the finding, described in the accompanying paper (Sarkar et al., 1979), that gramicidin interacts with the DNA binding site of RNA polymerase. Finally, it should be noted that the heptapeptide Z-L-Trp-(D-Leu-L-Trp)$_3$-NH(CH$_2$)$_2$OH, the octapeptide Z-(D-Leu-L-Trp)$_4$-NH(CH$_2$)$_2$OH, and the nonapeptide Z-L-Val-(D-Leu-L-Trp)$_4$-NH(CH$_2$)$_2$OH are more effective than gramicidin in restoring the normal spore phenotype and in inhibiting RNA polymerase. The fact that completely synthetic oligopeptides can effectively substitute for the natural product is of considerable interest and opens the way for the development of affinity-label probes to study the mechanism of action of gramicidin both in vivo and in vitro.

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Studies on the Mechanism and Specificity of Inhibition of Ribonucleic Acid Polymerase by Linear Gramicidin†

Nilima Sarkar, Donna Langley, and Henry Paulus*

ABSTRACT: In order to define the mechanism by which linear gramicidin modulates transcription during sporulation of *Bacillus brevis*, we have examined the kinetics and the specificity of inhibition of ribonucleic acid (RNA) synthesis by the peptide antibiotic. The inhibition by gramicidin was competitive with respect to the duplex deoxyribonucleic acid (DNA) template, indicating interference with the binding of DNA to the enzyme. Gramicidin inhibition was observed with both procaryotic and eucaryotic RNA polymerases, but not with DNA polymerase, suggesting that the gramicidin binding site was conserved during evolution and probably corresponds to the DNA binding site of RNA polymerase. σ factor had no significant effect on the inhibition by gramicidin. At high DNA concentrations, inhibition was incomplete even at high gramicidin concentrations. Direct evidence for a gramicidin-resistant mode of transcription was obtained by titrating the DNA template with actinomycin D. When ~75% of transcription was blocked by the drug, the residual RNA synthesis was completely refractory to inhibition by gramicidin. This effect was seen only with natural DNA templates and not with synthetic polynucleotides or denatured DNA, suggesting that it involves specific classes of DNA sequences. Unlike actinomycin D, other drugs which inhibit RNA synthesis by complexing with DNA could not modulate gramicidin inhibition. The most simple interpretation is that gramicidin inhibition occurs selectively at certain classes of promoters which are also most sensitive to blockage by actinomycin D. It appears that by interacting with the DNA binding site, gramicidin can selectively modulate the affinity of RNA polymerase for different promoters.

The studies described in the preceding paper demonstrated that the biological function of gramicidin is not due to its ionophoretic properties but seems rather to be related to its ability to inhibit RNA synthesis. It was therefore of interest to study in some detail the effect of the antibiotic on the transcription process. We had shown earlier that gramicidin inhibits the interaction of RNA polymerase with DNA but has no effect on RNA chain initiation and elongation (Paulus & Sarkar, 1976; Sarker et al., 1977). In this paper, we present a more detailed investigation on the kinetics and specificity of inhibition of RNA synthesis by gramicidin. Our results suggest that the peptide antibiotic interacts with a DNA binding site on RNA polymerase.

Materials and Methods

Enzymes. RNA polymerase was purified from exponentially growing cells of *Bacillus brevis* ATCC 8185 as described

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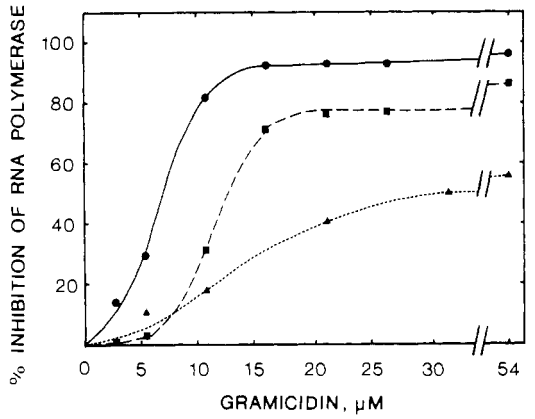

FIGURE 1: Inhibition of RNA synthesis by gramicidin at different DNA concentrations. RNA polymerase (0.5 μg) from *B. brevis* was assayed with 0.0025 A_{260}/mL (●), 0.01 A_{260}/mL (■), or 0.04 A_{260}/mL (▲) of *B. brevis* DNA at various concentrations of gramicidin.

previously (Paulus & Sarkar, 1976). Wheat germ RNA polymerase II was purchased from Miles. RNA polymerase from *Escherichia coli* MRE 600 was obtained from Boehringer as the holoenzyme and resolved into core enzyme and σ factor by chromatography on phosphocellulose as described by Burgess & Travers (1971). DNA polymerase I from *E. coli* was the gift of Dr. C. C. Richardson.

DNA Templates. DNA from *B. brevis* and from bacteriophage ϕ_e was isolated by phenol extraction (Marmur, 1961) and further purified by CsCl density gradient centrifugation. DNA from bacteriophage T7 was the gift of Dr. R. Kolodner. Salmon sperm DNA was obtained from Calbiochem and was denatured by titrating to pH 12.4 with 1 N NaOH at room temperature, followed by rapid neutralization after 10 min with 1 N HCl. Poly(dG-dC) was purchased from PL-Laboratories, and poly(dA-dT) was obtained from Boehringer.

Antibiotics. Gramicidin (85% gramicidin A, 10% gramicidin B, and 5% gramicidin C) was obtained from Nutritional, chromomycin A, distamycin A, and daunomycin were from Boehringer, and actinomycin D and ethidium bromide were from Calbiochem. Netropsin was a gift from Dr. E. L. Patterson, Lederle Laboratories, and Hoechst 33258 was donated by Dr. S. K. Sengupta. N^2-Benzylactinomycin D, 7-nitroactinomycin D, 3-ethyl-7-ethoxyactinomycin D, N^2-(10-aminodecyl)actinomycin D, and 1,9-(diethylaminoethylcarbamoyl)actinocin were synthesized and generously provided by Dr. S. K. Sengupta.

Other Materials. [5-^{3}H]UTP and [5-^{3}H]CTP were purchased from New England Nuclear.

RNA Polymerase Assay. Bacterial RNA polymerases were assayed as described previously (Sarkar et al., 1977), and wheat germ RNA polymerase was assayed as described by Jendrisak & Burgess (1975). Gramicidin was added as a solution in ethylene glycol, and an equivalent concentration of solvent (5% by volume) was added to all incubation mixtures. RNA synthesis was always initiated by the addition of RNA polymerase.

Results

Effect of DNA Concentration on Gramicidin Inhibition. At low DNA concentrations RNA synthesis was almost completely inhibited by relatively low concentrations of gramicidin, whereas higher concentrations of the antibiotic were required for inhibition when the DNA concentration was increased (Figure 1). The response to gramicidin was strikingly sigmoid, but it is not clear whether this is a real phenomenon or an artifact due to nonspecific adsorption of small amounts of the antibiotic to the walls of the reaction vessel or one of the components of the incubation mixture. At very high concentrations of DNA, complete inhibition was not observed even at high levels of gramicidin. Double-reciprocal plots of the effect of DNA concentration on RNA synthesis in the absence and presence of gramicidin indicated that the antibiotic inhibited competitively with respect to DNA for all duplex DNA templates studied, except with poly(dG-dC) where inhibition was of a mixed type (parts A–E of Figure 2). In contrast, inhibition of transcription of single-stranded DNA by gramicidin was largely uncompetitive (Figure 2F).

Inhibition of RNA Polymerases from Different Sources. Like the RNA polymerase from *B. brevis*, *E. coli* RNA polymerase as well as RNA polymerase II from wheat germ was subject to competitive inhibition by gramicidin when double-stranded DNA was used as template (parts A and C of Figure 3). On the other hand, the response of these enzymes to gramicidin was quite different when denatured DNA was transcribed. No inhibition was seen under these conditions with *E. coli* RNA polymerase (Figure 3B), while wheat germ RNA polymerase was inhibited in a competitive manner (Figure 3D). No significant inhibition by gramicidin was seen with *E. coli* DNA polymerase I (Figure 4).

Effect of σ Factor on Gramicidin Inhibition. E. coli RNA polymerase was resolved into core enzyme and σ factor by chromatography on phosphocellulose. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed no σ factor in the preparation of RNA polymerase core thus obtained, under conditions where 0.05 equiv would easily have been detected (not shown). As shown in Figure 5, there was no significant difference in the sensitivity to gramicidin between RNA polymerase core and holoenzyme. As with the holoenzyme, the core RNA polymerase was inhibited competitively with respect to DNA (not shown).

Modulation of Gramicidin Inhibition by Actinomycin. In the presence of actinomycin D or certain of its derivatives, the ability of gramicidin to inhibit the residual RNA synthesis was strikingly modified. Figure 6 shows the titration of RNA synthesis with N^2-benzylactinomycin D in the absence and presence of gramicidin. At 0.5 μg/mL of the actinomycin derivative, RNA synthesis was reduced to 20% but was refractory to inhibition by gramicidin. At higher actinomycin concentrations, RNA synthesis progressively declined to zero and was actually stimulated by the addition of gramicidin. The effect of increasing gramicidin concentration at a level of actinomycin which produced 70% inhibition of transcription is shown in Figure 7. Under conditions where gramicidin alone inhibited RNA synthesis up to 60%, the peptide antibiotic had no effect when added in combination with actinomycin.

The modulation of gramicidin inhibition by actinomycin D was seen both with *B. brevis* and *E. coli* RNA polymerases, provided a natural DNA template was used (Table I). The effect was seen regardless of whether the incorporation of [^{3}H]UMP or [^{3}H]CMP was studied and was therefore not simply due to the restriction of transcription to AT-rich regions of the DNA template. When single-stranded DNA or the synthetic polynucleotides poly(dA-dT) and poly(dG-dC) were used as templates, actinomycin D had no effect on gramicidin inhibition. If the effect of actinomycin D on gramicidin inhibition had been due to some kind of interaction of actinomycin D with gramicidin, no such template specificity would have been expected.

The results summarized in Table II show that a number of actinomycin D analogues which were modified in the actinocin

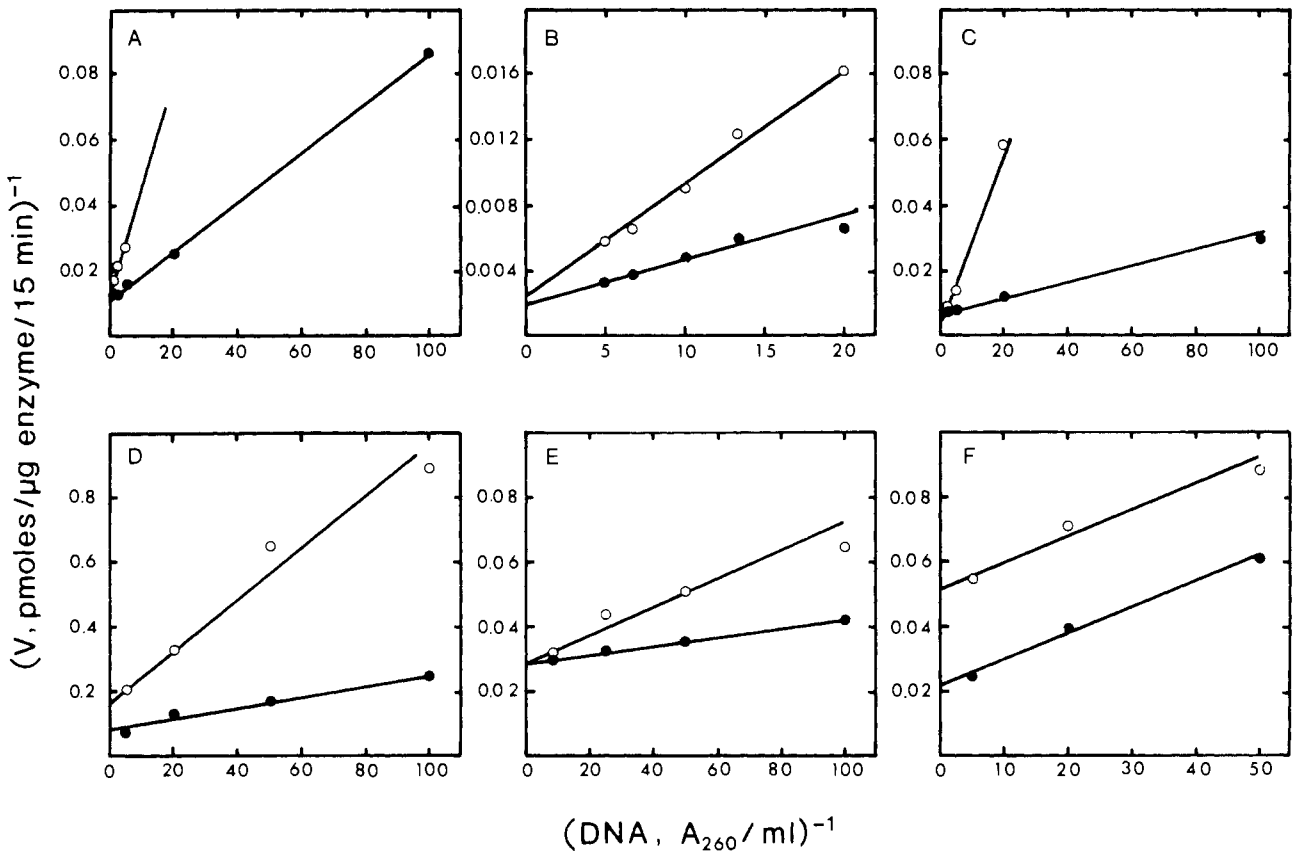

FIGURE 2: Double-reciprocal plots of the effect of DNA concentration on the rate of RNA synthesis in the absence and presence of gramicidin. RNA polymerase (0.5 μg in parts A, B, C, E, and F and 0.9 μg in part D) from *B. brevis* was assayed at the DNA concentrations indicated in the absence (●) or presence (○) of gramicidin (27 μM in part A and 54 μM in parts B–F). The following DNA templates were used: (A) *B. brevis* DNA; (B) bacteriophage ϕ_e DNA; (C) bacteriophage T7 DNA; (D) poly(dG-dC); (E) poly(dA-dT); and (F) denatured salmon sperm DNA.

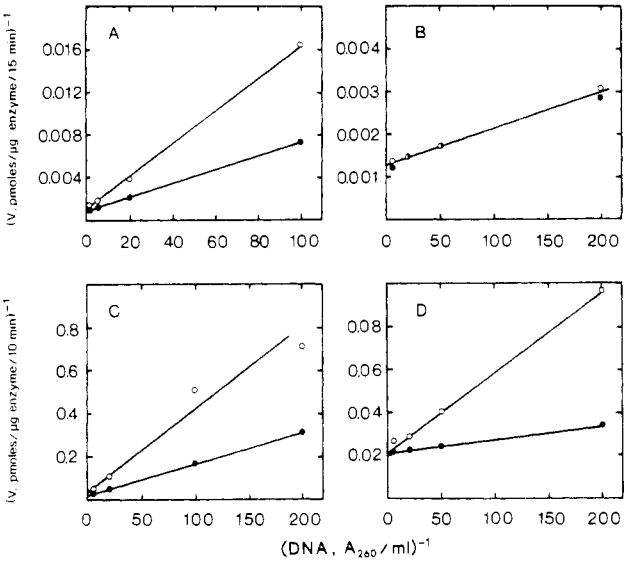

FIGURE 3: Effect of gramicidin on RNA synthesis by RNA polymerases from *E. coli* and wheat germ. Double-reciprocal plots of the effect of DNA concentration on RNA synthesis by (A) 0.1 or (B) 0.01 μg of *E. coli* RNA polymerase and by (C) 1.36 or (D) 0.27 μg of wheat germ RNA polymerase II, in the absence (●) or presence (○) of 54 μM gramicidin. The following DNA templates were used: (A) bacteriophage T7 DNA; (B and D) denatured salmon sperm DNA; and (C) calf thymus DNA.

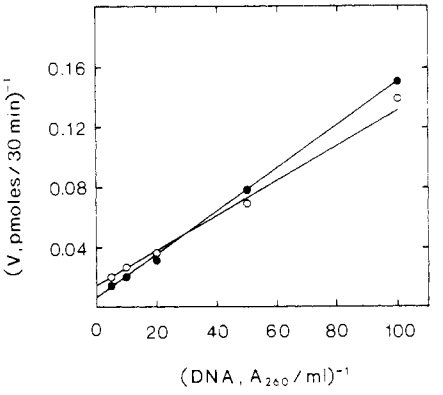

FIGURE 4: Effect of gramicidin on DNA synthesis by *E. coli* DNA polymerase I. DNA synthesis was measured in the presence of 0.05 units of DNA polymerase I, 20 μM [^{3}H]dTTP, 33 μM each dATP, dCTP, and dGTP, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 25 μg/mL of gelatin, "activated" salmon sperm DNA (Sigma) as indicated, and no (●) or 54 μM (○) gramicidin.

chromophore were able to modulate gramicidin inhibition, even in cases where the ability to bind to DNA as measured by inhibition of RNA synthesis was reduced more than 100-fold. On the other hand, an actinocin derivative which lacked the peptide chains and all other DNA binding or intercalating agents tested had no such effect.

Discussion

The effect of gramicidin on RNA synthesis at different DNA concentrations manifested itself in double-reciprocal plots that intersected on the ordinate. Such a kinetic behavior could arise either from interaction of gramicidin with DNA or from the binding of gramicidin to RNA polymerase in a way which interferes with DNA binding. Because gramicidin is known not to bind to DNA (Ristow et al., 1975), the first

Table I: Effect of Enzyme and Template Source on the Modulation of Gramicidin Inhibition of RNA Synthesis by Actinomycin D

RNA polymerase (μg)	DNA template (A_{260} units)	[^{3}H]UMP incorpn (pmol/15 min) with[a]				% inhibn by G	
		none	G	A	A + G	−A	+A
B. brevis (0.5)	*B. brevis* (0.02)	16.7	8.7	4.5[b]	5.2[b]	48	(−14)
B. brevis (0.5)	*B. brevis* (0.02)	41.0[c]	20.6[c]	8.6[b,c]	12.8[b,c]	50	(−49)
B. brevis (0.9)	ϕ_e phage (0.064)	309	186	105[b]	107[b]	40	(−3)
B. brevis (0.5)	T7 phage (0.4)	105	58	47[d]	45[d]	45	4
E. coli (0.05)	*B. brevis* (0.02)	16.5	14.1	2.8[b]	4.5[b]	15	(−59)
E. coli (0.1)	T7 phage (0.02)	62	44	12.5[e]	16.2[e]	29	(−29)
B. brevis (0.9)	poly(dG-dC) (0.018)	25.0[c]	9.6[c]	12.9[c,f]	7.7[c,f]	61	40
B. brevis (0.5)	poly(dA-dT) (0.015)	26	17	24[e]	15[e]	35	38
B. brevis (0.5)	denatured salmon sperm (0.2)	35	23	34[e]	19[e]	34	44

[a] G = gramicidin (54 μM); A = actinomycin. [b] 0.15 μg/mL of actinomycin D. [c] Incorporation of [^{3}H]CMP. [d] 0.03 μg/mL of actinomycin D. [e] 0.1 μg/mL of actinomycin D. [f] 0.9 μg/mL of actinomycin D.

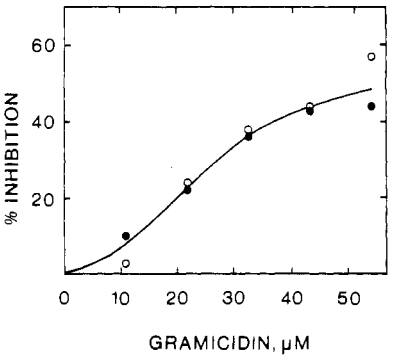

FIGURE 5: Inhibition by gramicidin of RNA polymerase holoenzyme and core. *E. coli* RNA polymerase holoenzyme (0.016 μg) (●) and core enzyme (0.16 μg) (○) were assayed in the presence of 0.004 A_{260}/mL of calf thymus DNA at the gramicidin concentrations indicated.

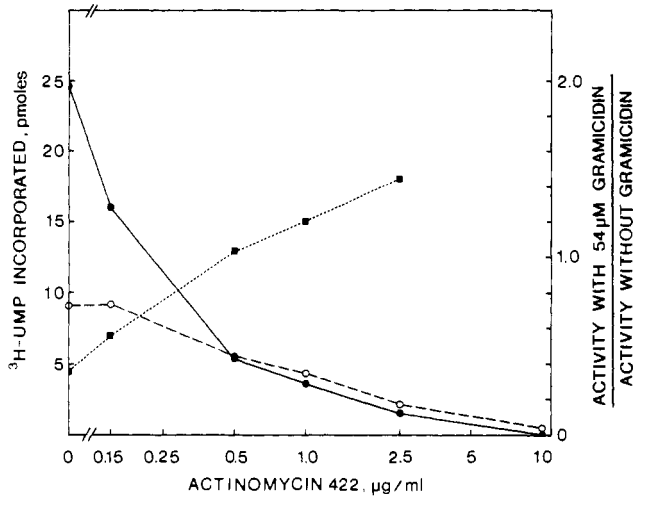

FIGURE 6: Effect of actinomycin D and gramicidin on the activity of RNA polymerase. RNA polymerase (0.45 μg) from *B. brevis* was assayed with *B. brevis* DNA (0.05 A_{260}/mL) at the concentrations of N^2-benzylactinomycin D indicated in the absence (●) and presence (○) of 54 μM gramicidin. (■) Activity ratio in the presence and absence of gramicidin.

possibility can be excluded and the competitive inhibition of RNA polymerase by gramicidin must therefore be due to the direct interaction of gramicidin with the enzyme. Such an interaction could occur directly at the DNA binding site or at an allosteric site which modulates the affinity for DNA. Even though these possibilities cannot be distinguished kinetically, comparison of the sensitivity to gramicidin of various RNA polymerases should reveal whether the gramicidin binding site is unique to *B. brevis* or whether it has been conserved during evolution. It was found that gramicidin

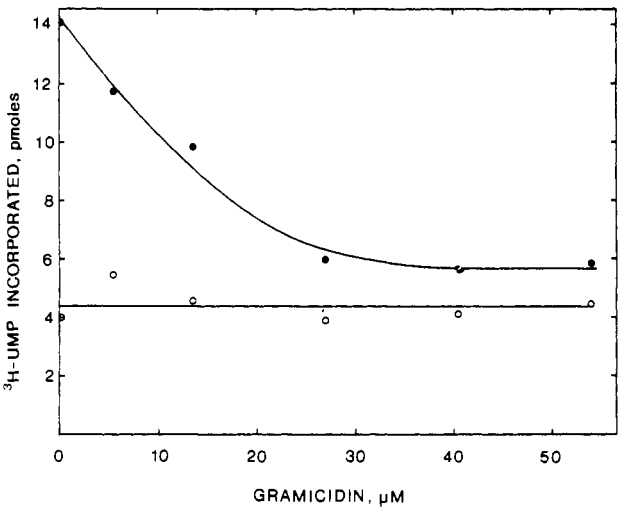

FIGURE 7: Effect of actinomycin D on the inhibition of RNA synthesis by gramicidin. RNA polymerase (0.45 μg) from *B. brevis* was assayed with *B. brevis* DNA (0.05 A_{260}/mL) at the concentrations of gramicidin indicated in the absence (●) and presence (○) of 1 μg/mL N^2-benzylactinomycin D.

Table II: Effect of DNA Complexing Agents on Inhibition of RNA Synthesis by Gramicidin[a]

CA[b] (μg/mL)	% inhibn	% inhibn by gramicidin (54 μM)	
		−CA	+CA
actinomycin D (0.15)	73	48	(−14)
N^2-benzylactinomycin D (1)	74	38	(−27)
7-nitroactinomycin D (1.8)	76	35	(−81)
3-ethyl-7-ethoxy-actinomycin D (8.5)	43	46	0
N^2-(10-aminodecyl)-actinomycin D (20)	80	38	(−38)
1,9-(diethylaminoethyl-carbamoyl)actinocin (10)	75	37	55
chromomycin A (1)	78	47	68
distamycin A (0.4)	60	42	42
netropsin (1)	86	48	45
daunomycin (1)	75	45	53
ethidium bromide (5)	84	50	62
Hoechst 33258 (3)	73	49	43

[a] RNA synthesis was assayed with *B. brevis* RNA polymerase (0.5 μg) and *B. brevis* DNA (0.2 μg/mL) in the absence and presence of the DNA complexing agent at the concentration indicated and without and with 54 μM gramicidin. [b] CA = complexing agent.

inhibited also the RNA polymerase from *E. coli* as well as RNA polymerase II from wheat germ, indicating that the antibiotic binds to a site common to RNA polymerases from diverse species and therefore most likely essential to RNA

polymerase function. This would presumably be the DNA binding site.

It should be noted that with single-stranded DNA as template, the pattern of inhibition was more complex and depended on the source of RNA polymerase. With the enzyme from *B. brevis*, inhibition was predominantly uncompetitive, suggesting that gramicidin has a higher affinity for the enzyme–DNA complex than for the free enzyme. This requires at least two DNA binding sites on RNA polymerase, a conclusion which is supported by the studies of Pfeffer et al. (1977) on the interaction of heparin with the RNA polymerase–DNA complex. In fact, our earlier finding that gramicidin destabilizes the binary complex between RNA polymerase and DNA provides direct evidence for a gramicidin binding site on this complex (Sarkar et al., 1977). However, with duplex DNA templates, the affinity of gramicidin for this second site must be relatively weak in order to account for the competitive inhibition observed under those conditions. The affinity of gramicidin for *B. brevis* RNA polymerase would thus increase in the following order: enzyme–duplex DNA complex, free enzyme, and enzyme–single stranded DNA complex. The observation that the patterns of gramicidin inhibition with single-stranded DNA as template were different with RNA polymerases from *E. coli* and wheat germ would suggest that the binding of gramicidin to the enzyme–single-stranded DNA complex is relatively much weaker with these RNA polymerases.

It may at first sight be unexpected that an uncharged polypeptide would interact with a polynucleotide binding site on an enzyme. The results with gramicidin analogues described in the preceding paper (Paulus et al., 1979) have shown that the interaction of gramicidin with RNA polymerase depends primarily on the carboxyl-terminal portion of the antibiotic, containing the highly nonpolar repeating sequence D-leucyl-L-tryptophan. Energetic considerations and model building have indicated that a plausible structure of Z-(D-Leu-L-Trp)$_4$-NH(CH$_2$)$_2$OH in aqueous solution is a helix in which the indole side chains are stacked in the interior nearly perpendicular to the helix axis (W. Veatch, unpublished experiments). The helical parameters are very similar to those of a single-stranded deoxyribopolynucleotide in the helical "B" form (Hingerty & Broyde, 1978). Moreover, the hydrogen-bonding groups of the peptide backbone are in a similar orientation relative to the planar indole moieties as the hydrophilic phosphodiester groupings are relative to the nucleotide bases. The accommodation of these chemically diverse substances by the same site on RNA polymerase is therefore sterically quite plausible. In this context, it should be noted that glutamic acid–tryptophan copolymers are quite potent inhibitors of RNA polymerase, presumably by binding to the polynucleotide site, and that the inhibition is greatest when the polymers contain glutamic acid and tryptophan in equimolar amounts (Krakow, 1974).

The presence of σ factor had no effect on the inhibition by gramicidin, suggesting that the σ subunit does not influence the binding of the antibiotic to RNA polymerase. This is of interest because the relationship of σ factor to the DNA binding site is still obscure. σ is not required for the binding of RNA polymerase to DNA, but it reduces the affinity of the enzyme for nonpromoter regions (Hinkle & Chamberlin, 1972), facilitates the formation of the "open" promoter complex (Hinkle & Chamberlin, 1970; Zillig et al., 1970), and alters the specificity of transcription (Travers et al., 1978). Whether these effects are produced by direct interaction with DNA or by conformational changes in RNA polymerase is not clear (Chamberlin, 1976), and cross-linking studies of RNA polymerase to DNA have produced conflicting results (Hillel & Wu, 1978; Okada et al., 1978). Our results clearly show that σ subunit is not the site of gramicidin binding.

In view of the fact that the inhibition of RNA polymerase by gramicidin is competitive with respect to duplex DNA, one would expect differential effects of the antibiotic on the transcription at low- and high-affinity promoters. With whole bacterial DNA as template, where one deals with a broad spectrum of different promoters, it would ordinarily be difficult to distinguish various promoter classes in terms of their sensitivity to gramicidin. However, there are two circumstances in which a selective effect of gramicidin might be seen even with complex DNA templates. One is under conditions where DNA is in large molar excess over RNA polymerase and all enzyme present can be saturated with high-affinity promoters. We found that high DNA concentrations resulted not only in an increase in the amount of gramicidin required for 50% inhibition, as expected for competitive inhibition, but also in a decrease in the maximum extent of inhibition (Figures 1 and 7). This could have been due to the limited solubility of gramicidin, but another explanation would be that transcription at some high-affinity promoters is almost completely resistant to gramicidin.

Another circumstance in which one might expect to see the selectivity of gramicidin inhibition would be a situation where transcription of a whole class of genes is specifically blocked. This was achieved by the use of actinomycin D, an agent which binds selectively to GC base pairs of the DNA template (Goldberg & Friedman, 1971). Our results showed that as RNA synthesis was restricted by the addition of low levels of actinomycin D, an increasingly greater proportion became resistant to inhibition by gramicidin (Figure 6). It appears that actinomycin D preferentially inhibits transcription that is most sensitive to gramicidin and that gramicidin-resistant transcription is relatively insensitive to actinomycin D. This means that gramicidin, like actinomycin D, inhibits transcription most strongly at sites containing GC base pairs, while transcription at promoter regions composed primarily of AT base pairs is relatively resistant to inhibition by gramicidin. Since the formation of the stable RNA polymerase–DNA complex appears to involve the opening of the DNA duplex (Chamberlin, 1976) and therefore occurs most readily at sites of low local melting temperature, i.e., AT-rich sequences, it appears that gramicidin-resistant transcription involves relatively high-affinity promoters. This accords with our interpretation of incomplete inhibition by gramicidin at high DNA concentrations, discussed earlier. Indeed, at relatively high DNA concentrations, the amount of transcription which was insensitive to inhibition by gramicidin in the presence of actinomycin agreed well with the residual transcription seen at high gramicidin concentrations in the absence of actinomycin D (Figure 7).

Our interpretation of the effect of actinomycin D on the inhibition by gramicidin was supported by experiments on its dependence on the DNA template. Actinomycin D modulated gramicidin inhibition only when natural DNA templates were used but not with single-stranded DNA or the synthetic polymers poly(dA-dT) and poly(dG-dC), suggesting the involvement of specific promoters. Moreover, the observation that the transcription of actinomycin-resistant templates, such as denatured DNA and poly(dA-dT), was nevertheless sensitive to gramicidin excluded the trivial explanation that the effect of actinomycin D was due to restriction of RNA synthesis to single-stranded or AT-rich regions of the DNA

template. Further evidence against this possibility came from the observations that actinomycin reduced the inhibition of RNA synthesis also when CTP was the radioactive precursor instead of UTP (Table I) and that complete inhibition could be seen at higher concentrations of actinomycin (Figure 6).

It is of interest to compare the effect of actinomycin D on gramicidin inhibition with that of other DNA complexing agents. The modification of the actinocin chromophore, even when it reduced the ability to bind to DNA more than 100-fold, had no effect on the ability to modulate gramicidin inhibition. On the other hand, an actinocin derivative without peptide moieties had no effect on gramicidin inhibition even though it could still inhibit RNA synthesis. Other drugs which inhibit RNA synthesis by binding to DNA, including chromomycin which interacts specifically with GC base pairs (Behr et al., 1969), were unable to modulate gramicidin inhibition. This suggests that the effect of actinomycin D, at the low concentrations used in our experiments, depends on subtle differences in the nucleotide sequence of various classes of promoters rather than on the overall base composition. In this context, it should be noted that unlike all other agents studied in Table II, actinomycin D is a peptide antibiotic, produced by *Streptomyces antibioticus* during the transition from vegetative growth to sporulation (Katz & Weissbach, 1963). It is tempting to speculate that actinomycin D has evolved to fulfill the same function in the producing organism as the peptide antibiotic gramicidin. If this function consists of inhibiting the expression of vegetative genes whose products are not essential for sporulation (Sarkar & Paulus, 1972), it would not be surprising if actinomycin D had evolved to inhibit transcription, albeit by a different mechanism, at the same class of promoters which are sensitive to gramicidin.

The results presented in this paper reveal what may seem a remarkable lack of specificity in the action of gramicidin. The peptide antibiotic inhibits not only the RNA polymerase from *B. brevis* but also the enzymes from *E. coli* and wheat germ, a eucaryote. Instead of interacting at a specific regulatory site, gramicidin modulates the activity of RNA polymerase by competing for the DNA binding site. If the antibiotic had a specific regulatory function in bacterial sporulation, one might have expected a much more specific mechanism of action to have evolved. However, consideration of the evolutionary constraints on the regulation of bacterial sporulation shows that a more specific mode of regulation could hardly have evolved. This is because the mechanisms that emerged to control sporulation had to be carefully selected as not to interfere with normal growth, and any drastic changes, such as the evolution of a specific site on RNA polymerase for a polypeptide, were almost certainly precluded. It is much more likely that a peptide evolved which could interact with an already existing site on RNA polymerase in a manner as to modulate its activity in the desired direction. The most simple solution would be competition for the DNA binding site which, as we have seen, can lead to the selective inhibition of transcription at specific classes of promoters. On account of the fact that the structure of promoters and of the DNA binding site of RNA polymerase has been conserved during evolution, as shown by the observation that *E. coli* RNA polymerase can initiate specific transcription on eucaryotic chromatin, it would be expected that gramicidin should have similar effects on RNA polymerases from diverse sources. The mode of action of gramicidin on RNA polymerase is thus consistent with the postulated regulatory role in bacterial sporulation. However, a final confirmation of this hypothesis must come from studies of the effect of the peptide antibiotic on the transcription of isolated genes which encode specific vegetative or sporulation functions.

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