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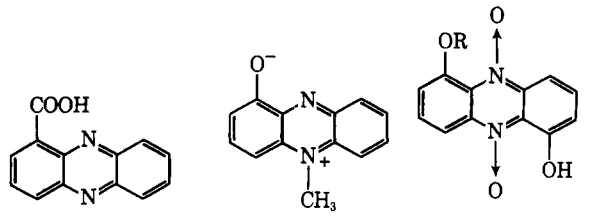
Interaction of Phenazines with Polydeoxyribonucleotides*

Ulrich Hollstein† and Robert J. Van Gemert, Jr.

ABSTRACT: The interaction of several phenazine antibiotics (phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocyanin, 1,6-dimethoxyphenazine and its mono- and di-*N*-oxide, 1,6-dihydroxyphenazine, iodinin, and myxin) with DNA was studied by visible spectrophotometry. There were two types of binding for these derivatives: a strong binding (association constants 10^4–10^6 range) and a weaker binding (association constants 10^3–10^4 range). There was no base specificity for phenazine-1-carboxamide and pyocyanin; however, the binding strength decreased for phenazine-1-carboxamide if DNA was substituted by poly(d(A-T))·poly(d(A-T)) or poly(dC)·poly(dG). Binding was weakened by increasing ionic strength. No binding to single-stranded polydeoxyribonucleotides was detected. These results are in agreement with an intercalative model which has also been proposed for other tricyclic planar aromatic antibiotics. The phenazine derivatives inhibit DNA template-controlled RNA synthesis whereby iodinin approaches the inhibitory intensity of actinomycin. It is suggested that the inhibition, at least in part, is due to intercalation.

Phenazine derivatives have been known for many years from microbial as well as synthetic origins. Many of about 20 known microbial metabolites possess antibiotic activity (Miller, 1961). Antiviral and antitumor activity has been detected in numerous synthetic phenazine derivatives (Endo *et al.*, 1966; Katagiri *et al.*, 1967). Of particular interest is the recently discovered antibiotic myxin which has been reported to possess an unusually broad antimicrobial spectrum (Peterson *et al.*, 1966). A few representative phenazine derivatives are shown in Chart I. There is a striking similarity between the planar aromatic phenazine skeleton and that of the acridines, phenoxazines (actinomycins), and other planar tricyclic antibiotics such as chromomycin, daunomycin, and ethidium bromide. Studies of these antibiotics led to an intercalative model for a ligand–DNA complex (Ward *et al.*,

CHART I



phenazine-1-carboxylic acid | pyocyanin | R = H, iodinin; R = CH_3, myxin

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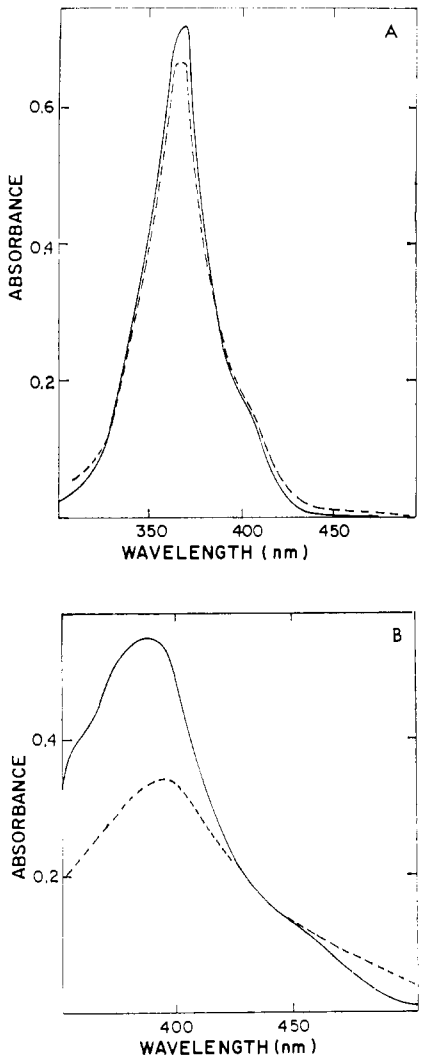

FIGURE 1: Absorption spectrum of phenazine derivative in the absence (——) and presence (----) of excess calf thymus DNA. (A) Phenazine-1-carboxylic acid, $c_t = 8.94 \times 10^{-6}$ M, $c_{DNA} = 2.91 \times 10^{-3}$ monoM; 0.001 M acetate buffer, pH 6.0, in 5% ethanol. (B) Phenazine-1-carboxamide, $c_t = 6.80 \times 10^{-5}$ M, $c_{DNA} = 3.04 \times 10^{-3}$ monoM; 0.001 M acetate buffer, pH 6.0, in 5% ethanol.

1965; Blake and Peacocke, 1968; Müller and Crothers, 1968). It was of interest, therefore, to investigate whether the mechanism of the antibiotic action of phenazine derivatives could be explained in an analogous fashion. We report here the effect of various polynucleotides on the electronic spectra of several phenazine derivatives. The binding parameters are calculated for the apparent complex formation. In addition, the effect of the antibiotics on DNA-controlled transcription is studied.

Materials and Methods

Phenazine-1-carboxylic acid was prepared from 1-methylphenazine (Hollstein, 1968) by oxidation with chromium trioxide (Clemo and McIlwain, 1934). Its amide was obtained *via* the acid chloride (Kögl and Postowsky, 1930). Photochemical oxidation of phenazine methosulfate (McIlwain, 1937) yielded pyocyanin. Applying the Wohl–Aue reaction, 1,6-dimethoxyphenazine was secured (Yoshioka and Kidani, 1952). This key substance was used in the synthesis of its mono- and di-*N*-oxide (Sigg and Toth, 1967) and of 1,6-dihydroxyphenazine (Yoshioka and Kidani, 1952). Iodinin was prepared from 1,6-dihydroxyphenazine with *m*-chloroperbenzoic acid, and myxin was obtained by partial methylation of iodinin (Weigele and Leimgruber, 1967). All products were extensively purified by recrystallization or chromatography on Florisil until they showed constant melting point and thin-layer chromatographic purity and until their electronic spectra corresponded to those reported in the literature.

Polynucleotides and Enzymes. Calf thymus DNA, Na salt, type I (Sigma Chemical Co.), was used throughout this investigation. Poly(d(A-T))·poly(d(A-T)), poly(dC)·poly(dG), and poly(d(A,C,T,G))[1] 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.

Preparation of Solutions. Stock solutions of 10^{-3}–10^{-4} M, having absorbances in the range 2–5, were prepared of all phenazines in sodium acetate buffer (10^{-3} M, pH 6.0). In the case of the sparingly soluble phenazines, addition of ethanol (up to 5%) was necessary to achieve sufficient solubility. Stock solutions of 10^{-2}–10^{-3} M of all polynucleotides were prepared in 10^{-3} M Na^+ (buffer or NaCl). The RNA polymerase solution contained 5.3 mg/ml with 2630 units/mg (Ratliff *et al.*, 1967). For the inhibition runs, it was suitably diluted 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.

Optical measurements were carried out at 26° in a Cary Model 14R spectrophotometer with a repetitive scan assembly. Difference spectra were obtained with a set of 2 × 10 mm tandem cells. All other spectra were taken in cylindrical 10-, 50-, or 100-mm cells (operational volumes 3, 15, and 30 ml, respectively) or in square 10-mm micro cells (operational volume 1 ml). The validity of Beer's law was checked and confirmed for all phenazines in the operational concentration range of 10^{-5}–10^{-6} M. This measurement also yielded, after leasts-square analysis, an accurate ϵ scale for the spectral curve.

Solutions with varying phenazine to polynucleotide ratios were prepared in two ways. (A) A set of tubes was filled with constant volumes (1–2 ml) of phenazine stock solution, varying amounts of standard polynucleotide solution (0–2 ml, ~0.02 M), and enough buffer to make up a constant volume (3–5 ml). From the difference spectrum of the first tube (no polynucleotide) read against the last tube (excess polynucleotide), the wavelength at maximum, $\Delta\epsilon$, was determined. The base-line value at $\Delta\epsilon_{max}$, usually 0.01–0.02 absorption unit, was determined from the spectrum of polynucleotide (same concentration as last tube) against the buffer. At intermediate polynucleotide concentrations, corrections were made for proportionated base-line values. The solution in each tube was read against the buffer at the wavelength corresponding to $\Delta\epsilon_{max}$. (B) In some runs a 50- or 100-mm cell was filled with a suitable concentration of phenazine and read against the buffer. From a 5-ml microburet, known quantities of standard polynucleotide solution were added to the cell and, after thorough mixing, the cell was read against the buffer. Base-line values and values for λ at $\Delta\epsilon_{max}$ were determined as above.

Determination of Binding Parameters. Assuming that the phenazines act as ligands entering a complex with the polynucleotide and that this is a reversible process, the equilib-

[1] The following abbreviations have been used: poly(d(A,C,T,G)), single-stranded DNA having a random base sequence; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

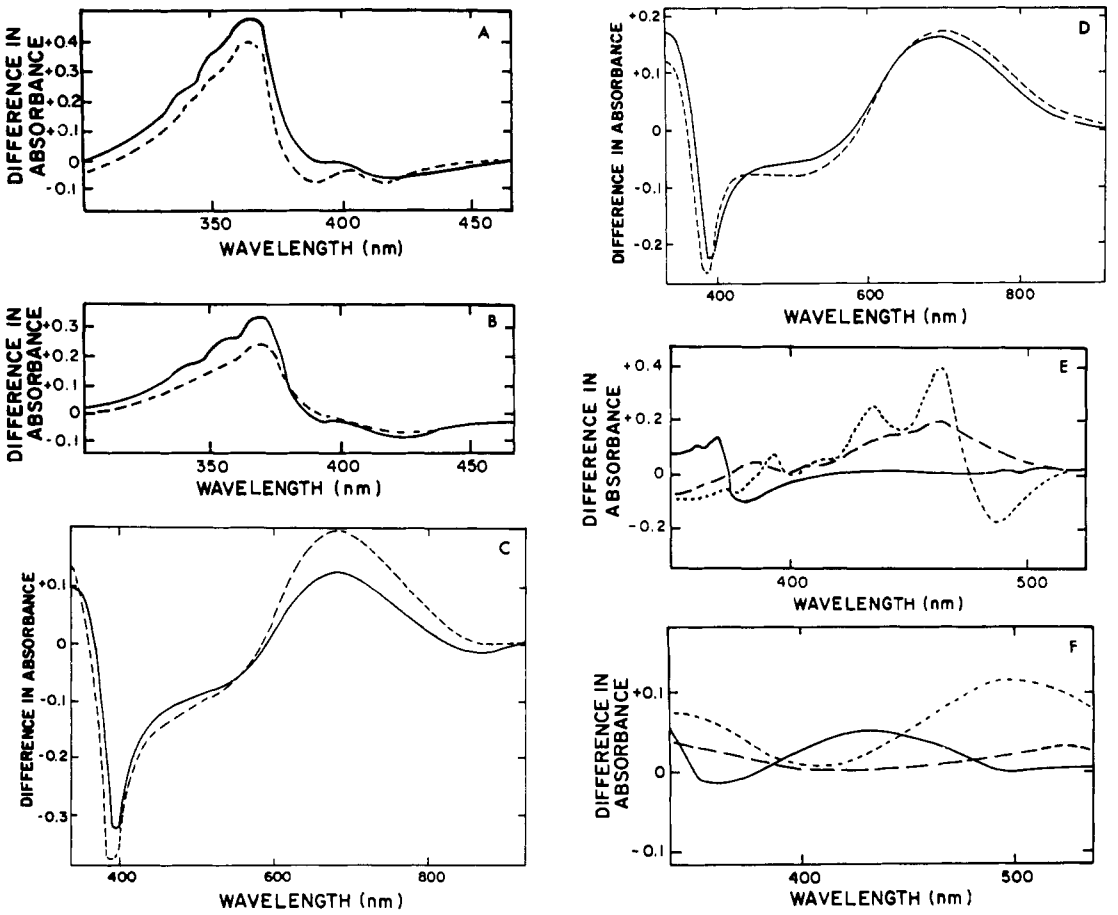

FIGURE 2: Difference absorption spectrum of phenazine derivative in the absence and presence of excess polynucleotide. (A) Phenazine-1-carboxamide, $c_t = 1.70 \times 10^{-4}$ M (4% ethanol); plus calf thymus DNA, $c_t = 9.00 \times 10^{-3}$ monoM (———); plus poly(d(A-T))·poly(d(A-T)), $c = 9.00 \times 10^{-3}$ monoM (-----); 0.01 M acetate, pH 6.0. (B) Phenazine-1-carboxamide, $c_t = 1.70 \times 10^{-4}$ M (4% ethanol); plus calf thymus DNA, $c = 2.58 \times 10^{-3}$ monoM (———); plus poly(dC)·poly(dG), $c = 2.58 \times 10^{-3}$ monoM (-----); 0.01 M acetate, pH 6.0. (C) Pyocyanin, $c_t = 1.29 \times 10^{-4}$ M; plus calf thymus DNA, $c = 9.00 \times 10^{-3}$ monoM (——); plus poly(d(A-T))·poly(d(A-T)), $c = 9.00 \times 10^{-3}$ monoM (-----); 0.01 M acetate, pH 6.0. (D) Pyocyanin, $c_t = 1.29 \times 10^{-4}$ M; plus calf thymus DNA, $c = 2.58 \times 10^{-3}$ monoM (———); plus poly(dC)·poly(dG), $c = 2.58 \times 10^{-3}$ monoM (----); 0.01 M acetate buffer, pH 6.0. (E) 1,6-Dimethoxyphenazine, $c_t = 1.49 \times 10^{-4}$ M (3% ethanol) (——); 1,6-dimethoxyphenazine mono-*N*-oxide, $c_t = 1.18 \times 10^{-4}$ M (3% ethanol) (-----); 1,6-dimethoxyphenazine di-*N*-oxide, $c_t = 6.85 \times 10^{-5}$ M (5% ethanol) (.....); calf thymus DNA, $c = 7.25 \times 10^{-3}$ monoM; 0.001 M acetate buffer, pH 6.0. (F) 1,6-Dihydroxyphenazine, $c_t = 6.43$ 10^{-5} M (5% ethanol), $c_{DNA} = 7.45 \times 10^{-3}$ monoM (——); iodinin, $c_t = 7.88 \times 10^{-6}$ M (5% ethanol), $c_{DNA} = 2.33 \times 10^{-3}$ monoM (-----); myxin, $c_t = 2.29 \times 10^{-5}$ M, $c_{DNA} = 1.67 \times 10^{-3}$ monoM (. . . .); 0.001 M acetate buffer, pH 6.0.

rium ligand + polynucleotide $\rightleftarrows$ complex has an association constant

$$K = \frac{c_b}{c_f(B' - c_b)} \quad (1)$$

where c_b = concentration of bound ligand = concentration of complex, c_f = concentration of free ligand, and B' = maximum available binding sites on the polynucleotide chain. Furthermore, we call the total concentration of the ligand $c_t = c_f + c_b$, the ratio of bound molecules per monomeric nucleotide $c_b/c_{monomers} = r$, and the maximum available binding sites per monomeric nucleotide $B'/c_{monomers} = B$. Equation 1 can then be written in a more practical form $r/c_f = K(B - r)$ which allows a plot of r/c_f *vs.* r, giving a straight line with slope $-K$ and intercept B.

In the case of two types of binding with sufficient difference in binding energy, two linear regions may be discerned with slopes $-K_1$ and $-K_2(K_1 \gg K_2)$ and corresponding intercepts B_1 and $B_1 + B_2$ (Blake and Peacocke, 1968). If many types of binding exist, a curve without any discernible linear regions may result and only an approximate range of K and B values may be deduced.

Values for c_b and c_f were obtained spectrophotometrically. For a mixture of bound and free ligand: $A^\lambda = \epsilon_f^\lambda c_f + \epsilon_b^\lambda c_b$, where A^λ = absorbance at a given wavelength, and ϵ_f^λ and ϵ_b^λ are the molecular extinction coefficients at the wavelength for the free and bound ligand, respectively. With $c_t = c_f + c_b$ and $\epsilon_f^\lambda - \epsilon_b^\lambda = \Delta\epsilon^\lambda$, this can be written as $c_b = (\epsilon_f^\lambda c_t - A^\lambda)/\Delta\epsilon^\lambda$. Measurement of A^λ at different monomeric nucleotide concentrations yields a set of values for r/c_f and r.

Determination of Inhibition. A set of tubes each containing a mixture of 10 μl of 7.45×10^{-3} M calf thymus DNA, 0.37 ml of a premix consisting of 10 ml of 0.1 M HEPES (pH 7.9), 0.1 ml of 1.0 M $MgCl_2$, 0.25 ml of 0.1 M $MnCl_2$, 1.0 ml of 0.01 M [^{14}C]ATP, 2.0 ml of 0.005 M CTP, 2.0 ml of 0.005 M GTP, 2.0 ml of 0.005 M UTP, and 1.0 ml of H_2O, with 200 nmoles of each triphosphate/0.37 ml, 20 μl of four-times-diluted RNA polymerase, varying amounts of diluted phenazine stock solution (0–100 μl) and enough water to make 0.5 ml, was incubated at 37° for 30 min.

TABLE I: Hypochromic and Bathochromic Effects of Excess Polynucleotide on Visible Peak.

Phenazine Derivative	Polynucleotide	Ionic Strength	Hypochromic Effect at λ_{max} (%)	Bathochromic Effect at λ_{max} (nm)
Phenazine-1-carboxylic acid	DNA	0.2	0	0
Phenazine-1-carboxylic acid	DNA	0.001	7	1
Phenazine-1-carboxamide	DNA	0.2	0	0
Phenazine-1-carboxamide	DNA	0.001	38	2
Phenazine-1-carboxamide	DNA	0.01	36	2
Phenazine-1-carboxamide	Poly(d(A-T))·poly(d(A-T))	0.01	37	3
Phenazine-1-carboxamide	Poly(dC)·poly(dG)	0.01	21	2
Pyocyanin	DNA	0.2	0	0
Pyocyanin	DNA	0.001	12	14
Pyocyanin	DNA	0.01	11	12
Pyocyanin	Poly(d(A-T))·poly(d(A-T))	0.01	12	14
Pyocyanin	Poly(dC)·poly(dG)	0.01	11	14
1,6-Dimethoxyphenazine	DNA	0.2	0	0
1,6-Dimethoxyphenazine	DNA	0.001	13	0
1,6-Dimethoxyphenazine mono-*N*-oxide	DNA	0.001	28	0
1,6-Dimethoxyphenazine di-*N*-oxide	DNA	0.001	30	0
1,6-Dihydroxyphenazine	DNA	0.001	28	1
Iodinin	DNA	0.001	53	0
Myxin	DNA	0.001	14	1
N-Methylphenazinium cation[a]	DNA	0.001	56	4
Actinomycin[b]	DNA	0.01	26	6
9-Aminoacridine[c]	DNA	0.1	45	6

[a] Ishizu *et al.* (1969). [b] Müller and Crothers (1968). [c] Drummond *et al.* (1959).

Each solution was transferred quantitatively with a little distilled water to a Whatman GFB glass fiber filter paper disk (diameter 5 mm, thickness 0.67 mm). The disks were soaked at 5° for 15 min in a solution containing 5% trichloroacetic acid and 1% sodium pyrophosphate. 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 in a toluene scintillator solution in a Packard scintillation counter.

A control was run without the phenazine, and all counting data were corrected for a blank which contained no enzyme. In order to reach sufficiently high concentrations, up to 5% ethanol was allowed for those phenazines which were little soluble in water. Control runs with varying concentrations of ethanol showed no inhibition up to this ethanol concentration.

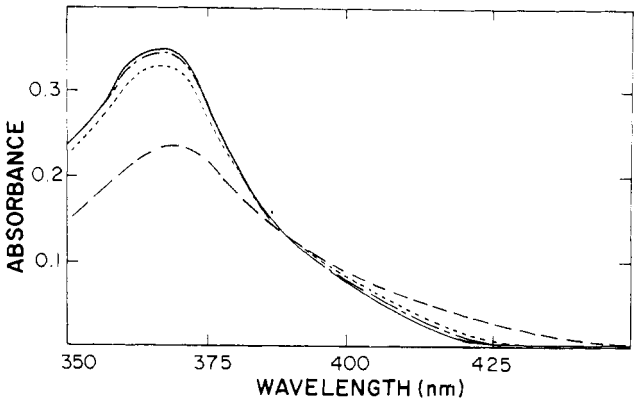

FIGURE 3: Absorption spectrum of phenazine-1-carboxamide, c_t = 3.95×10^{-5} M (——); plus excess calf thymus DNA, c = 8.17×10^{-3} monoM (-----); plus excess heat denatured (~80% single stranded) DNA, c = 8.17×10^{-3} monoM (. . . . .); plus excess poly(d(A,T,C,G)), c = 1.09×10^{-3} monoM (-·-·-); 0.001 M acetate buffer, pH 6.0.

Results

The absorption spectra of various phenazine derivatives were measured in the absence and presence of excess calf thymus DNA. Phenazine-1-carboxylic acid showed a negligible change, but phenazine-1-carboxamide, pyocyanin, 1,6-dimethoxyphenazine, its mono- and di-*N*-oxide, 1,6-dihydroxyphenazine, iodinin, and myxin showed hypochromic shifts of 12–53% and bathochromic shifts of 1–14 nm if measured at ionic strengths of 0.001 (Figures 1 and 2). These shifts were slightly reduced at an ionic strength of 0.05; however, at an ionic strength of 0.2, any change between the spectrum without and with DNA was imperceptible (Table I).

The effect of calf thymus DNA on the absorption spectrum was compared with that of synthetic polynucleotides containing either adenine and thymine only [poly(d(A-T))·poly(d(A-T))] or guanine and cytosine only [poly(dC)·poly(dG)]. For phenazine-1-carboxamide and pyocyanin, only negligible differences could be detected (Figure 2A–D) in this comparison.

In order to study the importance of double strandedness

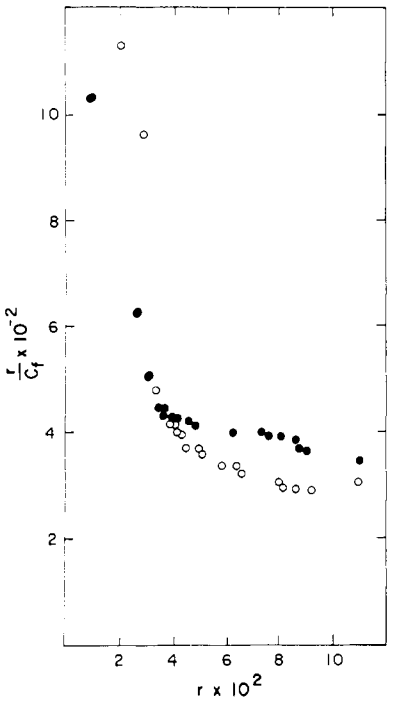

FIGURE 4: Plot of r/c_f *vs.* r for the interaction of phenazine derivative with polynucleotide; acetate buffer, pH 6.0. Phenazine-1-carboxamide with calf thymus DNA; $\mu = 0.001$ (○), $\mu = 0.05$ (●).

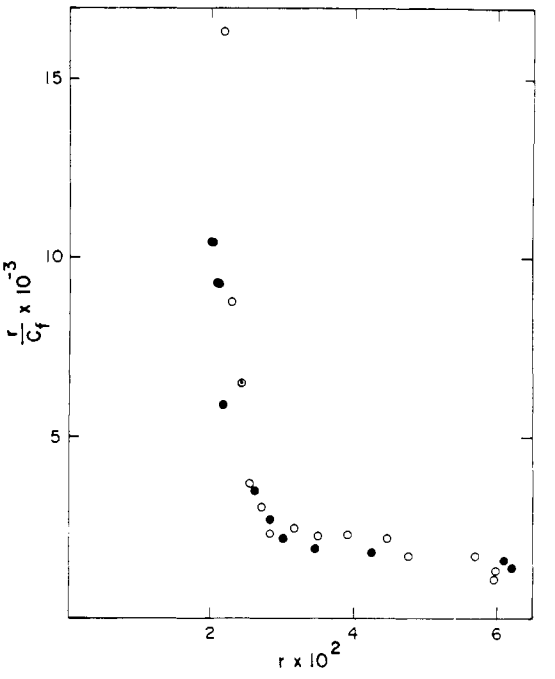

FIGURE 6: Plot of r/c_f *vs.* r for the interaction of phenazine derivative with polynucleotide; acetate buffer, pH 6.0. Pyocyanin with calf thymus DNA, $\mu = 0.001$, calculated at 378 nm (○) and at 670 nm (●).

on binding of phenazine-1-carboxamide, the effect of calf thymus DNA was compared with that of heat-denatured calf thymus DNA and of synthetic poly(d(A,T,C,G)). Heat-denatured DNA, which contained according to its own extinction coefficient about 80% single strands, caused a-hypochromic shift of one-fifth of that observed with double-stranded DNA. Synthetic poly(d(A,T,C,G)) which, because of its random base sequence, is expected to be void of double-stranded regions failed to lower the absorption of all (Figure 3).

The binding parameters K_1, K_2, B_1, and B_2 were derived

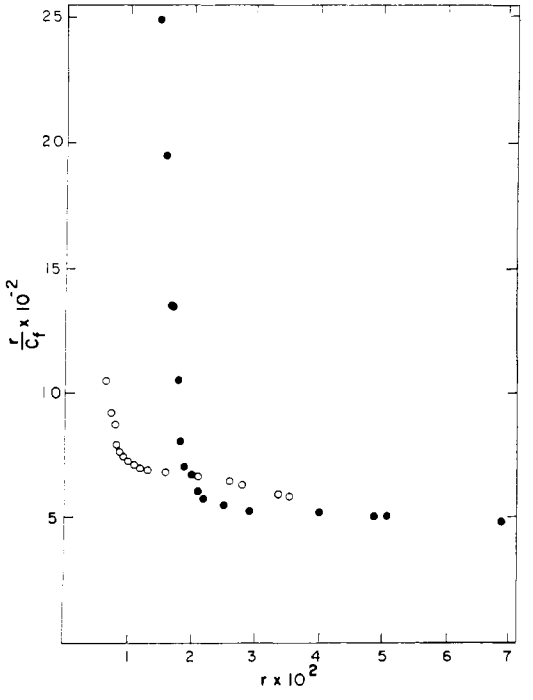

FIGURE 5: Plot of r/c_f *vs.* r for the interaction of phenazine derivative with polynucleotide; acetate buffer, pH 6.0. Phenazine-1-carboxamide with poly(d(A-T)·poly(d(A-T)), $\mu = 0.05$ (○); with poly(dC)·poly(dG), $\mu = 0.05$ (●).

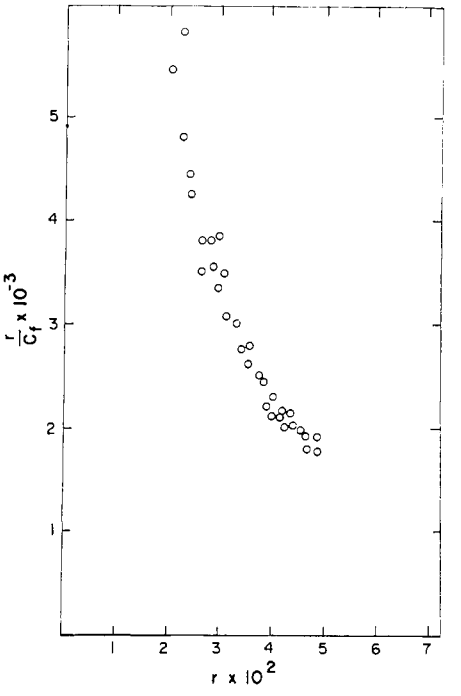

FIGURE 7: Plot of r/c_f *vs.* r for the interaction of phenazine derivative with polynucleotide; acetate buffer, pH 6.0. 1,6-Dihydroxyphenazine with calf thymus DNA, $\mu = 0.001$.

TABLE II: Binding Parameters for Various Phenazine–Polynucleotide Complexes.

Phenazine	Polynucleotide	μ	K_1	K_2	B_1	B_2
Phenazine-1-carboxamide	DNA	0.001	2×10^6	4×10^4	0.03	0.06
Phenazine-1-carboxamide	Poly(d(A-T))·poly(d(A-T))	0.05[a]	1×10^6	4×10^4	0.03	0.06
Phenazine-1-carboxamide	Poly(dC)·poly(dG)	0.05[a]	1×10^5	3×10^3	0.01	0.2
Phenazine-1-carboxamide	DNA	0.05[a]	2×10^5	1×10^3	0.02	0.2
Pyocyanin	DNA	0.001	4×10^4	1×10^3	0.05	0.2
1,6-Dihydroxyphenazine[b]	DNA	0.001	$\sim 10^5$	$\sim 10^3$	~0.05	~0.1
Iodinin	DNA	0.001	9×10^6	$\sim 3 \times 10^5$	0.01	0.2
Myxin	DNA	0.001	2×10^7	2×10^5	0.02	0.03

[a] Compared at higher ionic strength because T_m of poly(d(A-T))·poly(d(A-T)) at $\mu = 0.001$ is 19°, but 47° at $\mu = 0.05$. [b] No linear region was discernible.

from r/c_f *vs.* r plots (Figures 4–8) and are tabulated in Table II. Except for 1,6-dihydroxyphenazine, two linear regions could be observed in each case with association constants differing about two orders of magnitude. The stronger binding (K_1) for the various compounds measured was associated with an average separation ranging from 15 to 50 base pairs (B_1), while the weaker binding (K_2) involved 3–25 base pairs. A slight decrease in K_1 was observed for phenazine-1-carboxamide when the ionic strength was raised from 0.001 to 0.05. Substitution of calf thymus DNA by either poly(d(A-T))·poly(d(A-T)) or poly(dC)·poly(dG) lowered both K_1 and K_2 for phenazine-1-carboxamide by one order of magnitude and raised the number of weak binding sites (B_2) by a factor of 3. Pyocyanin showed binding (K_1) by 2 orders of magnitude lower than the other phenazines measured at the same ionic strength.

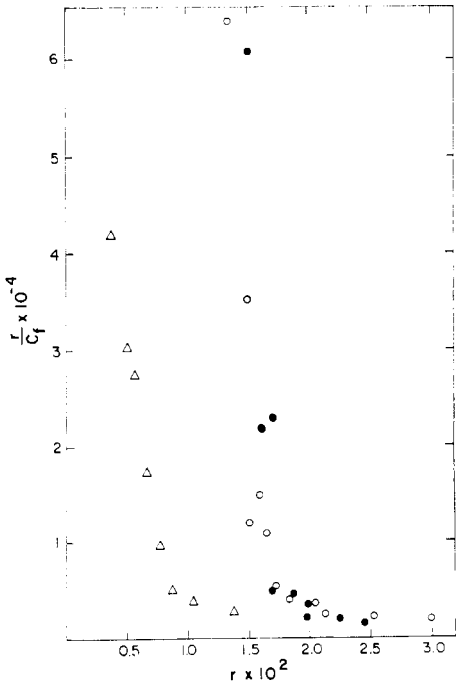

FIGURE 8: Plot of r/c_f *vs.* r for the interaction of phenazine derivative with polynucleotide; acetate buffer, pH 6.0. Myxin with calf thymus DNA, $\mu = 0.001$, calculated at 505 nm (○) and at 345 (●); iodinin with calf thymus DNA, $\mu = 0.001$ (△).

The influence of phenazine derivatives on DNA-controlled RNA synthesis was studied by measuring the incorporation of labeled ribonucleotides in the presence of varying amounts of phenazine derivative. Because of large differences in solubility of the phenazine derivatives, the curves (Figure 9) can be compared only in the lower concentration regions. The remarkably low solubility of iodinin (2 μg/ml of water and 3 μg/ml of 5% ethanol) allowed measurement only in the 10^{-6} M range. The curves show a strong inhibitory effect for iodinin and 1,6-dihydroxyphenazine as compared with phenazine-1-carboxamide and pyocyanin. The 1,6-dimethoxy derivatives represented much weaker inhibition.

Experiments to study the possible binding of phenazine derivatives to RNA polymerase by equilibrium dialysis are being carried out. Preliminary results indicate that, in the case of phenazine-α-carboxamide, no phenazine–enzyme complex is formed.

Discussion

It appears from the spectrophotometric analyses that phenazine derivatives bind to native DNA. The shape of all calculated binding isotherms suggests at least two binding types: a stronger complex with relatively few binding sites per deoxyribonucleic acid nucleotide, and a weaker complex where the binding sites per nucleotide approach the maximum of one. Association constants for the two binding types differ about 2 orders of magnitude or 2–3 kcal/mole. Association constants are of the same order as those found for aminoacridines (10^6), ethidium bromide (10^6 (Waring, 1965)), actinomycin (10^6–10^7 (Müller and Crothers, 1968)), and the methylphenazinium cation (10^5 (Ishizu *et al.*, 1969)). However, the number of binding sites per nucleotide (B_1, B_2) found in this study was less than in the cited examples.

While the weaker type of binding with polynucleotides has been explained for other tricyclic aromatic antibiotics as a complex formation on the surface of the polynucleotide, it has been proposed that the stronger binding is of an intercalative type (Lerman, 1961) whereby the aromatic system is held between two successive bases, presumably through interaction of the π electrons of the ligand with the parallel bases. Recent developments have shown that the aminoacridine–DNA complex can best be described by a model whereby the ligand is intercalated between successive bases of a single-stranded polynucleotide (Blake and Peacocke, 1968). Alternatively, the actinomycin complexes (Müller

and Crothers, 1968) fit a model whereby intercalation takes place between successive base pairs in a double-stranded polynucleotide.

In the present study, the spectra of ligand–polynucleotide mixtures show hypochromic–bathochromic shifts characteristic for π-complex formation. Table I shows a comparison with other tricyclic aromatic antibiotics. The shifts are strongly dependent on ionic strength and completely disappear at an ionic strength of 0.2. Such dependence may be interpreted in two ways: either there is a shielding effect by the inorganic cations on the negative sites of the polynucleotide strand (*i.e.*, the phosphate which otherwise is the site of binding), or the inorganic cations decrease through a shielding effect the repulsion of successive phosphates, rendering the intercalation of a ligand between successive bases or base pairs more difficult. The former situation has been assumed for aminoacridines where the binding parameters for single- and double-stranded DNAs are similar. For the phenoxazones such as actinomycin, however, the ionic strength dependence led to the latter interpretation.

The interaction of one of the presently studied phenazine derivatives (phenazine-1-carboxamide) with single-stranded polynucleotide (poly(d(A,C,T,G)) as well as denatured DNA) was found to be negligible, and it must be assumed that, at least in this case, a double-stranded polynucleotide is required for binding, suggesting a binding of the intercalative type.

The antibiotic phenazine-1-carboxylic acid showed a very small hypochromic effect in the presence of DNA. Possibly this compound, which at pH 6.0 is in the anion form, is hindered in its approach to the polyanionic polynucleotide.

The antibiotics chromomycin and oligomycin (Ward *et al.*, 1965), as well as actinomycin (Cerami *et al.*, 1967), require the 2-amino group for binding on a purine nucleus (*i.e.*, they require the presence of guanine in native DNA). More recently it was found (Wells and Larson, 1970) that the mere presence of guanine in DNA is not a sufficient condition for binding of actinomycin; the nature of the bases adjacent to guanine seems to be of importance. We did not find such base specificity for binding of phenazine-1-carboxamide and pyocyanin to native DNA: the hypochromic effect for these phenazine derivatives is essentially the same whether measured in admixture with native DNA, poly(d(A-T))·poly(d(A-T)), or poly(dC)·poly(dG). However, the binding parameters (K and B) for the phenazine-1-carboxamide–poly(d(A-T))·poly(d(A-T)) or poly(dC)·poly(dG) complex differ considerably from the phenazine-1-carboxamide DNA complex. The strong and weak association constants (K_1, K_2) are one order of magnitude less, and the number of available stronger binding sites (B_1) shows a 3-fold increase. Again, this seems to indicate that internal binding is enhanced by succession of different base pairs (*e.g.*, A-T followed by C-G or G-C) rather than a succession of alternating (A-T, T-A) or a succession of identical (C-G) pairs. The population of the weaker, possibly external binding sites is then augmented at the cost of the internal binding sites.

All phenazine derivatives studied inhibit DNA template-controlled RNA synthesis, although they do this with widely varying intensity. Such inhibition could result from blocking of the template (*e.g.*, by intercalation) or by binding to the RNA polymerase or to a ribonucleoside 5′-triphosphate. In addition, the rates of association–dissociation equilibrium with DNA are of importance. The faster the complex can dissociate into free DNA and antibiotic, the shorter is the time during which the enzyme is prevented from continuing synthesis along the DNA template. Indeed, the high biological

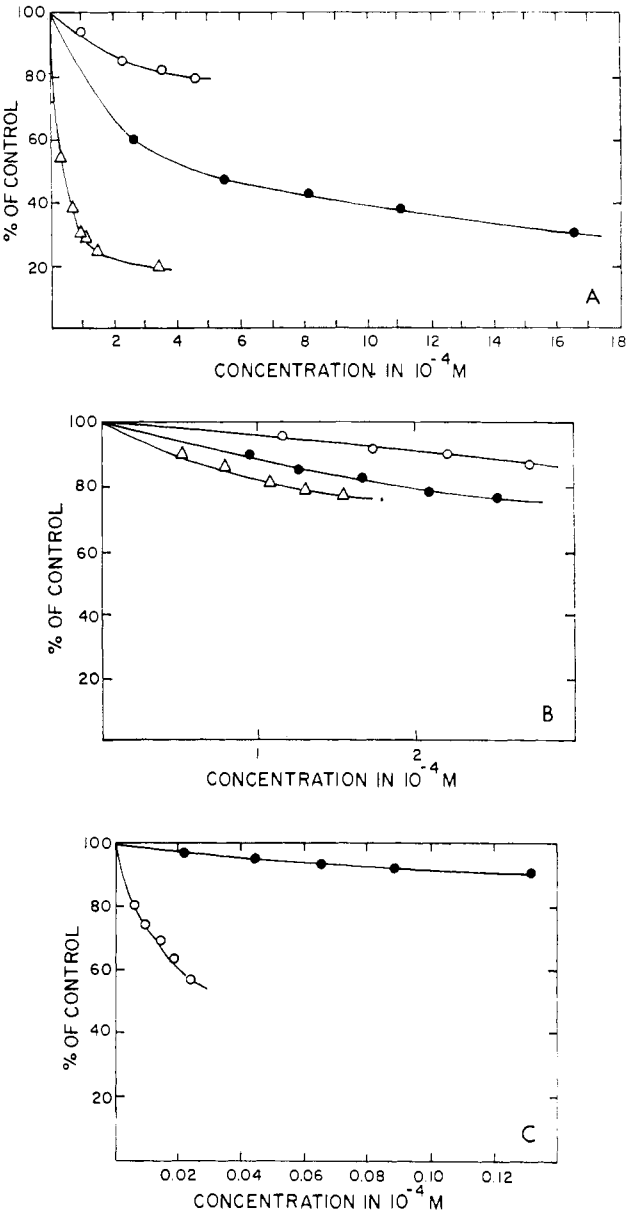

FIGURE 9: Effect of phenazine derivative on RNA synthesis primed by calf thymus DNA (the extent of the curves limited by the solubility of the phenazine derivative). (A) Phenazine-1-carboxamide (○); pyocyanin (●); and 1,6-dihydroxyphenazine (△). (B) 1,6-Dimethoxyphenazine (○); 1,6-dimethoxyphenazine mono-*N*-oxide (●); and 1,6-dimethoxyphenazine di-*N*-oxide (△). (C) Iodinin (○) and myxin (●).

activity of actinomycin may well be due to its slow dissociation rate (Müller and Crothers, 1968). For comparison it was found convenient to express the antibiotic concentration on a 10^{-4} M scale (Figure 5) and to indicate that concentration at which 50% of the control was remaining ($c_{1/2}$). Table III shows a comparison of the values found for various phenazine derivatives to other DNA complexing antibiotics.

Except for iodinin, the inhibitory effect of phenazine derivatives is weak. The strong effect of iodinin is in agreement with the fact that, in spite of its low solubility in water (2 μg/ml), it still exhibits marked antibiotic activity. The inhibitory effect of myxin is of the same order as that found in an *in vivo* study with *E. coli* (Lesley and Behki, 1967), where it has been speculated that myxin inhibits DNA

TABLE III: Inhibition of RNA Synthesis.

Antibiotic	$C_{1/2}$ (mole/l.)[a]	Reference
Phenazine-1-carboxamide	$>10^{-3}$	
Pyocyanin	4×10^{-4}	
1,6-Dimethoxyphenazine	$>10^{-3}$	
1,6-Dimethoxyphenazine mono-*N*-oxide	$>10^{-3}$	
1,6-Dimethoxyphenazine di-*N*-oxide	$>10^{-3}$	
1,6-Dihydroxyphenazine	4×10^{-5}	
Iodinin	4×10^{-6}	
Myxin	$\sim 10^{-4}$	
Echinomycin	4×10^{-7}	Ward *et al.* (1965)
Actinomycin	2×10^{-7}	Goldberg *et al.* (1962)
Actinomycin	4×10^{-6}	Hartmann *et al.* (1963)

[a] Concentration at which 50% of control remains.

synthesis without being bound to DNA but by direct action on the DNA-polymerizing enzyme. Our study indicates that myxin binds to DNA and, although inhibition of RNA synthesis may not be due entirely to the DNA complex, the association may be reasonably assumed to be a contributory factor.

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