# Inhibition of Cell-Free Protein Synthesis by Poly(9-vinyladenine), Poly(1-vinyluracil), and the Corresponding Vinyl Copolymer†

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ABSTRACT: The synthesis of poly(9-vinyladenine-*co*-1-vinyluracil) and the effects of poly(9-vinyladenine), poly(1-vinyluracil), and the copolymer on protein synthesis are described. Although the vinyl analogs did not serve as template, they were potent inhibitors of complementary polynucleotide-stimulated [$^{14}$C]aminoacyl-tRNA binding to ribosomes and [$^{14}$C]-amino acid incorporation into polypeptides. Poly(9-vinyladenine) completely inhibited the poly(U) stimulated binding of [$^{14}$C]Phe-tRNA to ribosomes and [$^{14}$C]phenylalanine incorporation into polypeptides. Likewise, poly(1-vinyluracil) totally inhibited poly(A) stimulated [$^{14}$C]lysine incorporation. However, the copolymer of 9-vinyladenine and 1-vinyluracil was less effective in inhibiting both reactions.

Although many nucleic acid analogs with modified base or carbohydrate moieties have been studied (Roy-Burman, 1970), there have been only a few attempts to prepare compounds with alterations in the phosphodiester backbone (Halford and Jones, 1968; Eckstein and Gindl, 1969). Nucleic acid analogs with the sugar–phosphate backbone replaced by a backbone containing only carbon–carbon linkages have been prepared (Pitha *et al.*, 1971, and references therein). These vinyl polymers differ from polynucleotides by the absence of negatively charged phosphate groups, shorter distances between the bases, and the lack of steric regularity. These differences exclude complete base pairing in any complex of a vinyl polymer with a polynucleotide.

Since the complex of poly(vC) with poly(I) shows antiviral activity (Pitha and Pitha, 1971) and the vinyl polymers interact with the complementary bases in polynucleotides, it was of interest to explore the possible effects of vinyl polymers as templates or inhibitors in a cell-free protein synthesis system. No similar study with this type of analog has been reported except for the recent investigation of polyacrylate and carboxymethylate analogs of nucleic acids (Cowling *et al.*, 1971). Several physicochemical studies were undertaken to correlate the observed biological activity of the vinyl analogs. In addition to the previously prepared vinyl homopolymers, we prepared and studied the corresponding copolymer poly(vA,vU).[1]

## Material and Methods

*Escherichia coli* tRNA and uniformly labeled [$^{14}$C]phenylalanine, -lysine and -valine of specific activities 455, 310, and 260 $\mu$Ci $\mu$mole$^{-1}$ were purchased from Schwarz-Mann, Orangeburg, N. Y. Frozen *E. coli* B cells were obtained from General Biochemicals. Pyruvate kinase and phosphoenol pyruvate (trisodium salt) were obtained from Sigma Chemical Co. Poly(A), poly(U), and poly(A,U) were purchased from Miles Laboratories. The poly(A) was purified by repeated ethanol precipitation to yield high molecular weight material. Homopolynucleotide concentrations were calculated from their absorbance and are reported as moles of base residues. The following extinction coefficients were used for poly(A) 10,100 at 259 nm (pH 7.0), for poly(U) 9430 at 261 nm (pH 7.5) (Holcomb and Tinoco, 1965; Blake *et al.*, 1967), for poly(vA) 9000 at 256 nm (pH 7.0), and for poly(vU) 6000 at 264 nm (pH 7.0) (Pitha *et al.*, 1970, 1971). One $A_{260}$ unit is the quantity of material in 1 ml which will yield an absorbance of 1.0 at 260 nm in a 1-cm path-length cuvet.

*Preparation of Poly(vA,vU).* A suspension of freshly recrystallized 1-vinyl-4-ethoxy-2-pyrimidinone (91 mg) and 9-vinyladenine (51 mg) in distilled water (10 ml) was flushed with nitrogen for 10 min. Then the reaction flask, equipped with a rubber septum cap, was immersed in boiling water. After the suspension dissolved, 1 mg of potassium persulfate in 1 ml of water was injected. Thirty minutes later the mixture was cooled, and 80 ml of ethanol was added. Centrifugation and drying yielded 47 mg of polymer. All bands of the infrared spectrum (KBr pellet) could be attributed to poly(9-vinyladenine-*co*-1-vinyl-4-ethoxy-2-pyrimidinone), and no monomers were detected in the product. Ethoxy groups were removed by dissolving the polymer in 15 ml of 1 N HCl and stirring for 24 hr at room temperature. The resulting suspension was dissolved by adding base. Undissolved material was removed by centrifugation at 16,000*g* for 10 min. The supernatant was neutralized with HCl and the resulting suspension was exhaustively dialyzed against distilled water and recentrifuged. Poly(vA,vU) is soluble in water or dilute salt solutions, but it precipitates at high salt concentrations. The clear supernatant was used as a stock solution (uv maximum at 259 nm, absorbance 160). A similar procedure was used to prepare the other polymers with the exception of poly(vU) which was synthesized in better yields by polymerization in tetrahydrofuran (Pitha *et al.*, 1970).

Base ratios of the products were determined from infrared spectra measured in KBr pellets. Absorptions at characteristic wave numbers, 1690 cm$^{-1}$ for poly(vU) and 1575 cm$^{-1}$ for poly(vA), were compared to the corresponding absorp-

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[1] Abbreviations used are: poly(vA), poly-9-vinyladenine; poly(vU), poly-1-vinyluracil; poly(vA,vU), the copolymer of 9-vinyladenine and 1-vinyluracil.

TABLE I: Polymerization of 9-Vinyladenine and 1-Vinyl-4-ethoxy-2-pyrimidinone.

| Monomer Ratio Pyrimidine:Purine | Concn (mg/ml) | Yield (%) | Uracil Residues in Polymer (%) | Solubility of Polymer[a] |
|---|---|---|---|---|
| 0:1 | 10 | 83 | 0 | s w, s ac |
| 1:4 | 23 | 59 | 44 | i w (0.5),[b] s ac, s d |
| 1:1 | 19 | 26 | 58 | s w, i ac, s d |
| 2:1 | 20 | 33 | 62 | s w (160),[b] i ac |
| 1:0 | 10 | 23 | 100 | s w (130),[b] i ac |

[a] s (soluble), i (insoluble), w (water), ac (dilute aqueous acid), d (dilute aqueous alkali). [b] Absorbancy of solution at uv maximum (1-cm cuvet).

tions in a number of standards, prepared by mixing vinyl homopolymer solutions. The use of homopolymers is critical. Uridine and adenine were also tested as standards but exhibited grossly different widths of the critical bands.

*Spectral Measurements.* Interactions between polymers were studied by the continuous variation method (Job, 1928) at 0.1 mM (base) total concentration in buffered solutions (50 mM sodium chloride–10 mM sodium cacodylate, pH 7.0) using ultraviolet spectra. Eleven reaction mixtures, equidistant in the Job graph, were routinely followed for 1 week at room temperature. For the study of equilibria 1 and 2, 1 mM $MgCl_2$ was added to the buffer. In the melting experiments the temperature was increased by 1°/min, ultraviolet and optical rotatory dispersion spectra were measured at total polymer concentrations ranging from $5 \times 10^{-5}$ to $1 \times 10^{-4}$ M (base), and mixtures were equilibrated for a period longer than 1 day.

*Aminoacylation of tRNA. E. coli* aminoacyl-tRNA synthetase was prepared from the 105,000*g* postribosomal supernatant, which was buffered in 10 mM Tris-HCl (pH 7.8), 5.6 mM 2-mercaptoethanol, 14 mM $MgCl_2$, and 60 mM KCl. Protamine sulfate (1%) was added until no more precipitate formed; the precipitate was removed by centrifugation at 16,000*g* for 10 min. The material which precipitated with ammonium sulfate (pH 7.5) between 30 and 70% saturation was collected by centrifugation at 16,000*g* for 30 min. The precipitate was dialyzed against 0.1 M Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5.6 mM 2-mercaptoethanol, and 50% glycerol for 3 hr and stored in small aliquots at −90°. The reaction mixture for the preparation of [$^{14}$C]aminoacyl-tRNAs contained in 1 ml: 0.1 M Tris-HCl (pH 7.8), 10 mM KCl, 10 mM $MgCl_2$, 5.6 mM 2-mercaptoethanol, 2 mM ATP, 5 μCi of [$^{14}$C]amino acid, 20–50 $A_{260}$ units of tRNA, and aminoacyl-tRNA synthetase (1.2 mg of protein). The mixture was incubated at 37° for 15 min, then 0.1 volume of 1 M sodium acetate (pH 5.0) was added, followed by phenol extraction. The [$^{14}$C]aminoacyl-tRNA was precipitated with ethanol and stored at −90°.

*Ribosomal Binding Assay.* Ribosomes were prepared according to Nirenberg (1963), except that the *E. coli* B cells were lysed by sonication (Insonator Model 1000, Savant Instruments). The binding assay was that of Nirenberg and Leder (1964), and the incubation mixture (0.05 ml) contained: 0.1 M Tris-HCl (pH 7.2), 50 mM KCl, 20 mM $MgCl_2$, 2.6 $A_{260}$ units of ribosomes, 20–50 μmoles of [$^{14}$C]aminoacyl-tRNAs, and polynucleotides, trinucleotides, and vinyl polymers as specified in the figure legends. Codon polynucleotide and inhibitors were always preincubated for 10 min at 24° before the addition of ribosomes. Incubation was carried out at 24° for 20 min, followed by dilution with 3 ml of buffer and filtration on Millipore membranes. Radioactivity was measured in a Nuclear-Chicago Mark II liquid scintillation spectrometer.

*Assay of [$^{14}$C]Amino Acid Incorporation into Polypeptides.* The reaction mixture (0.25 ml) contained: 0.1 M Tris-HCl (pH 7.8), 14 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 0.2 mM GTP, 7.5 mM phosphoenolpyruvate, 2.5 μg of pyruvate kinase, 5.6 mM 2-mercaptoethanol, 100 μM each of 19 [$^{12}$C]amino acids (minus the appropriate [$^{14}$C]amino acid), 0.025 μCi of [$^{14}$C]amino acid, and 0.59 mg of S-30 protein. Template polynucleotides and inhibitors were always preincubated for 10 min at 24° before the addition of S-30 fraction. Polynucleotide and inhibitor concentrations are described in the figure legends. The reaction mixture was incubated at 37° for 15 min. The reaction was stopped with the addition of 3 ml of 10% trichloroacetic acid for polyphenylalanine or with 3 ml of 5% trichloroacetic acid–0.25% sodium tungstate reagent (pH 2.0) for polylysine synthesis (Gardner *et al.*, 1962; Algranati and Lengyel, 1966). Reaction mixtures were then incubated at 90° for 20 min, chilled on ice for 30 min, vigorously agitated, and filtered on Millipore membranes with four washes of 5% trichloroacetic acid for polyphenylalanine synthesis or with trichloroacetic acid–0.25% soidum tungstate for polylysine.

## Results

*Vinyl Polymers and Their Interactions.* In addition to the known homopolymers, we prepared a new series of copolymers of 1-vinyluracil and 9-vinyladenine. In aqueous solutions with persulfate initiation, 9-vinyladenine polymerizes with higher yields than the protected pyrimidine monomer and also predominates in the products (Table I). In a further study, we concentrated on the product with 62% uracil residues. This copolymer is soluble in water, and thus interstrand base pairing is not the dominant mode of association.

The amount of intrastrand base pairing in dilute aqueous solutions of this polymer was investigated by monitoring the changes in hypochromism caused by adding urea (up to 5 M) or by increasing the temperature (up to 85°). In both cases the spectral changes were under 5% of the total absorption, and thus intrastrand base pairing is not extensive. The interactions with other polymers were investigated, but no hypochromic changes were detected in systems consisting of vinyl copolymer, and either poly(U) or poly(A) or poly(vU) or poly (vA). All these results are consistent with the interpretation that in the copolymer a small number of entropically favored intrastrand base pairs enforce a conformation which is unsuitable for any extensive base pairing with other polymers. Consequently, the complexes, if any at all are formed, have only a small amount of intercomponent base pairs, and both components are rather independent. The properties of poly(vA,vU) thus contrast with the easily observable interactions of poly(vA) or poly(vU). Both homopolymers form hypochromic complexes with the specificity determined entirely by base pair formation (Pitha *et al.*, 1970, 1971; Pitha and Pitha, 1970).

In addition to these specificities, data on the position of equilibria in reactions 1 and 2 were required for an under-

TABLE II: Effect of Vinyl Polymers upon [$^{14}$C]Aminoacyl-tRNA Binding to Ribosomes.[a]

| Template (μmole of Base Residues) | Inhibitor (μmole of Base Residues) | [$^{14}$C]Aminoacyl-tRNA Bound | | | |
|---|---|---|---|---|---|
| | | Phe 29.8 pmoles | | Lys 24.2 pmoles | |
| | | pmoles | Δpmoles | pmoles | Δpmoles |
| None | None | 0.334 | 0 | 0.765 | 0 |
| Poly(U) (0.0388) | None | 18.3 | 18.0 | | |
| Poly(U) (0.0388) | Poly(vA) (0.103) | 0.899 | 0.565 | | |
| Poly(U) (0.0388) | Poly(vA,vU) (0.198) | 13.8 | 13.5 | | |
| Poly(A,U) (0.0389) | None | 8.80 | 8.47 | | |
| Poly(A,U) (0.0389) | Poly(vA) (0.370) | 1.31 | 0.976 | | |
| Poly(A,U) (0.0389) | Poly(vU) (0.525) | 7.85 | 7.52 | | |
| Poly(vU) (0.0250) | None | 0.644 | 0.310 | | |
| Poly(vU) (0.250) | None | 0.703 | 0.369 | | |
| Poly(A) (0.0294) | None | | | 8.29 | 7.53 |
| Poly(A) (0.0294) | Poly(vU) (0.0833) | | | 0.629 | −0.136 |
| Poly(A) (0.0294) | Poly(vA,vU) (0.198) | | | 5.61 | 4.85 |
| (A-A-A) (0.0160) | None | | | 4.94 | 4.18 |
| (A-A-A) (0.0160) | Poly(vU) (0.0833) | | | 2.75 | 1.99 |
| (A-A-A) (0.0160) | Poly(vA,vU) (0.198) | | | 3.94 | 3.18 |
| Poly(vA) (0.0123) | None | | | 0.919 | 0.154 |

[a] The incubation mixture (0.05 ml) contained: 0.1 M Tris-HCl (pH 7.2), 50 mM KCl, 20 mM $MgCl_2$, 2.6 $A_{260}$ units of ribosomes and inhibitors, and template and [$^{14}$C]aminoacyl-tRNA as shown. Inhibitors and templates were preincubated at 24° for 10 min before the addition of ribosomes and [$^{14}$C]aminoacyl-tRNA. The incubation was at 24° for 20 min, and the remaining conditions as described in Methods.

standing of the inhibitory effects of vinyl polymers on protein synthesis. These equilibria were studied by three methods.

$$\text{poly(A)}\cdot 9\text{poly(vU)} + 2\text{poly(U)} \rightleftharpoons \text{poly(A)}\cdot 2\text{poly(U)} + 9\text{poly(vU)} \quad (1)$$

$$2(\text{poly(vA)}\cdot\text{poly(U)}) + 1\text{poly(A)} \rightleftharpoons \text{poly(A)}\cdot 2\text{poly(U)} + 2\text{poly(vA)} \quad (2)$$

Mixtures corresponding to the extreme positions of equilibria 1 and 2 differ very distinctly in their melting behavior. The polynucleotide complex melts cooperatively at 70°, while the analog complexes have broad melting ranges, $T_m$ at 55° for poly(vA) and $T_m$ at 59° for poly(vU). When equilibrium mixtures for the systems 1 and 2 were prepared, using approaches from both sides, they showed a simple cooperative melting with a $T_m$ of 70°. For further studies of equilibria at room temperature spectral methods were used. The ultraviolet spectra for the extreme positions of equilibria were calculated. System 1 shows a distinct difference in the 252- to 264-nm region, as may be expected from the low hypochromism of the poly(vU) complex (Pitha *et al.*, 1970). For system 2, the 280- to 290-nm region is more useful. Here, the analog complex is hypochromic compared to the components (Pitha and Pitha, 1970), while the polynucleotide complex is hyperchromic (Massoulie *et al.*, 1965). Spectra of equilibrated mixtures were found to be practically identical to the calculated combinations containing the polynucleotide complex and free vinyl polymer. As a third approach, the optical rotatory dispersion spectra were used. The spectrum of poly(A)·2 poly(U) was unchanged by the addition of either poly(vA) or poly(vU). Thus, all three methods failed to detect a serious amount of analog complex in equilibria 1 and 2.

*Effect of Vinyl Polymers on Triplet and Polynucleotide Stimulated* [$^{14}$C]*Aminoacyl-tRNA Binding to Ribosomes.* In view of the structural similarities of vinyl polymers with polynucleotides, it was of interest to study the possible function of the vinyl polymers as templates or inhibitors for ribosomal binding of [$^{14}$C]aminoacyl-tRNA. Table II shows that, although the vinyl polymers did not function as template, they did inhibit [$^{14}$C]aminoacyl-tRNA binding that was stimulated by the complementary polynucleotides. Poly(vA) completely inhibited the poly(U)-stimulated [$^{14}$C]Phe-tRNA binding to ribosomes, while the inhibition with poly(vA,vU) was only 26%. Likewise, poly(vU) completely inhibited the poly(A)-directed [$^{14}$C]Lys-tRNA binding to ribosomes in contrast to poly(vA,vU) which inhibited the binding only to 36%. However, poly(vU) and poly(vA,vU) decreased the triplet (A-A-A)-stimulated [$^{14}$C]Lys-tRNA binding to ribosomes only partially. Similarly, using a randomly ordered heteropolymer, poly(A,U), as template, the attachment of [$^{14}$C]Phe-tRNA to ribosomes was completely inhibited by poly(vA), but poly(vU) was relatively ineffective in that system.

Further, the stoichiometry of the inhibition of [$^{14}$C]aminoacyl-tRNA ribosomal binding by complementary polynucleotides and vinyl polymers was investigated. Poly(A) and poly(vA) inhibition of poly(U)-stimulated binding of [$^{14}$C]Phe-tRNA to ribosomes is shown in Figure 1. Complete inhibition with poly(A) was achieved at a mole ratio of approximately 2 moles of uracil/mole of adenine residues, and the poly(vA) inhibition was essentially complete at a 1 to 1 mole ratio. Poly(U) and poly(vU) inhibition of poly(A)-stimulated [$^{14}$C]Lys-tRNA binding is shown in Figure 2, and complete inhibition with poly(U) occurred at a 1 to 1 mole ratio. The inhibition by poly(vU) did not exhibit clear-cut stoichiometry, and essentially complete inhibition was achieved at a mole

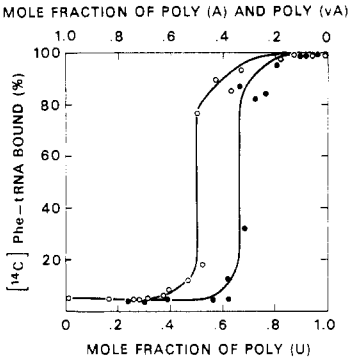


FIGURE 1: Inhibition of poly(U)-directed binding of [$^{14}$C]Phe-tRNA to ribosomes by poly(A) and poly(vA). The incubation mixture contained 37.0 nmoles of poly(U) and 29.8 pmoles of [$^{14}$C]-Phe-tRNA, and the remaining conditions are described in Table II. Inhibition by poly(A) (●) and poly(vA) (○).

ratio of 3 uracils/adenine residue. Both complementary vinyl polymer and polynucleotide inhibition required a preincubation with template before the ribosomes were added to the reaction system.

*Effect of Vinyl Polymers on Polynucleotide-Stimulated* [$^{14}$C]*Amino Acid Incorporation into Polypeptides.* Both poly(A) and poly(vA) inhibited the poly(U)-directed [$^{14}$C]phenylalanine incorporation into polypeptides. These results are plotted as reciprocal velocity *vs.* reciprocal poly(U) concentration (Figure 3). The plot was linear with the least-squares product-moment correlation coefficient greater than 0.9 for each type of inhibition. The inhibition was competitive for both poly(A) and poly(vA). Poly(vA,vU) was inhibitory to poly(U)-stimulated [$^{14}$C]phenylalanine incorporation into polypeptides only when used in high concentrations, while poly(A) and poly(vA) inhibition occurred at much lower concentrations (Table III). Poly(U) and poly(vU) inhibition of poly(A)-directed [$^{14}$C]lysine incorporation into polypeptides is shown in Figure 4. Both poly(U) and poly(vU) produced complete inhibition at a 1 to 1 mole ratio of uracil to adenine residues.

An experiment was designed to determine if the vinyl analogs compete with polynucleotide for ribosomal binding sites (Figure 5). In this experiment, poly(vU) neither served as template nor did it compete with poly(U) for ribosomal binding sites. On the contrary, poly(vA) completely inhibited

TABLE III: Inhibition of Poly(U)-Stimulated Incorporation of [$^{14}$C]Phenylalanine into Polypeptides by Poly(vA,vU).[a]

| Poly(vA,vU) (μmoles of Base Residues) | [$^{14}$C]Phe Incorpd[b] (pmoles) | % Inhibn |
|---|---|---|
| None | 13.6 | 0 |
| 0.0394 | 14.2 | 0 |
| 0.788 | 7.74 | 43 |
| 1.31 | 3.83 | 72 |

[a] The conditions are described in the legend to Figure 3, except that poly(vA,vU) was used as inhibitor. [b] The incorporation in the absence of poly(U) was 3.36 pmoles.

the poly(U)-directed [$^{14}$C]phenylalanine incorporation into polypeptides.

## Discussion

The failure of the vinyl polymers to stimulate the ribosomal binding of [$^{14}$C]aminoacyl-tRNAs is probably due to their lack of negatively charged phosphate groups. The absence of poly(vU) competition for poly(U) binding sites on the ribosomes, as was revealed in a competition experiment, is probably a manifestation of the fact that the vinyl polymers are not bound to the ribosomes. On the other hand, physicochemical studies have shown that the vinyl analogs are displaced from polynucleotide–vinyl polymer complexes by the complementary polynucleotide, and the possibility that the vinyl analogs form weak, possibly nonspecific, complexes with ribosomes cannot be excluded. However, even if vinyl polymers were bound to the ribosomes, it is doubtful that they would be effectively translated because the distances between bases in the vinyl polymers are shorter than those in the anticodon on the tRNA.

The inhibition of triplet- and polynucleotide-directed [$^{14}$C]-aminoacyl-tRNA binding by complementary polynucleotides or vinyl polymers proceeds by forming a complex with the polynucleotide prior to binding to the ribosomes. This

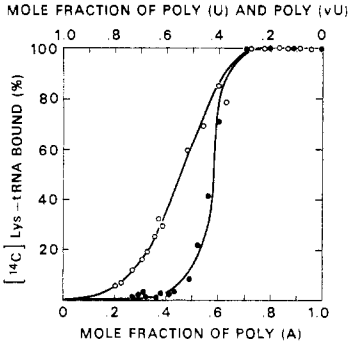


FIGURE 2: Inhibition of poly(A)-directed binding of [$^{14}$C]Lys-tRNA to ribosomes by poly(U) and poly(vU). The incubation mixture contained 8.88 nmoles of poly(A) and 24.2 pmoles of [$^{14}$C]Lys-tRNA, and the remaining conditions are described in Table II. Inhibition by poly(U) (●) and poly(vU) (○).

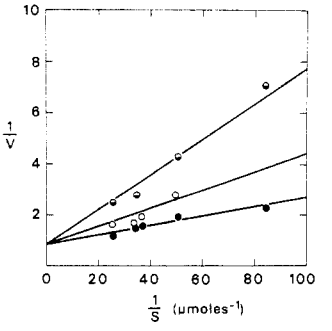


FIGURE 3: Double-reciprocal plots of the inhibition of poly(U)-stimulated incorporation of [$^{14}$C]Phe into polypeptides by poly(A) and poly(vA). The incubation was stopped after 15 min and the remaining conditions are as described in Methods. *S* is the micromolar concentration of poly(U) and *V* is the pmoles of [$^{14}$C]Phe incorporated per min. Poly(U)-stimulated [$^{14}$C]Phe incorporation (●), inhibition by 4.9 nmoles of poly(A) (○), by 8.22 nmoles of poly(vA) (◒).

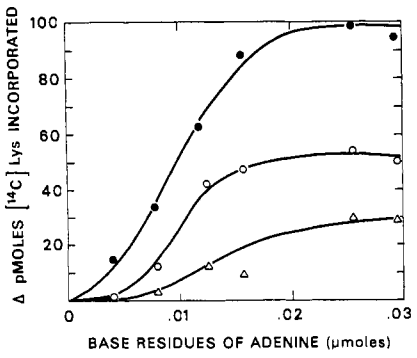


FIGURE 4: Inhibition of poly(A)-stimulated incorporation of [$^{14}$C]-lysine into polypeptides by poly(U) and poly(vU). Experimental conditions are as described in Methods. Incorporation in the presence of poly(A) alone (●); inhibition by 4.38 nmoles of poly(vU) (○), by 3.88 nmoles of poly(U) (△).

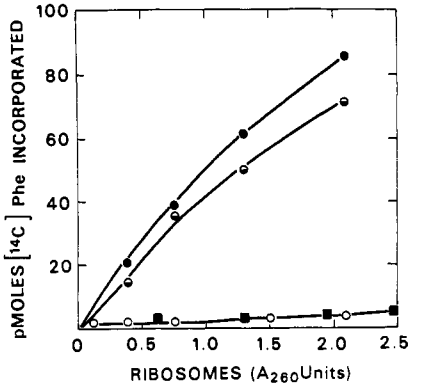


FIGURE 5: The effect of ribosome concentration of poly(U)-stimulated [$^{14}$C]phenylalanine incorporation in the presence of poly(vU) and poly(vA). Experimental conditions are as described in the legend to Figure 3, except that 36.8 nmoles of poly(U), aminoacyl-tRNA synthetase (0.19 mg of protein), 5 $A_{260}$ units of tRNA and ribosomes (as indicated) were used. Incorporation in the presence of 87.5 nmoles of poly(vU) without poly(U) (○), poly(U) alone (◒), poly-(U) and 87.5 nmoles of poly(vU) (●), poly(U) and 46.3 nmoles of poly(vA) (■).

type of inhibition using the complementary polynucleotide has been reported previously (Nirenberg and Matthaei, 1961; Moller and von Ehrenstein, 1963; Williamson *et al.*, 1967). At pH 7.0, poly(A) and poly(U) can form both a 1 to 1 complex and a complex containing 2 uracil residues/adenine residue; the type of complex formed is controlled by the mole ratio of poly(A) to poly(U) added (Felsenfeld and Miles, 1967; Blake *et al.*, 1967). The polynucleotide inhibition is in precise agreement with this stoichiometry. Poly(U)-stimulated ribosomal binding of [$^{14}$C]Phe-tRNA was inhibited with poly(A) at a 1 to 2 ratio of adenine to uracil. In the reverse case using poly(A) as template for [$^{14}$C]Lys-tRNA binding, the inhibition was essentially complete at a 1 to 1 ratio. As predicted by physicochemical studies (Pitha *et al.*, 1971), poly(vA) also inhibited the poly(U)-directed [$^{14}$C]Phe-tRNA binding at a 1 to 1 ratio. However, the 1 to 3 ratio of adenine to uracil for the poly(vU) inhibition of poly(A)-directed [$^{14}$C]Lys-tRNA binding is not in agreement with the 1 to 9 stoichiometry as reported for the complete complex formation (Pitha *et al.*, 1970). This is probably an indication that formation of a complex of the maximal hypochromicity is not necessary to block the binding reaction.

It is interesting to note that a polynucleotide–polynucleotide complex is more stable than the corresponding complex of polynucleotide–vinyl polymer. Furthermore, the anticodon–trinucleotide interactions are more favored energetically (per base pair) than oligonucleotide–oligonucleotide interactions (Eisinger, 1971). But the anticodon–polynucleotide interaction involves only 3 base pairs, while the polynucleotide–vinyl polymer interaction contains many more. Hence, we observed nearly complete inhibition of polynucleotide-directed [$^{14}$C]aminoacyl-tRNA binding with the vinyl homopolymers. On the other hand, when the number of template–vinyl polymer base pairs is reduced, as in the case of the vinyl homopolymer (A-A-A)– or the poly(vA,vU)–polynucleotide interaction, only incomplete inhibition results. It is also possible that the complexed vinyl polymer may exert sterical stress to prevent even temporarily free parts of the polynucleotide from acting as template.

The study of vinyl polymers as inhibitors of cell-free protein synthesis not only revealed that these polymers are potent inhibitors, but also helped to elucidate the mechanism of the inhibition. The 1 to 1 stoichiometry of the poly(vU) inhibition of poly(A)-stimulated [$^{14}$C]lysine incorporation into polypeptides and the poly(vA,vU) inhibition of poly(U)-directed [$^{14}$C]phenylalanine incorporation favor the idea that it is not necessary to block all codon sequences in the polynucleotide template to inhibit protein synthesis. Further studies of possible vinyl polymer inhibition of natural mRNA-directed protein synthesis are in progress.

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# Synthesis of a Linear Gramicidin by a Combination of Biosynthetic and Organic Methods†

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ABSTRACT: Linear gramicidin is a pentadecapeptide with the sequence *N*-formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. Ribosome-free extracts from *Bacillus brevis* (ATCC 8185), on Sephadex G-200 filtration, yielded fractions free of aminoacyl-tRNA ligases which catalyzed ATP–$PP_i$ exchanges and covalent trichloroacetic acid stable binding of all amino acids incorporated in linear gramicidin. From these fractions $Cl_3CCOOH$-stable protein-bound peptides were prepared and, after alkali (pH 11) or peroxidative liberation, separated by thin-layer chromatography. Labeled valine, glycine, alanine, leucine, and tryptophan used alternatively with phenylalanine, *i.e.*, all the amino acids present in linear gramicidin, were incorporated into one of the peptides. The amino acid stoichiometry found in this peptide by double labeling as described in the text was Gly:Ala:Leu:Val:Phe, 1:2:4:4:4. Moreover, in agreement with the structure of linear gramicidin, alanine was found only in L configuration, valine in 50% D and 50% L, and leucine in the D configuration; all derived from L-amino acids used as biosynthetic precursors. Aminoethanolysis of this enzyme-bound intermediate, followed by organic formylation, yielded a product which comigrated with linear gramicidin in four solvent systems. These results indicate that biosynthetically a pentadecapeptide is formed which remains thioester linked to the enzyme and presumably is released enzymatically by aminoethanolysis. Crude extracts yielded a formylated peptide analogous to the one analyzed. The experiments suggest for the biosynthesis of linear gramicidin a mechanism analogous to that of gramicidin S and tyrocidine (Lipmann, F. (1971), *Science* 173, 875). However, a complete biosynthesis of linear gramicidin could not be achieved due to our inability to obtain extracts that linked the C-terminal aromatic amino acid to ethanolamine.

The linear gramicidins are antibiotics produced by the same strains of *Bacillus brevis* (ATCC 8185 or Dubos strain ATCC 10068) that produce tyrocidine (Hotchkiss, 1944). Figure 1 shows the structures of these pentadecapeptides, which differ only in the alternative incorporation of tryptophan, tyrosine, and phenylalanine as aromatic amino acids similar to the differences in the tyrocidines. The amino-terminal valine is formylated, and the carboxyl-terminal tryptophan is peptidically linked to ethanolamine. If glycine is considered an equivalent of a D-amino acid, the linear gramicidins consist throughout of alternating L- and D-amino acid residues, thereby placing constraints on the secondary structure (Urry *et al.*, 1971).

The development of cell-free systems for the synthesis of GS[1] and Ty made it possible to decipher the mechanism of biosynthesis of these cyclic decapeptides by nonribosomal systems (Saito *et al.*, 1970; Kurahashi *et al.*, 1969; Bredesen *et al.*, 1968; Lipmann *et al.*, 1971). These studies showed that the amino acids are activated by ATP on complementary enzymes of a molecular weight roughly proportional to the number of amino acids activated. From the resulting aminoacyladenylate the amino acid is then transferred to an enzymic sulfhydryl where it is bound covalently as thioester (Kleinkauf and Gevers, 1969). In both GS and Ty the N-terminal phenylalanine is activated and racemized by the smallest enzyme, mol wt 100,000. Reaction between it and the larger enzyme carrying the other amino acids initiates polymerization to peptide intermediates that remain thioester linked to enzymes until released by cyclization (Gevers *et al.*, 1969; Ljones *et al.*, 1968). Pantetheine, covalently bound to the larger enzyme proteins (Kleinkauf *et al.*, 1970; Gilhuus-Moe *et al.*, 1970), appears to mediate, by alternating transthiolation and transpeptidation, the elongation of successive peptidyl additions to enzyme–thioester-linked amino acids (Kleinkauf *et al.*, 1971).

The present experiments describe a soluble enzyme system that performs a partial LG biosynthesis. The mode of amino acid activation parallels that for GS and Ty biosynthesis. Thioester-linked glycine and alanine, exclusively present in LG, were already found in trichloroacetic acid precipitates during studies of Ty biosynthesis in the same extracts used here for studying LG biosynthesis (Roskoski *et al.*, 1970a). We have been able to identify an enzyme-bound pentadecapeptide intermediate of LG. After chemical ethanolaminolysis from its thioester link to denatured enzyme and chemical formylation, a compound was isolated that appeared to be identical with authentic LG. In crude extracts enzymatic N-terminal formylation was obtained; however, enzymatic conjugation of the carboxyl terminal with ethanolamine to complete the biosynthesis was not achieved.

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[1] Abbreviations used are: LG, linear gramicidin; GS, gramicidin S; Ty, tyrocidine.