different spatial arrangement. The results we have obtained are consistent with the possibility that a relatively large number of antibody specificities could be generated by different ways of combining relatively few distinct heavy chains with relatively few distinct light chains, as was suggested in the previous paper (Painter *et al.*, 1972). We know of no evidence to indicate whether or not such a process is important in the natural generation of antibody specificity.

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Inhibition of Ribonucleic Acid Synthesis by Myxin†

Ulrich Hollstein* and Patrick L. Butler

ABSTRACT: The inhibition of DNA template-controlled RNA synthesis has been studied kinetically in the absence and presence of the antibiotic myxin. The rates as function of concentration of the four ribonucleotides, one varied at a time, have been measured and could be expressed in a Michaelis–Menten equation. Myxin inhibits the RNA chain growth by affecting the rate terms for CTP and GTP, but not for ATP and UTP. The control of myxin on the inhibition of incorporation of C and G is equally effective. The base-specific behavior of myxin is compared to that of other antibiotics.

The antibiotic myxin, isolated from a *Sorangium* species, has been reported to possess an unusually broad antimicrobial spectrum. It is capable of inhibiting growth of a wide variety of microorganisms including gram-positive and -negative bacteria, fungi, actinomycetes, and yeasts (Peterson *et al.*, 1966). Myxin has been synthesized following several unambiguous and independent routes allowing the assignment of its structure 1-hydroxy-6-methoxyphenazine 5,10-dioxide (Sigg and Toth, 1967; Weigele and Leimgruber, 1967). The *in vivo* effect of the antibiotic has been found to consist of an inhibition of DNA synthesis and RNA synthesis, and a reduction of protein synthesis. It was speculated that the activity of myxin was due to interaction with the DNA-polymerizing enzyme without being bound to DNA (Lesley and Behki, 1967). Recently we have found that phenazine antibiotics, including myxin, inhibit the *in vitro* RNA synthesis and that these compounds bind to DNA. Therefore, we concluded that the association of antibiotic with DNA, presumably through intercalation, is a contributory factor, if not the sole cause, in the inhibition of DNA tem-

† From the Department of Chemistry, The University of New Mexico, Albuquerque, New Mexico 87106, and from the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received September 27, 1971.* This work was performed partially under the auspices of the U. S. Atomic Energy Commission while U. H. was an Associated Western Universities Faculty Participant at Los Alamos.

plate-controlled RNA synthesis (Hollstein and Van Gemert, 1971). Consequently it was of interest to study the effect of myxin on DNA-controlled transcription in more detail. Thus, we have studied the kinetics of the propagation step of RNA synthesis (Anthony *et al.*, 1966) by varying the concentration of the four nucleoside triphosphates, one at a time, in the absence and presence of myxin.

Materials and Methods

Materials. The nucleoside triphosphates ATP,[1] CTP, GTP, and UTP as well as [^{14}C]ATP were purchased from Calbiochem. Calf thymus DNA, Na salt, type I, Sigma Chemical Co., was used throughout this investigation. Poly[d(A-T)]·poly[d(A-T)] and poly(dC)·poly(dG) were obtained from Dr. R. L. Ratliff, and *Escherichia coli* RNA polymerase was obtained from Dr. D. A. Smith, Biomedical Research Group, Los Alamos Scientific Laboratory, Los Alamos, N. M. Myxin was a gift from Hoffman-LaRoche. It had a melting point of 137° and $\lambda_{max}^{H_2O}$ of 347 nm (ϵ 6000) and 510 nm (ϵ 6500) in accordance with values reported in the literature (Edwards and Gillespie, 1966; Sigg and Toth, 1967; Weigele and Leimgruber, 1967).

Solutions. The following aqueous stock solutions were prepared. [CTP] = [GTP] = [UTP] = 10^{-3} M. [[^{14}C]ATP] = 10^{-3} M with a specific activity of 10 μCi/μmole. [DNA] = 3.93×10^{-3} mono-M in 0.001 M NaCl, as determined with ϵ(P) = 6600. RNA polymerase (2630 units/mg; 5.3 mg/ml) (Ratliff *et al.*, 1967) was kept at −20°. Before each run a suitable quantity was diluted eightfold with a solution containing 0.01 M Tris (pH 7.9), 0.1 M $MgCl_2$, 0.01 M mercaptoethanol, 5×10^{-5} M EDTA, and 1 mg/ml of bovine serum albumin. The myxin solutions were prepared in relatively large amounts of water with a few per cent ethanol. Ethanol and some water was evaporated *in vacuo* and the remaining suspension was filtered through a fine-porosity, fritted glass filter funnel. Concentrations were determined spectrophotometrically at 347 or 510 nm. Two stock solutions (3.31×10^{-6} and 5.78×10^{-6}) were used.

Kinetic Measurements. Kinetic experiments were performed by keeping three of the four ribonucleotide concentrations constant while varying the concentration of the fourth downward from the other three. For each run 100 μl of a solution consisting of 10 ml of 0.1 M Hepes (pH 7.9), 0.1 ml of 1.0 M $MgCl_2$, and 0.25 ml of 0.1 M $MnCl_2$ was placed in each of five tubes. To each was added 50 μl of the stock solutions of those nucleotides, whose concentration was to be kept constant with the exception of CTP. Quantities of 50, 40, 30, 20, and 50 μl (control tube) of the nucleotide whose concentration was to be varied were then added. The volumes in tubes 2, 3, and 4 were adjusted to those of tubes 1 and 5 by addition of water. The DNA stock solution (10 μl) and the freshly eight-times diluted RNA polymerase solution (20 μl) were added. In the control tube (5) 20 μl of water was substituted for 20 μl of RNA polymerase.

The mixtures were allowed to equilibrate for 15 min at 37°. In order to wash any polymerase which has not initiated RNA synthesis off the DNA, 100 μl of 2 M $(NH_4)_2SO_4$ was added. It was followed by 100 μl of a myxin stock solution or water. The final $(NH_4)_2SO_4$ concentration is at this point 0.4 M. The solutions were again incubated at 37° for 15 min to ensure equilibrium. Propagation of RNA synthesis was started by addition of 50 μl of the CTP stock solution, or, in the case where the concentration of this ribonucleotide was to be varied, by addition of 50, 40, 30, 20, and 50 μl to each of the five tubes. At suitable time intervals 100-μl aliquots were withdrawn, immediately transferred to a Whatman GFB glass-fiber filter paper disk (diameter 5 mm, thickness 0.67 mm), and quickly added to a cold solution containing 5% trichloroacetic acid and 1% sodium pyprophosphate in which they were soaked for 15 min at 5°. The time elapsed between the addition of CTP and contact with trichloroacetic acid solution was noted. Adherent liquid was removed by suction on a Büchner funnel and the disks were washed on the funnel three times with cold 5% trichloroacetic acid–1% sodium pyrophosphate solution and three times with a 1:1 mixture of ethanol and ethyl ether. After drying for 20 min at 80–100° the disks were counted while suspended in a toluene scintillator solution using a Packard scintillation counter. All counting data were corrected for the reading of the control solution (tube 5) which contained no enzyme. The efficiency of the counter was determined using an aliquot of the [^{14}C]ATP solution absorbed on a glass-fiber filter disk and dried. The mole fractions of ribonucleotides incorporated were assumed to be 0.28 for ATP and UTP and 0.22 for CTP and GTP in accordance with the base composition of calf thymus DNA (Sober, 1968). From these data the total amount of RNA synthesized could be calculated and plotted *vs.* time. Initial rates of RNA synthesis were read from the initial slopes of these graphs.

Results

The kinetic data can be interpreted by using a theoretical rate equation similar to one derived earlier (Bremer, 1967). The average incorporation rate of a ribonucleotide α per unit time is: $v_\alpha = 1/t_\alpha$, where t_α is the average time of incorporation for ribonucleotide α. With f_α denoting the mole fraction of ribonucleotide α in the synthesized RNA, the total average incorporation rate is: $v = 1/t = 1/\Sigma_\alpha f_\alpha t_\alpha$, where t is the average incorporation time for any ribonucleotide. Thus, the total rate v is the formation of the product RNA chain per time unit per polymerase·DNA·RNA complex and can be equated to: $k_1k_3[\alpha]/(k_2 + k_3 + k_1[\alpha])$, where k_1 and k_2 are the forward and reverse rate constants for the (enzyme–complex)–substrate formation, k_3 is the rate constant for the formation of the product RNA from the foregoing complex, and $[\alpha]$ is the concentration of the substrate ribonucleotide α (Mahler and Cordes, 1966).

Substitution of K for $(k_2 + k_3)/k_1$ yields for the rate per ribonucleotide α

$$v_\alpha = \frac{1}{t_\alpha} = \frac{k_{3\alpha}[\alpha]}{K_\alpha + [\alpha]}$$

and for the overall rate

$$\frac{1}{v} = t = \sum_\alpha \left(\frac{f_\alpha(K_\alpha + [\alpha])}{k_{3\alpha}[\alpha]} \right) = \sum_\alpha \left(\frac{f_\alpha K_\alpha}{k_{3\alpha}[\alpha]} + \frac{f_\alpha}{k_{3\alpha}} \right)$$

or, with $f_\alpha K_\alpha / k_{3\alpha} = A_\alpha$ and $f_\alpha / k_{3\alpha} = B_\alpha$

$$\frac{1}{v} = t = \sum_\alpha \left(\frac{A_\alpha}{[\alpha]} + B_\alpha \right)$$

[1] Abbreviations used are: ATP, UTP, GTP, and CTP, ribonucleoside 5′-triphosphates of adenine, uracil, guanine, and cytosine, respectively; poly[d(A-T)]·poly[d(A-T)], double-stranded DNA whose individual strands have the alternating base sequence; poly(dC)·poly(dG), double-stranded DNA constructed from single strands of polydeoxycytidylic acid and polydeoxyguanylic acid.

TABLE I: Rate Calculations from Figure 1.[f]

	r	$v \times 10^7$ M min^{-1}	Ribonucleotide Molecules Incorpd/min per Chain	Inc Time for 1 Ribonucleotide Molecule min ($=t(r)$)	$t(r = 1)/t(r = 0)$
CTP	0	3.40[a]	70	0.0144	
[CTP] = 3.33×10^{-5}	0	2.61[a]	53	0.0188	4.23
CTP	1	0.803[e]	16.4	0.0610	20.9
[CTP] = 3.33×10^{-5}	1	0.125[e]	2.6	0.392	
GTP	0	3.25[b]	67	0.0151	
[GTP] = 3.33×10^{-5}	0	1.92[b]	39	0.0255	4.33
GTP	1	0.750[e]	15.3	0.0654	16.4
[GTP] = 3.33×10^{-5}	1	0.117[e]	2.40	0.417	
ATP	0–1	3.25[c]	66	0.0151	
UTP	0–1	3.17[d]	65	0.0154	

[a] From Figure 1C. [b] From Figure 1B. [c] From Figure 1A. [d] From Figure 1D. [e] Calculated by extrapolation. [f] [ATP] = [CTP] = [GTP] = [UTP] = 9.44×10^{-5} M, unless otherwise indicated. [DNA] = 7.42×10^{-5} mono-M = 4.9×10^{-9} M.

If the concentrations of three of the four ribonucleotides are kept constant and the concentration of the fourth, say GTP, is varied a Lineweaver–Burk plot $1/v$ *vs.* 1/[GTP] results in a straight line

$$\frac{1}{v} = t = \frac{A_G}{[GTP]} + \left\{B_G + \sum_{\alpha=A,C,U}\left(\frac{A_\alpha}{[\alpha]} + B_\alpha\right)\right\}$$

where A_G is the slope and the expression in parentheses denotes the $1/v$ axis intercept.

In order to be able to study the inhibiting effect of myxin on the incorporation of ribonucleotides it is assumed that this effect is linearly related to the bound ligand per base pair ratio r. If t_α^0 and t_α^1 represent the incorporation time of ribonucleotide α in the absence ($r = 0$) and the hypothetical maximal presence ($r = 1$) of the ligand, respectively, the incorporation time for α at r is: $t_\alpha^r = t_\alpha^0 + r(t_\alpha^1 - t_\alpha^0)$, and the average incorporation time of any ribonucleotide is

$$t^r = \sum_\alpha f_\alpha t_\alpha^0 + r\sum_\alpha f_\alpha(t_\alpha^1 - t_\alpha^0)$$

Substitution into the Lineweaver–Burk equation yields if, again [GTP] is varied

$$\frac{1}{v(r)} = t^r = \frac{A_G^0 + r(A_G^1 + A_G^0)}{[GTP]} + \left\{B_G^0 + r(B_G^1 - B_G^0) + \sum_{\alpha=A,C,U}\frac{A_\alpha^0 + r(A_\alpha^1 - A_\alpha^0)}{[\alpha]} + B_\alpha^0 + r(B_\alpha^1 - B_\alpha^0)\right\}$$

where the expression in parentheses represents the $1/v$ axis intercept. Thus, the reciprocal rate is a linear function of the reciprocal of the ribonucleotide concentration to be varied, and the slope as well as the $1/v$ axis intercept is a linear function of r. Moreover, at constant substrate concentrations, the reciprocal rate is a linear function of r.

Rate data are presented in Figure 1 in a double-reciprocal plot of rate *vs.* one variable ribonucleoside triphosphate concentration while the other three are kept constant. As predicted by the theory the functions are linear. The data are pictured for three r values, *viz.*, 0.0, 0.00842, and 0.0147. While the slope is invariable for ATP and UTP, the CTP and GTP plots show an increase of slope with r. Thus, myxin affects the rate terms for the incorporation of CTP and GTP but not for ATP and UTP. That the slopes are also a linear

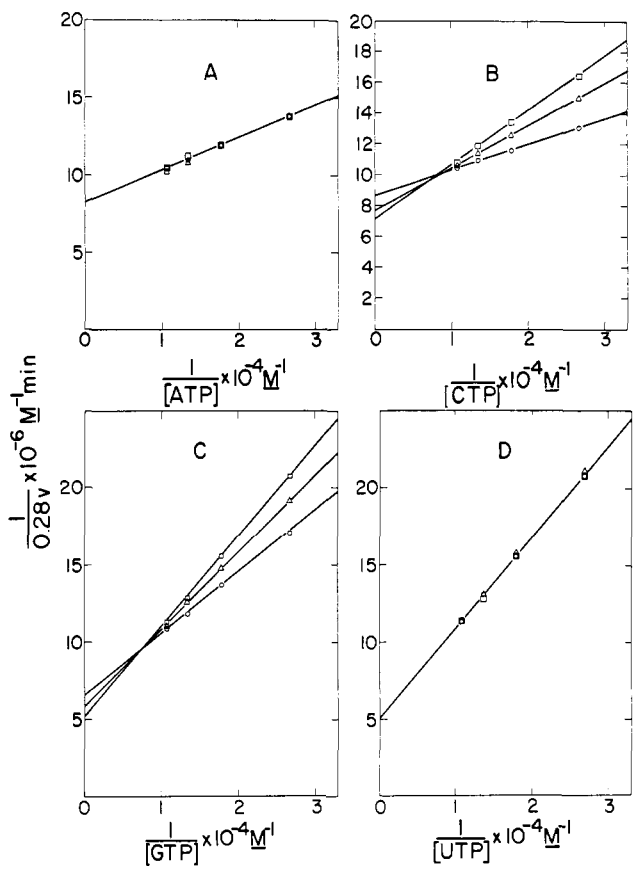

FIGURE 1: Inverse of initial rate *vs.* the inverse of substrate concentration. [ATP] = [CTP] = [GTP] = [UTP] = 9.44×10^{-5} M. [DNA] = 7.42×10^{-5} mono-M = 4.9×10^{-9} M (based on an assumed average molecular weight of 5×10^6). [Crude RNA polymerase] = 5.0×10^{-8} M. $r = 0.0$ (○), $r = 0.00842$ (△), $r = 0.0147$ (□). (These values are calculated on the basis of previous determinations under the present conditons.)

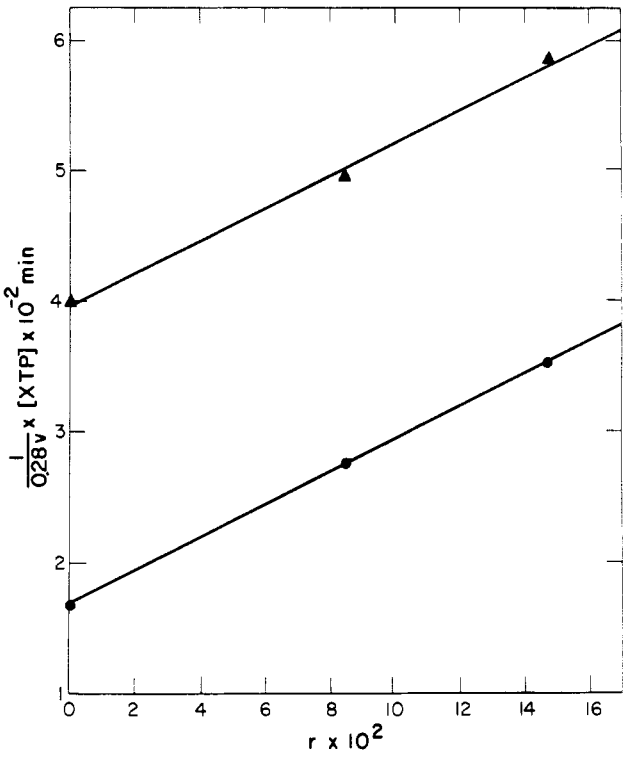

FIGURE 2: Slope of double reciprocal plot ($1/v$ *vs.* $1/$[ribonucleoside triphosphate], Figure 1) *vs.* r. [GTP] = 9.44×10^{-5} M, [CTP] = 3.78×10^{-5} M (●); [CTP] = 9.44×10^{-5} M, [GTP] = 3.78×10^{-5} M (▲).

function of r, as required by the theory, is showin in Figure 2. As predicted by the theory the inverse initial rates are also linear with r (Figure 3).

The data presented can be used to calculate a number of parameters and constants which govern the kinetics of the RNA synthesis under the present conditions and in the absence and presence of myxin. Evidence has been presented earlier for T_7 DNA that under the experimental conditions utilized here, there is one growing RNA chain per polydeoxyribonucleotide molecule (Hyman and Davidson, 1970). Assuming that the present case is analogous, the rate in terms of ribonucleotides per time unit per chain may be calculated. The data from Figure 1 have been used for these calculations and the results are tabulated in Table I.

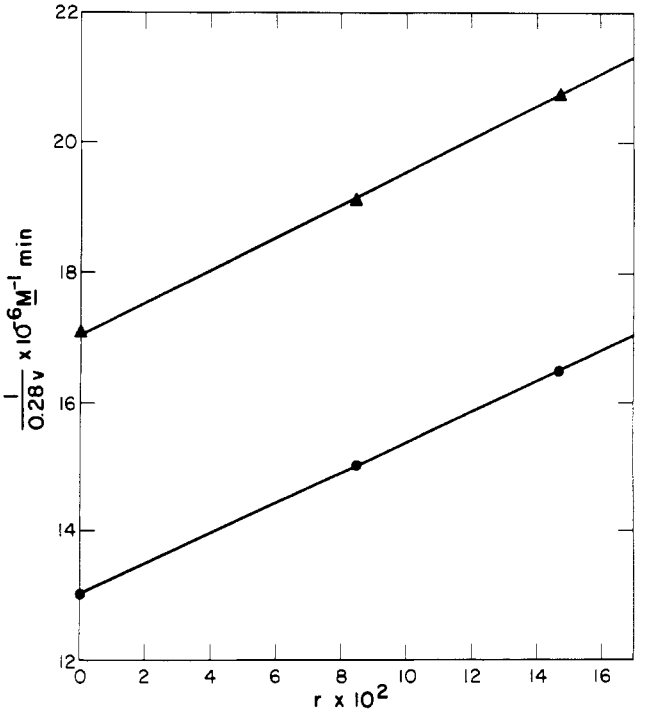

FIGURE 3: Inverse initial rates from Figure 1 *vs.* r. [ATP] = [GTP] = 9.44×10^{-5} M, [CTP] = 3.78×10^{-5} M (●); [ATP] = [CTP] = [UTP] = 9.44×10^{-5} M, [GTP] = 3.78×10^{-5} M (▲).

It can be seen that when myxin is absent and the concentrations of all four ribonucleotides are equal, *viz.*, 9.44×10^{-5} M, the rates calculated from Figure 1A–D agree with each other within experimental error. The average value is $3.3 \pm 0.1 \times 10^{-7}$ M min^{-1} and the average number of ribonucleotide molecules incorporated per min per chain is 67 ± 3. The ratio of incorporation time per ribonucleotide molecule for the hypothetical state of one myxin molecule per base pair and zero myxin molecules per base pair has been calculated from Figure 1B,C for the condition where all four ribonucleotide concentrations are equal. The two values, 4.33 and 4.23, are in agreement within experimental error. However, if this ratio is calculated for situations where either [CTP] or [GTP] differs from the concentrations of the other three ribonucleotides, these values are different, *viz.*, 16.4 and 20.9. That the latter ratios should indeed be different can be derived from the earlier presented rate equation, as follows

$$\frac{t(r=1)}{t(r=0)} = 1 + \frac{\dfrac{A_\beta^1 - A_\beta^0}{[\beta]} + B_\beta^1 - B_\beta^0 + \sum_{\alpha \neq \beta}\left(\dfrac{A_\alpha^1 - A_\alpha^0}{[\alpha]} + B_\alpha^1 - B_\alpha^0\right)}{\dfrac{A_\beta^0}{[\beta]} + B_\beta^0 + \sum_{\alpha \neq \beta}\left(\dfrac{A_\alpha^0}{[\alpha]} + B_\alpha^0\right)}$$

With $[\beta] \neq [\alpha]$ it can be seen that the ratio depends on whether the subscript β refers to CTP or GTP, even if $[\beta]$ = [CTP] = [GTP] since $(A_C^1 - A_C^0)/[\beta] \neq (A_G^1 - A_G^0)/[\beta]$ and also $A_C^0/[\beta] \neq A_G^0/[\beta]$. The vertical intercepts of the double-reciprocal plots in Figure 1 represent in the absence of myxin

$$\sum_\alpha B_\alpha + \sum_{\alpha'} \frac{A_{\alpha'}}{[\alpha']}$$

where α refers to A, C, G, and U, and α' refers to ribonucleotides with constant concentration only. The individual slopes are given by $A_{\alpha'}$. Consequently, for the calculation of the second term of the vertical intercept expression all four slopes are needed. Table II presents the measured slopes, intercepts and calculated second terms.

In the absence of myxin ($r = 0$) the average of the $\Sigma_\alpha B_\alpha$ values is -0.4 to -0.5 min which may considered to be zero within experimental error. Therefore, with this approximation the reaction rate is proportional to the ribonucleotide concentrations within the concentration limits of the present experiments. Also, after neglect of the term $\Sigma_\alpha B_\alpha$ the constants A_α are proportional to r. This simplification renders possible a calculation of the rate constant $A_\alpha^1/f_\alpha = K_\alpha^1/k_{3\alpha}^1$ at the hypothetical state where $r = 1$ by extrapolation as shown in Table II. It may be noted that these values, *viz.*, 786×10^{-7} and 814×10^{-7} M min for CTP and GTP are equal within experimental error. In a similar study with ac-

TABLE II: Rate Calculations from Figure 1. $[\alpha] = 9.44 \times 10^{-5}$ M.

Ribonucleotide Triphosphate	f_α	r	Slope $\times 10^7$ M min $= A_\alpha = (f_\alpha K_\alpha/k_{3\alpha})$	$(K_\alpha/k_{3\alpha}) \times 10^7$ M min	$\Sigma_{\alpha'}{}^a(A_{\alpha'}{}^b/[\alpha'])$ min	Vertical Intercept $\times 10^2$ min	$\Sigma_\alpha{}^a B_\alpha{}^c = \Sigma_\alpha(f_\alpha/k_{3\alpha})$ min
ATP	0.28	0	2.8	10.2	1.6	1.1	−0.5
CTP	0.22	0	2.3	10.4	1.7	1.2	−0.5
GTP	0.22	0	5.5	24.9	1 4	0 9	−0.5
UTP	0.28	0	8.1	28.9	1.1	0.7	−0.4
CTP	0.22	0.0147	4.8	21.8			
		1.0	173[d]	786			
GTP	0.22	0.0147	8.1	36.3			
		1.0	179[d]	814			

[a] α' refers to ribonucleotides with constant concentration only, α refers to all four ribonucleotides. [b] Calculated from the other three A_α values. [c] Vertical intercept minus $\Sigma_\alpha(A_{\alpha'}/[\alpha'])$. [d] Calculated by extrapolation.

tinomycin values of 833×10^{-7} and 517×10^{-7} M min for CTP and GTP, respectively, were found (Hyman and Davidson, 1970).

The specific interaction of myxin with guanine and cytosine is also apparent from the visible spectrum of myxin in the presence of DNA and polydeoxyribonucleotides containing 0 and 100% C·G pairs. Table III shows hypochromicities measured at the 508 nm peak of myxin. Under the conditions of the experiment the effect of the 100% C·G polymer is twice that of calf thymus DNA and of the 0% C·G polymer.

The inhibition of RNA synthesis by myxin was furthermore tested by measuring the inhibitory effect as a function of G·C content of the template polymer. The data are presented in Figure 4. The template activity of a polydeoxyribonucleotide containing 100% C·G pairs is about three times more susceptible to myxin inhibition than the template activity of a polymer with 0% C·G pairs. The activity of calf thymus DNA is found at an intermediate level.

Discussion

The results of the kinetic experiments reveal that myxin acts by affecting the rate terms for the incorporation of CTP and GTP into RNA, but not for the incorporation of ATP and UTP. The decrease found for the overall incorporation rate of the four ribonucleotides in the presence of myxin (Hollstein and Van Gemert, 1971) did not allow for a distinction between the kinetic behavior of the individual ribonucleotides. However, the present study shows that it is the CTP and GTP incorporation step which is slowed down in the presence of the antibiotic, while the ATP and UTP incorporation step remains unaltered. In agreement with these results it could be demonstrated that the inhibitory effect of myxin increases with the C·G content of the template.

The specific properties of C and G with regard to myxin are also reflected in the twice as large degree of hypochromicity which poly(dC)·poly(dG) afflicts on myxin as compared to the effect of calf thymus DNA. That poly[d(A-T)]·poly[d(A-T)] causes under the same conditions a hypochromicity equal to that of DNA may be interpreted as a type of binding to A and T which affects electronic transition probabilities but not incorporation rates of U and A, respectively. Apparently the presence of C·G pairs is not essential for the binding of myxin. These results are reminiscent of studies which showed that streptonigrin binds in equal amounts to calf thymus DNA and poly[d(A-T)]·poly[d(A-T)] but with at least a twofold increase of binding to poly(dC)·poly(dG) (Mizuno and Gilboe, 1970).

Differences in binding capacity of the A·T and the C·G

TABLE III: Hypochromicity of Myxin.

Myxin (M)	Polydeoxyribonucleotide	% Hypochromicity at $\lambda_{max} = 508$ nm $\frac{A_0 - A}{A_0} \times 100$
1.35×10^{-5}	8.04×10^{-4} M DNA	22
1.35×10^{-5}	7.85×10^{-4} M poly[d(A-T)]·poly[d(A-T)]	24
1.35×10^{-5}	7.95×10^{-4} M poly(dC)·poly(dG)	46

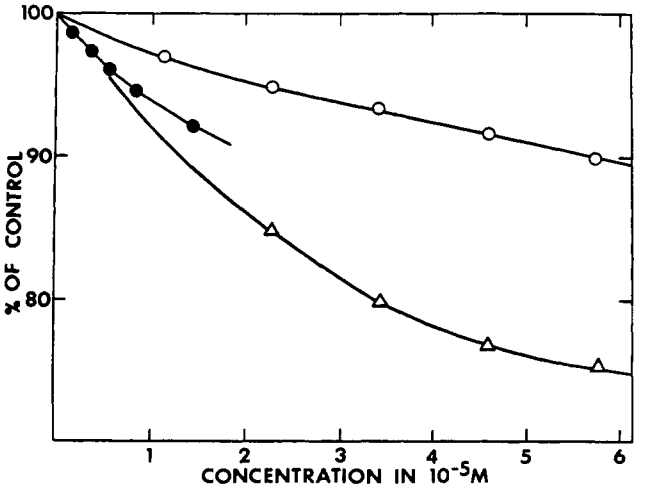

FIGURE 4: Effect of myxin on RNA synthesis primed by poly[d(A-T)]·poly[d(A-T)] (○), calf thymus DNA (●), and poly(dC)·poly(dG) (△). Experimental conditions and the DNA data points are from Hollstein and Van Gemert (1971).

couple have also been detected with other phenazine antibiotics (Hollstein and Van Gemert, 1971). Similar differences have been reported for the interaction of aminoacridines with synthetic and natural polydeoxyribonucleotides with different C·G contents, measured spectrophotometrically (Chan and McCarter, 1970). Also the effect of the antibiotic anthramycin on the binding reactivity of polydeoxyribonucleotides is related to their C·G content, as evidenced by buoyant density measurements (Kohn and Spears, 1970). Chromomycin, mithramycin, and olivomycin inhibit RNA synthesis only if the governing template comprises guanine (Ward *et al.*, 1965). The presence of guanine or, more general, a 2-aminopurine is believed to be essential for the interaction of actinomycin with polydeoxyribonucleotides (Cerami *et al.*, 1967). While the binding of actinomycin to a C·G pair has been interpreted as a charge-transfer complex stabilized by a unique fit of electron density distributions in the C·G and in the actinomycin chromophore (Müller and Crothers, 1968) later work revealed that the presence of G is not essential for binding and that a specific base sequence and thus, conformation rather than a specific base is a prerequisite for interaction (Wells and Larson, 1970). The same has been concluded with regard to chromomycin A_3 (Hayasaka and Inoue, 1969).

The present work shows that myxin is interacting with the C·G pair more strongly than with the other base pair of the intercalated structure. Unlike with actinomycin (Hyman and Davidson, 1970) the incorporation of C is inhibited by myxin with equal effectiveness as that of G (Table II). An electrostatic attraction between polar regions in the C·G pair and myxin may be postulated although the position of myxin relative to the C·G pair and the apparent symmetrical mode by which myxin affects C and G equally is not yet understood.

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