in the proximity of the elongation factor G gene. Episomal resistance in streptococcus is due to plasmids coding either for acetylating or for hydrolyzing enzymes.

A quite specific effect is that of type B components in bdellovibrio. These antibiotics prevent the fragmentation of the replicated body, if added during the first part of the replication cycle.

Multiplication of DNA phages in bacilli is sensitive to the inhibitory effect of virginiamycins. Single components block completely virus development and host cell lysis if added at the moment of infection, but this action decreases during the second part of the eclipse phase. In contrast, an irreversible inhibition of particle formation and lysis is produced at any moment of the viral cycle by a mixture of A and B compounds.

The induction of a lytic cycle in lysogenic cyanophyces was found to have three simultaneous requirements: light, repressor inactivation, and protein synthesis. Cells submitted to lytic induction in the presence of type A components do not produce viral particles; they can

TABLE 21. *Inhibitory action of virginiamycin components on growth, viability, and photosynthetic functions of protists*[a]

Biological functions	Bacteria	Cyanophyces	Algae
Growth			
Single components			
VM	RI	(R) I[b]	—[c]
VS	RI	NA	NA
Mixture, VM + VS	II	II	—
Viability			
Single components			
VM	NA	(R) I[b]	NA
VS	NA	NA	NA
Mixture, VM + VS	II	II	NA
Photosynthetic capacity			
Single components			
VM		(R) I	RI
VS		NA	
NA			
Mixture, VM + VS		II	II

[a] Three levels of inhibitory action are considered: irreversible inhibition (II), reversible inhibition (RI), and no action (NA).

[b] High levels of VM are lethal for cyanophyces.

[c] —, Growth of algae in true photoautotrophic media is supposed to be inhibited by VM (cf. text).

be multiplied indefinitely upon removal of the antibiotic, and retain the capacity of being induced successfully in the absence of inhibitors.

Antibiotics of A and B groups act at the level of 50S ribosomal subunits. The former type of inhibitors block elongation in vitro by preventing the EFTu-dependent binding of aminoacyl-tRNA to ribosomes and the peptidyltransferase-catalyzed peptide-bond formation between fMet-tRNA on the P site and either puromycin or aminoacyl-tRNA at the A site (Table 22). Initiation and elongation reactions which were tested in vitro were found unaffected by type B components, although polypeptide synthesis directed by some artificial and possibly natural messengers was found inhibited under certain conditions.

The stoichiometry of binding of both groups of inhibitors to 50S ribosomal subunits has been conclusively shown to be a monomolecular process. The association constant of A group compounds was found to be about one-eighth that of B group compounds (3.2×10^5 and 2.5×10^6 M^{-1}, respectively). The attachment of A type inhibitors to ribosomes causes a sixfold increase of the affinity of these particles for B type compounds. This finding offers a molecular explanation for the synergistic effect of the two groups of antibiotics in vivo.

TABLE 22. *Inhibitory action of virginiamycin components on peptide initiation and elongation in cell-free and suborganellar systems*

Biochemical reaction	Cell-free systems[a]		Suborganellar systems[a]	
	VM	VS	VM	VS
A. Initiation steps				
40S complex formation	UA	UA		
75S complex formation	UA	UA		
75S complex reactivity	I	UA		
B. Elongation steps				
Binding of aminoacyl-tRNA	I	UA		
Peptide bond formation	I	UA		
Translocation	UA	UA		
C. Protein synthesis				
Polyuridylic acid as template	I	I?	I	UA
MS2-RNA as template	I	I?		

[a] The in vitro inhibition of protein synthesis by virginiamycin components VM and VS was tested in cell-free systems from bacteria (*E. coli* and *B. subtilis*) and in suborganellar systems from plant chloroplasts. UA, Unaffected; I, inhibited.

The multiple effects of virginiamycins acting on bacterial and viral replication can be accounted for by the inhibition of protein synthesis at the 50S subunit level (Table 23). A possible exception is the block of DNA formation in cyanophyces, which is responsible for the lethal effect of type A components, and might be due to the peculiar structure of the genome of these microorganisms.

Virginiamycin-like antibiotics have found practical application in human and veterinary medicine. Although several experimental infections of laboratory animals were treated successfully by oral and parenteral administration of these antibiotics, their therapeutic use has been limited by their low hydrosolubility. Consequently, the main therapeutic application of these antibiotics has been the topical treatment of infectious foci of bones, articulations, teeth, and body cavities. The high sensitivity of *Staphylococcus pyogenes* and *Haemophilus pertussis* to these antibiotics has prompted their successful use in the therapy of dermatites and whooping cough.

Medical applications were neglected, however, in favor of the use of these drugs as food additives. At the present time, virginiamycins are widely used as growth promoters for poultry, swine, and cattle. Good improvement in feed utilization, lack of toxicity and of teratogenic capacity, poor readsorption, negligible tissue fixation, complete catabolism in the body, and high biodegradability in waste have made these compounds ideal helpers for livestock raising. These very properties have also justified their use in the prevention and treatment of enteric diseases

TABLE 23. *Metabolic alterations produced by virginiamycin components in bacteria*

Cell constituent	Virginiamycin action
DNA	Synthesis inhibited after long lag
RNA	
Polyribonucleotide chains	Polymerization increased (early effect); polymerization inhibited (late effect)
mRNA	Formation unaffected; decay prevented
rRNA	Synthesis of 16S and 23S species inhibited; undermethylation of rRNA precursors and decay increased
tRNA	Unknown
Proteins	
Polypeptide chains	Polymerization inhibited without delay
Specific proteins	Synthesis prevented
Chlorophyll	Synthesis inhibited after short lag

of farm animals, particularly of swine and ovine dysentery.

The study of virginiamycin-like antibiotics has helped to clarify the molecular mechanism of antibiotic action and resistance, to unravel the structure and functions of cell organelles and ribosomes, and to understand the regulatory processes underlying virus development and organelle morphogenesis.

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