Metabolism of Macromolecules in Bacteria Treated with Virginiamycin

By C. COCITO

*Laboratory of Molecular Genetics, Rega Institute,
University of Louvain, Belgium*

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SUMMARY

The two components of virginiamycin, M and S, separately exerted a reversible bacteriostatic activity on *Bacillus subtilis*. Their combination increased by a hundredfold the inhibitory activity of each factor and induced a loss of viability of bacteria. Such an irreversible step was preceded by a reversible phase, which was characterized by a long lag in colony formation.

Very short incubation with single virginiamycin components and their combination suddenly and completely blocked protein synthesis, whereas the rate of incorporation of labelled bases and nucleosides into polynucleotides was not altered appreciably unless protein formation was halted completely.

Nevertheless, some alterations of ribosomal RNA metabolism occurred very early after treatment with virginiamycin. The synthesis of 23S rRNA was specifically inhibited. Moreover, the degree of methylation of the rRNA which was made in the presence of the drug was lower than that of the controls. Also, the rRNA labelled in virginiamycin-treated cells was metabolically unstable. This indicates that formation and stability of rRNA, as well as the balance among rRNA species, depend on virginiamycin-sensitive protein synthesis.

Metabolism of pulse-labelled RNA was also altered in the presence of virginiamycin: its half-life was prolonged about sixfold by single components and eightfold by their combination. This was due to an increased turnover of rRNA and to prevention of messenger RNA decay.

It is concluded that peptide chain formation is the primary target of virginiamycins M and S (hence their synergistic antibiotic activity), that translation—not transcription—is prevented by these inhibitors, and that the alterations of nucleic acid metabolism are due to the halt of protein synthesis.

INTRODUCTION

Virginiamycin, an antibiotic which is produced by a mutant of *Streptomyces virginiae*, inhibits the growth of Gram-positive organisms *in vitro* and *in vivo* (Vanderhaeghe, van Dijk, Parmentier & De Somer, 1957). Its biological and chemical properties resemble those reported for other complex inhibitors which were described as Mikamycin, Ostreogrycin or E129 complex, PA114 factor, Pristinamycin or Pyostacin, Streptogramin and Vernamycin. The common feature of these antibiotics is that they contain two factors which, though exerting different degrees of inhibition on various micro-organisms, do possess a synergistic action in a given bacterium (Vazquez, 1966*a*).

Factor 'M' of virginiamycin, which is particularly active on micrococci, is a

macrocyclic lactone containing an oxazole ring (Fig. 1): it is probably identical to Ostreogrycin A (Vanderhaeghe *et al.* 1957; Delpierre *et al.* 1966). Factor 'S' of virginiamycin has a more powerful activity on bacilli and consists of a cyclopeptidic lactone ring (Fig. 1) (Vanderhaeghe & Parmentier, 1960) which resembles that of the B factor of Ostreogrycin complex (Eastwood, Snell & Todd, 1960) and of the I_A component of Pristinamycin (Jolles, Terlain & Thomas, 1965).

Virginiamycin S

Virginiamycin M

Fig. 1. Chemical structure of virginiamycin components.

The mechanism of the inhibitory activity of these complex antibiotics, which has been described respectively as bacteriostatic (Yamaguchi & Tanaka, 1964) and bactericidal (Chabbert & Acar, 1964; Videau, 1965; Murat & Pellerat, 1965), is still unclear. In several respects they resemble chloramphenicol, particularly in their ability to inhibit in some micro-organisms the incorporation of amino acids into polypeptides without altering the incorporation rate of radiophosphorus into polynucleotides (Yamaguchi & Tanaka, 1964).

However, a deeper investigation has revealed differences in the action of the two

components of streptogramin complex and chloramphenicol. Thus, for example streptogramin A (which corresponds to virginiamycin M) reduces the fixation of chloramphenicol to bacteria and ribosomes, and likewise chloramphenicol represses protein—not nucleic acid—formation in intact cells. Nevertheless, when tested *in vitro* on amino acid incorporation directed by synthetic polynucleotides, streptogramin A and chloramphenicol show different patterns of inhibition. On the other hand, streptogramin B (which resembles virginiamycin S) reduces the uptake of chloramphenicol by intact bacteria but does not compete with the chloramphenicol binding to ribosomes. Moreover, streptogramin B shows *in vitro* the same pattern of inhibition as chloramphenicol on homopolymer-directed amino acid incorporation, but in sensitive cells inhibits the accumulation of both nucleic acids and proteins (Vazquez, 1966a, b, c, d).

To reconcile such conflicting results one must postulate that streptogramin and chloramphenicol have more complex activities and interfere with several steps of the synthetic pathways of nucleic acids and proteins. The aim of this study was to elucidate the mechanism of action of virginiamycin complex in uninfected and virus-infected cells. The present and the accompanying paper deal respectively with the action of virginiamycin in *Bacillus subtilis* and in bacteria infected with the DNA-bacteriophage 2C. Purification of virginiamycins M and S and preparation of their labelled derivatives have made this investigation feasible.

METHODS

Abbreviations used: rRNA, mRNA and tRNA = ribosome, messenger and transfer RNA; HMW-RNA and LMW-RNA = high- and low-molecular weight RNA (chromatography fractions); Leu = leucine; Trp = tryptophan; Glc = glucose; U = uridine; T = thymidine; ECD = enzymic digest of casein; BSA = bovine serum albumin; TCA = trichloroacetic acid; EDTA = ethylene diaminetetraacetic acid; SDS = sodium dodecyl sulphate; DOC = sodium deoxycholate; MAK = kieselguhr coated with methylated albumin; Klett Units (K.U.) = $A_{660\text{ m}\mu} \times 10^3/2$; L and OS = media for *Bacillus subtilis*.

Micro-organisms. Three strains derived from *Bacillus subtilis* 168 were used in different experiments: (1) 168/6, wild type; (2) 168/2 leu⁻ and trp⁻; and (3) A26 U⁻ and trp⁻. *Escherichia coli* strain B furnished the RNA to be used as heterospecific marker for ultraviolet absorption.

Growth media. Stock cultures were grown in L medium containing 10 g. tryptone, 10 g. yeast extract, 5 g. NaCl, and 10^{-5}M -MnCl₂ per litre. Experiments employing either [³H]uridine (strains 168/2 and A26), [¹⁴C]amino acids (strains 168/2 and A26), or [¹⁴C]glucose (168/6) as tracers were carried out in a high-phosphate OS medium (Spizizen, 1958) supplemented with different amounts of Glc, ECD, Leu, Trp and U. Three different media (Young & Spizizen, 1963; Schaechter, 1963; Yudkin & Davis, 1965) were used to label cells with [³²P]phosphate: the level of [³¹P]phosphate was lowered to 10^{-3}M and replaced with 0.05 M-tris-HCl buffer (pH 7.4.)

Evaluation of bacterial numbers and viability. Bacterial concentration was evaluated by turbidimetric determination at 660 m μ in a Klett Summerson photoelectric colorimeter. Viability was determined by appropriate dilution in DSP (8.7 g. NaCl, 3.5 g. K₂HPO₄, 0.5 g. peptone, 10^{-6}M -MnCl₂ (pH 7.5) adjusted with HCl) and plating on L

agar; colonies were counted after different times of incubation at 37°. As a check on turbidimetric units and colony-forming counts, microscope counts (after fixation and staining with 5% I in 10% KI) and DNA content were previously established.

Isolation and disruption of bacteria. Three techniques were followed to stop metabolism while harvesting bacteria from growth medium: (1) NaN_3 (10^{-2} M) and rapid cooling followed by centrifugation; (2) precipitation with ice-cold TCA (0.5%, w/v, f.c.) and filtration on micropore filters; (3) precipitation with 95% ethanol at -18° .

Colorimetric determinations and radioactivity measurements were carried out directly on the acid and alkali extracts. Isolation and separation of macromolecules were carried out on homogenates obtained by mechanical disruption in a French-Aminco cell or by enzymic lysis with crystalline egg lysozyme in 0.15 M-NaCl, 0.1 M-tris-HCl (pH 7.4) + 0.02 M- NaN_3 . *Escherichia coli* RNA was extracted directly from intact organisms, with a hot phenol + detergent procedure (Cocito & De Somer, 1961; Cocito, 1963).

RNA extraction. Homogenates of *Bacillus subtilis* were treated with SDS (0.25%, w/v) and freshly redistilled phenol, the mixtures were centrifuged and the aqueous layers were re-extracted with phenol and ether. Suitable amounts of *Escherichia coli* RNA and NaCl (0.5 M) were added, and RNA was precipitated from 95% ethanol at -18° .

Ultracentrifugal fractionation of RNA. Solutions of RNA were layered on the top of 15 to 30% (w/v) gradients of sucrose in 0.10 M-NaCl, 0.2 M-acetic acid/Na acetate buffer (pH 5.2). Depending on the temperature of the centrifuge (10 to 23°) 0.1 to 2.5 mg. SDS/ml. were added. Fractionation was carried out either in a Spinco (rotor SW25.1, 23,000 rev./min.) or in an MSE ultracentrifuge (swing-out rotor 30, 30,000 rev./min.).

Radioactivity measurements. To ice-cold solutions of labelled nucleic acids or proteins 50 μg . bovine serum albumin and TCA (0.3 M) were added. After 30 min. of incubation in melting ice, the samples were filtered through 0.45 μ micropore membranes pre-soaked in 0.3 M-TCA for 18 hr at 4°. Precipitates were washed on the filters with ice-cold 0.3 M-TCA and air dried. Samples for ^3H , ^{14}C and ^{32}P were counted in a refrigerated liquid scintillation counter (Nuclear Chicago). Toluene containing 4 g. 2,5-diphenyloxazole (PPO) and 0.1 g. 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) per litre was used as scintillation fluid for solid samples, and Bray (1960) solution for liquid samples.

Colorimetric determinations. Phosphorus determinations were carried out with a modification of Fiske & SubbaRow procedure (Lindberg & Ernster, 1956). The colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used for proteins. DNA was determined with a modification of the Dische-Burton diphenylamine reaction (Giles & Myers, 1965). The colorimetric analysis of RNA was carried out with the orcinol method of Mejbaum modified by Ceriotti (1955).

Chemical fractionation of whole bacteria and cellular homogenates. Bacterial suspensions and homogenates were treated with TCA (0.5 M, 4°) and centrifuged. The pellets were dissolved in 0.1 M-NaOH. One sample of the solution was used for colorimetric estimation of proteins, and another for nucleic acid determination. The latter was performed by precipitation with HCl and 0.3 M-TCA at 4°, centrifugation, extraction of the sediment with HClO_4 for 15 min. at 90° and incubation in ice for 30 min. after addition of 2 mg. BSA. The precipitate of proteins was removed by

centrifugation and DNA and RNA were determined colorimetrically in the supernatant.

Purification of virginiamycins. Separation and purification of M and S factors from crude virginiamycin preparations was achieved on columns of silica gel (Vanderhaeghe *et al.* 1957; Vanderhaeghe & Parmentier, 1960). Crystalline preparations of the two factors (Gosselinckx, 1963) were employed through the entire work. The corresponding aqueous solutions were freshly prepared before each experiment.

Chemicals. Most of the radioactive products were obtained from the Belgian and French Departments of Radioisotopes of C.E.N. and the Radiochemical Centre, Amersham, England. Nucleic acid components were obtained from Calbiochem and Sigma, nucleases from Worthington, bovine serum albumin (fraction V) from Armour and Na laurylsulphate from T. Schuchard. The antibiotics actinomycin D and virginiamycin were gifts from Merck Sharp & Dohme (U.S.A.) and R.I.T. (Belgium) respectively: they are trade marked compounds of these two companies.

RESULTS

Action of virginiamycin on growth and viability of Bacillus subtilis. Growth curves of *Bacillus subtilis* 168/2 with increasing amounts of a single virginiamycin component are reported in Fig. 2. Factor S was more active than factor M, on a weight basis. Mixing the two virginiamycin components potentiated their antibiotic activity about 200-fold. In fact, a level of 0.5 µg./ml. of factors M and S induced an inhibitory effect comparable to that of 100 µg. of a single component.

The numbers of colony-forming units after incubation for different periods with virginiamycin components or their combination is reported in Table 1. Incubation of bacteria with high levels of single factors for less than 2 hr did not reduce their colony-forming capacity after plating on L agar (the viability decreased slowly as the cultures were maintained at 37° for longer periods). Mixing the two virginiamycins produced a double effect. For exposures shorter than 2 hr, the bacteria retained their viability, but colonies appeared on agar plates after an abnormal lag. Exposure times longer than this caused the colony-forming capacity to decrease rapidly. Hence, single factors had a bacteriostatic activity while together they were bactericidal, with an initial period in which the colony-forming capacity was altered.

Kinetics of amino acid incorporation into polypeptides. Incorporation of [¹⁴C]amino acids into acid-insoluble polypeptides was followed in organisms which were incubated with factors M, S or both. Both factors reduced the incorporation rate with little lag (Fig. 3) and the pattern of inhibition remained unchanged over a three-generation period. The rapidity of virginiamycin action was emphasized by slowing down the growth rate using auxotrophs grown in low amino acid medium. Under these conditions the inhibition by 2.5 µg. of M or S factors was more than 80% within 5 min. and amino acid incorporation was virtually halted within 1/25th of the generation time.

Rate of incorporation of labelled precursors into nucleic acids. Is polypeptide synthesis the direct target of the drug or the result of an inhibition of nucleic acid synthesis? The problem was investigated by following the rate of incorporation of labelled precursors into polynucleotides, in the presence of virginiamycins.

In the prototroph strain 168/6, virginiamycin M and a mixture of M and S

factors increased the incorporation rate of [^3H]thymidine into polydeoxyribonucleotides, and prolonged it beyond the limit of the control (Fig. 4). The same precursor was incorporated at a reduced rate upon incubation of the auxotroph mutant A26 with virginiamycins; this inhibition was negligible for the first 25 min. and increased as the time of contact with the drug was prolonged. The rate of incorporation of labelled uracil and uridine into polyribonucleotides was either unimpaired or increased during the first 15 min. of incubation with virginiamycin and decreased rapidly afterwards (Fig. 5).

Table 1. *Colony-forming units and time of colony formation after growth in the presence of virginiamycin components*

Strain and medium as in the legend for Fig. 2. Virginiamycin M (10 and 100 $\mu\text{g./ml.}$), S (same) and M + S (0.5 and 5 $\mu\text{g.}$ each component/ ml.): added at time 0 to the cultures. Viability test: samples taken at different intervals after addition of the antibiotic, immediately diluted and plated on nutrient agar. Visible colonies counted after 14, 24, and 48 hr at 37°.

Time of contact with virginiamycin (min.)	Time of incubation of agar plates (hr)	Virginiamycin concentration ($\mu\text{g./ml.}$)						
		= control	10 $\mu\text{g. M}_1$	100 $\mu\text{g. M}_1$	10 $\mu\text{g. S}$	100 $\mu\text{g. S}$	0.5 $\mu\text{g. M}_1$ + 0.5 $\mu\text{g. S}$	5 $\mu\text{g. M}_1$ + 5 $\mu\text{g. S}$
0	14	1.64×10^7						
	24	1.64×10^7						
10	14		2.56×10^7 *	2.48×10^7	4.88×10^7	3.40×10^7	—	—
	24		2.56×10^7 *	2.48×10^7	4.88×10^7	3.40×10^7	3.26×10^7	3.08×10^7 *
25	14	6.8×10^7						
	24	6.8×10^7						
30	14		3.90×10^7	4.40×10^7	4.10×10^7	5.70×10^7	—	—
	24						3.70×10^7	3.32×10^7
60	14		5.0×10^7	5.60×10^7	5.66×10^7	5.28×10^7	—	—
	24		5.0×10^7	5.60×10^7	5.66×10^7	5.28×10^7	2.60×10^7	2.36×10^7
65	14	14.0×10^7						
	24	14.0×10^7						
90	14		7.02×10^7	5.80×10^7	8.60×10^7	8.00×10^7	—	—
	24						2.20×10^7	3.04×10^7
120	14		9.0×10^7	5.80×10^7	8.04×10^7	7.70×10^7	—	—
	24		9.0×10^7	5.80×10^7	8.04×10^7	7.70×10^7	1.84×10^7	2.46×10^7
145	14		11.0×10^7	4.9×10^7	6.1×10^7	6.0×10^7	—	—
	24						1.82×10^7	1.60×10^7
170	14		7.0×10^7	3.5×10^7	4.4×10^7	3.2×10^7	—	—
	24		7.0×10^7	3.5×10^7	4.4×10^7	3.2×10^7	1.56×10^7	1.50×10^7

* Number of colonies of size comparable to that of the control, after incubation of nutrient agar plates for either 14 or 24 hr at 37°.

Synthesis of metabolically stable RNA. Though the halt of polypeptide synthesis cannot be accounted for by a block of polynucleotide chain formation, the effect of virginiamycin on proteins might still be caused by an inhibition of the synthesis of a single nucleic acid species. To test this possibility, RNA was labelled for 15 min. with [^3H]uracil (or uridine), extracted and fractionated by centrifugation in density gradients. In exponentially growing bacteria most of the label of a 15 min. pulse was incorporated into metabolically stable rRNA and tRNA (Fig. 6A).

The initial effect of virginiamycins showed a main radioactivity peak overlapping that of the 16S component, very little with 23S rRNA and more label in the 10S and 4S regions (Fig. 6B). After prolonged incubation with virginiamycins the 16S rRNA peak was still higher and broader than that of 23S rRNA, the proportion of RNA radioactivity in the 4S peak was higher than in the controls, and material of high specific activity which sedimented ahead of the rRNAs accumulated (Fig. 6C).

Fig. 2

Fig. 3

Fig. 2. Kinetics of growth in the presence of virginiamycin. Strain 168/2. Medium: OS, supplemented with 5 mg. Glc, 4 µg. Trp, 8 µg. Leu and 800 µg. ECD/ml. Virginiamycin S (1, 10 and 100 µg./ml.) added at time 0. Growth of the agitated culture at 37° followed turbidimetrically (K.U.).

Fig. 3. Kinetics of incorporation of amino acids into proteins. Strain A26. Medium: OS, supplemented with 5 mg. Glc, 4 µg. Trp, 4 µg. Leu, 200 µg. ECD, and 100 µg. U/ml. Virginiamycin: none (control) ○—○; 50 µg. factor M —△—; 50 µg. factor S —□—; 2.5 µg./ml. of both components —◇—, added at -1 min. to the cultures. Labelling: [¹⁴C]algal protein hydrolysate, specific activity 520.8 µc/mg., added at time 0 (0.417 µc/ml., f.c.). TCA-insoluble radioactivity collected on micropore filters and counted.

Integrated values of radioactivity of RNA fractions (which were pooled according to the scheme in Fig. 6A) are summarized in Table 2. During the first 15 min. of contact with virginiamycins M and M+S, incorporation of uracil into every type of RNA was increased. As the period of growth in the presence of virginiamycins was prolonged, however, the incorporation rate decreased progressively. In addition, the ratio 4S RNA/16S+23S RNA increased in proportion to the length of exposure to the drug, thus indicating a possible turnover of the high molecular weight fraction. Similar results were obtained by fractionation on MAK columns of RNA labelled with [³²P]orthophosphate.

Data reported in this section show that over-all synthesis of rRNA and tRNA was inhibited only after prolonged incubation with virginiamycin and exclude the possibility that the halt of protein synthesis might be due to a block of the transcription mechanism. Yet, virginiamycin induced a rapid alteration of RNA metabolism, as indicated by: (a) an early interference with the formation of 23S rRNA, (b) the accumulation of RNA with sedimentation coefficient higher than 23S, and (c) a probable turnover of rRNA. These alterations did not occur in rRNAs which were labelled before transfer to unlabelled medium containing virginiamycin (Fig. 7).

Fig. 4

Fig. 5

Fig. 4. Rate of labelling of DNA in a virginiamycin-treated prototroph. Strain 168/6. Medium: OS, supplemented with 5 mg. Glc and 800 µg. ECD/ml. Virginiamycin: none (control) $\circ$ — $\circ$; 50 µg. factor M $\triangle$ — $\triangle$; 50 µg. factor S $\square$ — $\square$; 2.5 µg./ml. of both components $\diamond$ — $\diamond$. Labelling: [^3H]thymidine, specific activity 17 c/mmole, added at 0 (A), 15 (B) and 30 (C) min. to the cultures (0.113 µc/ml., f.c.). Sampling: every 5 min. for 15 min. TCA-insoluble radioactivity collected on micropore filters in the presence of 100 µg. unlabelled thymidine and BSA/ml. and counted.

Fig. 5. Kinetics of incorporation of uracil into RNA. Strain and medium as in the legend for Fig. 3, but 800 µg. ECD/ml. Virginiamycin: none (control) $\circ$ — $\circ$; 50 µg. factor M $\triangle$ — $\triangle$; 50 µg. factor S $\square$ — $\square$; 2.5 µg./ml. of both components $\diamond$ — $\diamond$ added at -1 min. to the cultures. Labelling: [^3H]uracil, specific activity 12 c/mmole, added at time 0 (0.417 µc/ml., f.c.). TCA-insoluble radioactivity, precipitated in the presence of 100 µg. of unlabelled uracil and 100 µg. of BSA/ml. collected on micropore filters, and counted.

Turnover of pulse-labelled RNA in virginiamycin-treated cells. Bacteria were treated with virginiamycins, labelled with pulses of [^3H]uridine of 45 sec. and chased with an excess of unlabelled uridine and actinomycin (to prevent re-incorporation). At different times in the labelling and chasing periods, samples of the cultures were withdrawn, and the radioactivity incorporated into RNA was precipitated and measured.

Factor M apparently increased the level and inhibited the decay of pulse-labelled RNA: mean values of 90 and 420 sec. were calculated for the half-lives of pulse-labelled RNAs from control and M-treated bacteria respectively (Fig. 8). The half-life of RNA pulse-labelled in the presence of factor S was about 360 sec., whereas an eight-fold increase was caused by a combination of virginiamycin M and S.

Table 2. *Synthesis of rRNA and tRNA in the presence of virginiamycin*

Strain and medium as in the legend for Fig. 4. Virginiamycin: none (control); 50 μ g. factor M; 50 μ g. factor S; and 2.5 μ g. both factors/ml., added 3 min. before the isotopes. Labelling: [3 H]uracil (specific activity 12 c/mmole, 1.04 μ C/ml., f.c.) added at 15 min. interval to three sets of samples. Bacteria labelled with 15 min. pulses were disrupted by compression, treated with DNase and extracted with phenol. RNA was fractionated in a sucrose density gradient. Absorbance ($A_{265 \text{ m}\mu}$) was continuously monitored, and TCA-insoluble radioactivity of fractions, pooled according to the scheme of Fig. 6A, was collected on micropore filters and counted.

Virginia-mycin*				Radioactivity incorporated into RNA (acid-insoluble counts/min. $\times 10^{-3}$ [3 H]uracil $\times$ ml. $^{-1}$)								Ratios	
Factor	(μ g./ml.)	Label- ing†	Tur- bidity‡	>23S (A)	23S (B)	16S (C)	10S (D)	4S (E)	<4S (F)	Total (G)	% age var. total RNA (H)	rRNA tRNA (I)	
—	—	2-17	51	2.92 (3.0)	25.17 (26.0)	19.82 (20.5)	13.05 (13.5)	25.41 (26.3)	10.30 (10.6)	96.67 (100)	—	1.77	
M	50	—	35	10.65 (6.7)	33.79 (21.1)	31.28 (19.6)	25.76 (16.1)	45.49 (28.4)	12.59 (7.9)	159.56 (100)	+65.1	1.43	
S	50	—	34	4.65 (6.3)	12.97 (17.4)	15.93 (21.5)	10.26 (13.8)	26.18 (35.3)	4.25 (5.7)	74.24 (100)	-23.2	1.10	
MS	2.5	—	36	14.66 (6.3)	45.32 (19.3)	41.12 (17.6)	28.76 (12.3)	87.87 (37.5)	16.65 (7.1)	234.38 (100)	+142.5	0.98	
—	—	14-29	64	5.64 (3.5)	47.81 (29.9)	30.81 (19.3)	27.42 (17.1)	39.17 (24.5)	9.83 (6.2)	160.68 (100)	—	2.01	
M	50	—	49	4.40 (4.9)	13.67 (15.1)	23.13 (26.0)	17.66 (19.9)	25.31 (28.5)	4.73 (5.3)	88.90 (100)	-44.7	1.45	
S	50	—	48	4.12 (5.0)	14.66 (17.5)	15.95 (19.2)	14.32 (17.3)	29.60 (35.7)	4.38 (5.3)	83.03 (100)	-48.3	1.03	
MS	2.5	—	51	8.83 (5.1)	31.30 (17.7)	47.36 (26.8)	25.58 (14.5)	57.40 (32.5)	6.29 (3.6)	176.76 (100)	+10.1	1.37	
—	—	22-37	77	3.15 (1.5)	71.32 (34.1)	48.08 (23.1)	24.28 (11.6)	46.30 (22.2)	15.58 (7.5)	208.71 (100)	—	2.58	
M	50	—	57	10.57 (11.9)	22.55 (25.5)	21.29 (24.1)	7.41 (8.4)	18.44 (20.9)	8.06 (9.1)	88.32 (100)	-57.6	2.38	
S	50	—	58	6.91 (10.8)	15.83 (24.7)	12.17 (19.0)	7.30 (11.4)	17.95 (28.0)	3.93 (6.1)	64.09 (100)	-69.3	1.56	
MS	2.5	—	61	10.85 (23.8)	7.16 (15.7)	4.53 (10.0)	7.67 (16.9)	13.04 (28.6)	2.22 (4.9)	45.47 (100)	-78.2	0.90	

* Experimental conditions as in the legend for Fig. 8A. † Length of labelling period after addition of virginiamycin. ‡ Turbidity of the harvested culture. (H) Percentage variation of total acid-insoluble polynucleotides (G) with respect to the control. (I) (B+C)/E. In parentheses = percentage distribution of total RNA radioactivity (G) among RNA peaks.

Whilst there were differences in the turnover rates of different species of RNA, the ultracentrifugal patterns of RNA extracted from pulse-labelled virginiamycin-treated bacteria did not differ basically from those obtained in the absence of the anti-

Fig. 6. For legend see foot of facing page.

Fig. 7

Fig. 8

Fig. 7. Metabolic stability of RNA labelled before incubation with virginiamycins. Strain and medium as in the legend for Fig. 5. Labelling: growth for 2 generations in the presence of [^3H]uridine (specific activity 37.3 mc/mg., 5 $\mu\text{C}/\text{ml.}$, f.c.). Harvested bacteria were grown for two generations in unlabelled medium containing 200 $\mu\text{g.}$ of uridine/ml. To a sample of the culture 50 $\mu\text{g.}$ factor M/ml. were added, and growth was allowed to continue at 37°. Samples of the cultures were withdrawn at intervals for nucleic acid analysis, and a sample collected after 30 min. of contact with the antibiotic is shown. Other details as for Fig. 6.

Fig. 8. Synthesis and turnover of pulse-labelled RNA. Strain and medium as in the legend for Fig. 2. Control $\bigcirc-\bigcirc$; virginiamycin M 100 $\mu\text{g.}/\text{ml.}$ $-\triangle-$, added 10 min. before the isotopes. Labelling (time 0): [^3H]uracil (specific activity 12 c/mmole, 2.86 $\mu\text{C}/\text{ml.}$, f.c.). Chasing (at 45 sec.): 100 $\mu\text{g.}$ of unlabelled uracil and 10 $\mu\text{g.}$ actinomycin D/ml. TCA-insoluble radioactivity precipitated in the presence of 200 $\mu\text{g.}$ unlabelled uracil and 100 $\mu\text{g.}$ BSA/ml., collected on micropore filters and counted.

Fig. 6. Fractionation in sucrose density gradients of RNA labelled in the presence of virginiamycin. Strain and medium as in the legend for Fig. 4. A: no virginiamycin. B and C: 50 $\mu\text{g.}$ virginiamycin M. Labelling: [^3H]uracil (specific activity 12 c/mmole) added to the cultures (1.04 $\mu\text{C}/\text{ml.}$, f.c.) either 3 min. (A and B) or 30 min. (C) after virginiamycin. Bacteria were harvested by centrifugation 15 min. after addition of isotopes, disrupted by compression, treated with DNAase and extracted with phenol and ether. RNA was fractionated by centrifugation in sucrose gradient, absorbance at 260 $\text{m}\mu$ was continuously monitored, and TCA-insoluble radioactivity of the fractions was collected on micropore filters and counted.

biotic. In both cases most of the label of a 1 min. pulse was found in rRNA precursors, and little of it in tRNA, as shown by the overlapping of the curve of radioactivity and the 23S and 16S absorbance peaks. The main difference was that the amount of labelled precursors incorporated into the 23S rRNA peak was proportionally lower in the presence than in the absence of streptomycin.

Virginiamycin thus alters the turnover—not the formation—of pulse-labelled RNA, although some early alterations of the synthesis of 23S precursor RNA could be observed in bacteria incubated with the antibiotic.

Fig. 9. Ultracentrifugal fraction of methylated and total RNA, labelled and chased in the presence of virginiamycins. Strain A26. Medium: OS, supplemented with 5 mg. Glc, 4 μ g. Trp, 8 μ g. Leu, 40 μ g. U and 200 μ g. ECD/ml. A: control. B and C: 50 μ g. virginiamycin M/ml. (added at time 0). Eight min. pulses of [$6\text{-}^3\text{H}$]uracil (specific activity 10 c/mmole, 3.130 μ Ci/ml., f.c.) and [^{14}C]methyl-L-methionine (specific activity 24.6 mc/mmole, 0.156 μ Ci/ml., f.c.) given 3 min. later. At the end of labelling period one sample of the culture was cooled with dry ice and treated with NaN_3 (B); another was chased for 10 min. with 200 μ g. of uracil of methionine and 1 mg. of ECD/ml. (C). Harvested bacteria were lysed enzymically, digested with DNase, and extracted with SDS and phenol at 4°. RNA was precipitated and then fractionated as in the legend for Fig. 6 and TCA-insoluble ^{14}C (—□—) and ^3H (—○—) radioactivities of the fractions were counted and plotted together with the absorption tracing (—).

Metabolism of methylated RNA in the presence of virginiamycins. Data presented in the previous sections indicate that bulk RNA made in the presence of virginiamycins might be unstable and undergo turnover. More direct evidence for turnover was gathered by following the fate of methylated RNA. Since rRNA is methylated after formation of the chain (Borek, 1963; Gordon & Boman, 1964; Gordon, Boman & Isaksson, 1964), measure of methylated and total RNA after periods of labelling and chasing might furnish a direct proof of turnover without use of actinomycin D.

Exponentially multiplying organisms were incubated with single and both virginiamycins for different periods, labelled for 8 min. with [^{14}C]methyl-L-methionine (as

methyl group donor) and [³H]uracil (as total RNA marker), and either extracted or chased with an excess of unlabelled methionine and uracil. RNA was fractionated by ultracentrifugation, and the acid-insoluble ¹⁴C and ³H radioactivities of single fractions were measured.

Ultracentrifugal patterns of methylated and total RNA from labelled bacteria are shown in Fig. 9A, B and C. Control samples show two well-defined peaks of ¹⁴C and ³H radioactivities overlapping the 23S and 16S absorbance peaks of rRNA, and no evident sign of turnover upon chasing. In bacteria incubated for a few minutes with either virginiamycin, little or no 23S RNA was methylated nor did it appear to be ³H-labelled (Fig. 9B). Upon chasing—still in the presence of virginiamycin—some ¹⁴C radioactivity was released from the rRNA peaks and could be recovered in the low molecular weight region (Fig. 9C).

Table 3. *Metabolism of methylated RNA in the presence of virginiamycins*

Experimental conditions as in the legend for Fig. 9. Samples within each RNA peak were pooled according to the scheme of Fig. 6A. ¹⁴C and ³H TCA-insoluble radioactivity was measured.

			Radioactivity					Ratio ¹⁴ C/ ³ H in rRNA
Virginia- mycins (μ g./ml.)	Labelling* (min.)	Chasing (min.)	TCA-insol. cpm $\times 10^{-3}$ [¹⁴ C]methyl-L-methionine in RNA					
			23S	16S	10S	4S	< 4S	
—	2-10	—	4.12	5.48	2.25	6.87	8.06	0.380
M 50		10-20	5.02	7.20	1.96	6.78	6.53	0.372
		—	3.14	3.37	1.98	7.11	3.95	0.186
S 50		10-20	1.01	1.38	1.34	9.07	2.50	0.144
		—	3.34	3.91	2.90	11.04	1.41	0.187
M+S 5		10-20	1.33	2.36	1.87	10.08	1.03	0.176
		—	3.08	3.98	2.91	8.81	2.28	0.158
		10-20	3.46	2.80	2.74	6.37	2.72	0.163
—	15-23	—	4.67	4.90	2.45	7.81	4.40	0.313
M 50		23-33	6.47	7.32	3.11	13.05	4.37	0.301
		—	1.12	1.05	0.74	4.64	0.80	0.143
S 50		23-33	1.14	0.84	0.90	6.48	1.35	0.167
		—	1.36	1.56	1.03	4.75	0.91	0.161
M+S 5		23-33	1.07	2.58	1.36	7.29	1.24	0.235
		—	1.32	1.16	0.72	4.42	0.17	0.110
		23-33	0.77	0.55	0.61	4.05	4.05	0.123

* [¹⁴C]methyl-L-methionine and [6-³H]uracil.

Samples within each RNA peak were pooled according to the scheme of Fig. 6A, ¹⁴C and ³H radioactivities of each fraction were measured, and the results are tabulated in Table 3. It is clear that the rRNA which was methylated in the presence of virginiamycins underwent turnover upon chasing, an effect that was quite pronounced after short treatment with single virginiamycins. The inhibition of RNA synthesis occurring after prolonged incubation with the antibiotic obscured turnover at later times.

Thus rRNA (essentially 16S) which was made in the presence of virginiamycin decayed, yielding low molecular weight fragments which could be re-incorporated with high efficiency into rRNA in the presence of virginiamycin. In addition, the degree of methylation of the rRNA which was made in the presence of virginiamycin was lower than that of the controls, as indicated by its ¹⁴C/³H ratio (Table 3).

DISCUSSION

A mixture the two virginiamycins increases by 100-fold the antibiotic activity of the separate factors and has a bactericidal effect on *Bacillus subtilis*. Similar effects were observed with streptogramin (Chabbert & Acar, 1964), pristinamycin (Murat & Pellerat, 1965), and ostreogrycin (Vazquez, 1966*a*) in different micro-organisms. The irreversible loss of viability is preceded by a reversible alteration consisting of a long lag in colony formation on nutrient agar. A similar phenomenon was described by others and called 'bacteriopause' (Videau, 1965). This two-step effect can be explained by data which are presented in another paper of this series, which shows that virginiamycin components bind to cell structures engaged in protein formation. Conceivably, the block of a sufficiently great number of such units may cause an irreversible loss of viability, whereas the inactivation of a smaller proportion of them might allow some growth at slower rate, which causes the inhibitor to be 'diluted out' during subsequent generations. Also, the synergistic antibiotic activity of virginiamycin components can be explained by their common target and complementary action on protein synthesis.

Inhibition of protein formation by virginiamycin may be the result of a block of either transcription or translation: in the first case synthesis of both RNA and proteins will be prevented, whereas in the second case only RNA would be synthesized. Data presented in this paper show that short periods of incubation with virginiamycin, which are able to stop amino acid incorporation completely, have little or no effect on the formation of polynucleotide chains: a block of transcription can, thus, be ruled out. Such a conclusion, which agrees with previous reports on ostreogrycins (Yamaguchi & Tanaka, 1964; Vazquez, 1966*a*), has received further confirmation by a study of virginiamycin action in *Bacillus subtilis* infected with virus 2C. In this system, virginiamycin specifically blocks the synthesis of nucleic acid which depends on an active protein formation (Cocito, 1969).

Data of Table 2 and Fig. 5 show that formation of ribosomal RNA is stimulated by short incubation with virginiamycins and inhibited after complete block of protein synthesis. Similar observations were made by Kurland & Maaløe (1962) in *Escherichia coli* treated with chloramphenicol. These authors conclude that proteins are required for rRNA formation, not for tRNA synthesis, as well as for the regulation of the balance between RNA species. Data presented in this work are in very good agreement with their hypothesis.

Figure 7 shows that virginiamycins interfere with the decay of pulse-labelled RNA. Decay of mRNA does not depend on protein synthesis (since it occurs in the absence of polypeptide chain formation: Fan, Higa & Levinthal, 1964), but on the incorporation of messenger into, and its release from, the polysomes. The joint inhibition of messenger decay and protein synthesis by virginiamycin could then be explained by a block of translation at the polysome level, an interpretation that will be proved in a further paper (C. Cocito, to be published).

The usual labelling-chasing techniques have shown a limited turnover of the rRNA which is synthesized in the presence of chloramphenicol, presumably because of the high efficiency of re-utilization of breakdown products of decayed polynucleotides (Neidhardt & Gros, 1957). To circumvent this difficulty, Fan *et al.* (1964) and Nakada (1965) employed actinomycin D: they showed that chloramphenicol- and puromycin-RNAs are degraded to low molecular weight material when *de novo* RNA synthesis is prevented. Conclusions drawn by the combined use of inhibitors of transcription

and translation can be criticized, however; consequently, in the present work, use was made of a double labelling technique (Dubin & Elkort, 1965). Since unmethylated rRNA is likely to be synthesized during transcription, re-incorporation of a methylated fragment might not occur at all, or might occur with lesser efficiency than an unmethylated one. Data reported in Table 3 and Fig. 8 furnish a direct demonstration that the rRNA which is made in the presence of virginiamycin is unstable and undergoes turnover.

[¹⁴C]methyl group tracing in the control samples (Fig. 8) shows a higher peak in the 16S than in the 23S region. This is in agreement with the finding that 16S RNA contains 20% more methylated bases than 23S RNA (Gordon & Boman, 1964).

'Relaxed' and 'chloramphenicol' particles contain undermethylated rRNA and tRNA: for example, under threonine starvation the level of rRNA methylated is 60% and that of tRNA 90% of the control (Gordon *et al.* 1964). A similar conclusion can be drawn for virginiamycin. Since the stability of rRNA apparently depends on the degree of methylation, undermethylated rRNA is expected to be unstable and undergo turnover. Moreover, since mRNA, unlike rRNA, is not methylated, the hypothesis can be formulated that RNA function also depends on the degree of methylation (see Otaka, Osawa & Sibatani, 1964) and will be altered by virginiamycin.

Finally, the unstable RNA fraction indicated in this work as 'pulse-labelled' RNA presumably includes messenger RNA and decaying rRNA: virginiamycins increase the half-life of such 'pulse-labelled' RNA by inhibiting the decay of RNAs capable of a messenger function, and by rendering unstable the RNAs which are endowed with metabolic stability under physiological conditions. Similar observations were made by others with the RNA produced during methionine starvation by 'relaxed' mutants of *Escherichia coli* (Nakada, Anderson & Magasanik, 1964).

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