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Binding of quinoline analogues of echinomycin to deoxyribonucleic acid

Role of the chromophores

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Two novel antibiotics were isolated, designated compounds 1QN and 2QN respectively, having quinoline rings in place of one or both of the quinoxaline chromophores of echinomycin. Each removes and reverses the supercoiling of closed circular duplex DNA from bacteriophage PM2 in the fashion characteristic of intercalating drugs. For compound 1QN, the unwinding angle at I0.01 is almost twice that of ethidium, whereas for compound 2QN the value is indistinguishable from that of ethidium. Binding of both analogues produced changes in the viscosity of sonicated rod-like DNA fragments corresponding to double the helix extension found with ethidium, a feature characteristic of bifunctional intercalation by quinoxaline antibiotics. These results suggest that both compounds 1QN and 2QN behave as bifunctional intercalators but that compound 2QN produces only half the helix unwinding seen with compound 1QN and the natural quinoxalines. Binding curves for the interaction of both analogues with a variety of synthetic and naturally occurring nucleic acids were determined by solvent-partition analysis. Values for compound 2QN were also obtained by a fluorimetric method and found to agree well with the solvent-partition measurements. Compound 1QN bound most tightly to *Micrococcus lysodeikticus* DNA and, like echinomycin, exhibited a broad preference for (G + C)-rich DNA species. For compound 2QN no marked (G + C) preference was indicated, and the tightest binding among the natural DNA species studied was found with DNA from *Escherichia coli*. The two analogues also displayed different patterns of specificity in their interaction with synthetic nucleic acids. Compound 2QN bound to poly(dA-dT) slightly more tightly than to poly(dG-dC), whereas compound 1QN displayed a large (approx. 11-fold) preference in the opposite sense. There was evidence of co-operativity in the binding to poly(dA-dT). It may be concluded that the chromophore moieties play an active role in determining the capacity of quinomycin antibiotics to recognize and bind selectively to specific sequences in DNA.

Compounds 1QN and 2QN are semibiosynthetic derivatives of the naturally occurring quinoxaline antibiotic echinomycin (Fig. 1). Quinoxaline antibiotics, which are produced by certain varieties of streptomycetes, are characterized by a cross-bridged cyclic octapeptide dilactone containing both D- and L-amino acids to which are attached two quinoxaline-2-carboxylic acid chromophores. They form two families, the quinomycins and the triostins (Katagiri *et al.*, 1975; Waring, 1979), which differ only in the nature of the cross-bridge formed between the two *N*-methylcysteine residues in the peptide portion. The triostin group has a simple disulphide cross-bridge in place of the thioacetal cross-bridge of the quinomycins. Compounds 1QN and 2QN are quinomycins possessing the same peptide portion as echinomycin (quinomycin A), but have respectively one or both of the quinoxaline-2-carboxylic acid chromophores replaced by quinoline-2-carboxylic acid.

The quinoxalines are all powerful antimicrobial and antitumour agents, whose toxic effects have been attributed to their binding to the DNA of susceptible cells (Katagiri *et al.*, 1975; Waring & Makoff, 1974). Echinomycin was shown to intercalate bifunctionally into DNA at low (0.01 M) ionic strength (Waring & Wakelin, 1974) and was the first bifunctional intercalating agent ever to be char-

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acterized. The DNA-binding properties of a number of naturally occurring quinoxaline antibiotics have now been described (Waring & Wakelin, 1974; Wakelin & Waring, 1976; Lee & Waring, 1978*a*), as well as those of some synthetic analogues (Lee & Waring, 1978*b*). All the naturally occurring quinoxaline antibiotics, and the synthetic analogue des-*N*-tetramethyltriostin A ('TANDEM') have been shown to intercalate bifunctionally into DNA at low (0.01 M) ionic strength.

A bifunctional intercalator may be expected to display greater selectivity in its binding to DNA species of different nucleotide sequence than would a simple (monofunctional) intercalator (Waring & Wakelin, 1974). This property, plus the fact that a range of closely related natural and synthetic analogues are available, makes the quinoxaline antibiotics ideal probes with which to study, at the molecular level, the way(s) in which base sequence-specificity may be realized. Differences are indeed observed in the gross extents of binding as well as in the patterns of specificity displayed by different quinoxaline antibiotics (Lee & Waring, 1978*a*,*b*; Waring, 1979). There is no evidence, however, pertaining to the contribution, if any, of the quinoxaline chromophores to the overall selectivity of the antibiotics for different DNA base-sequences other than the observation that a synthetic analogue of the 'TANDEM' series having benzyloxycarbonyl substituents in place of the quinoxaline rings binds weakly to DNA, but apparently not by intercalation (Lee & Waring, 1978*b*). In the present paper detailed studies are reported on the binding of compounds 1QN and 2QN to a variety of naturally occurring and synthetic nucleic acids, and their binding characteristics are compared with those of the natural quinoxaline antibiotics.

Materials and methods

Compounds 1QN and 2QN were prepared in this laboratory by following procedures based on the methods of Yoshida *et al.* (1968). *Streptomyces echinatus* A8331 was obtained from Dr. J. Nüesch and Dr. K. Scheibli of CIBA–GEIGY, Basel, Switzerland. It was grown in liquid culture on a maltose minimal medium supplemented with 1 mM-quinoline-2-carboxylic acid added at inoculation time. The mycelium was collected after 10 days and extracted with chloroform and acetone. The antiobiotics were purified from the crude extract by preparative t.l.c. on silica gel with ethyl methyl ketone as eluent, followed by chromatography on a Sephadex LH-20 column. The two analogues were characterized by mass spectrometry, n.m.r. spectroscopy and by C, H and N analysis. Their purity was verified by chromatography in a variety of solvent systems, by analytical high-pressure liquid chromatography and by coil-planet centrifugation. Because of the asymmetry imparted to the quinomycin structure by the thioacetal cross-bridge (Fig. 1), compound 1QN probably consists of two different isomers, though it has not proved possible to separate them by the preparative techniques used.

Most experiments were conducted at 20°C in a Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer at pH 7.0 and *I* 0.01, designated 0.01 SHE buffer (Waring & Wakelin, 1974). Fluorescence experiments were conducted in an equivalent potassium Hepes buffer, pH 7.0 and *I* 0.01 (0.01 PHE buffer). For use in solvent-partition analysis the organic solvent isopentyl acetate/n-heptane (7:3, v/v) designated '70/30 IPA/heptane' was prepared as described in Lee & Waring (1978*a*).

Calf thymus DNA (highly polymerized sodium salt, type 1) and *Escherichia coli* DNA (type VIII) were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A. Bacterial DNA species were obtained by a modification of the method of Marmur (1961) as described in Lee & Waring (1978*a*). For the measurement of binding curves all high-molecular-weight DNA samples were sheared to a standard molecular weight by drawing a solution (2 mg/ml in 2.5 M-NaCl) 20 times into a 5 ml syringe through a no. 28 needle at 0°C. This produced fragments with a sedimentation coefficient of 18 S with a minimal content of single-stranded ends (Pyeritz *et al.*, 1972). After the shearing, the preparations were dialysed exhaustively against 0.01 SHE buffer, filtered through two Whatman GF/C glass-fibre filters and stored frozen at −22°C. Bacteriophage-PM2 DNA consisting of over 80% closed circular duplex molecules was prepared by the method of Espejo *et al.* (1969). Nucleic acid concentrations were based on an assumed value for $\varepsilon_{(P)260}$ (molar absorption coefficient with respect to nucleotides) of 6600 except for *Micrococcus lysodeikticus* DNA (6300) (Tubbs *et al.*, 1964).

Poly(dA-dT) was purchased from Boehringer Corp. (London), London W.5, U.K., and poly-(dG-dC) from P–L Biochemicals, Milwaukee, WI, U.S.A. Both synthetic polymers were readily soluble in 0.01 SHE and 0.01 PHE buffers and were used as supplied without further purification. Concentrations were based on the values of $\varepsilon_{(P)}$ given by Wells & Wartell (1974).

Absorption coefficients

The molar absorption coefficients of the compounds in '70/30 IPA/heptane' and 50% (v/v) dimethyl sulphoxide were measured by direct weighing. Because of the low solubility of these antibiotics in 0.01 SHE buffer absorption coefficients could not be determined by direct weighing. Consequently, solutions of the antibiotic in '70/30 IPA/

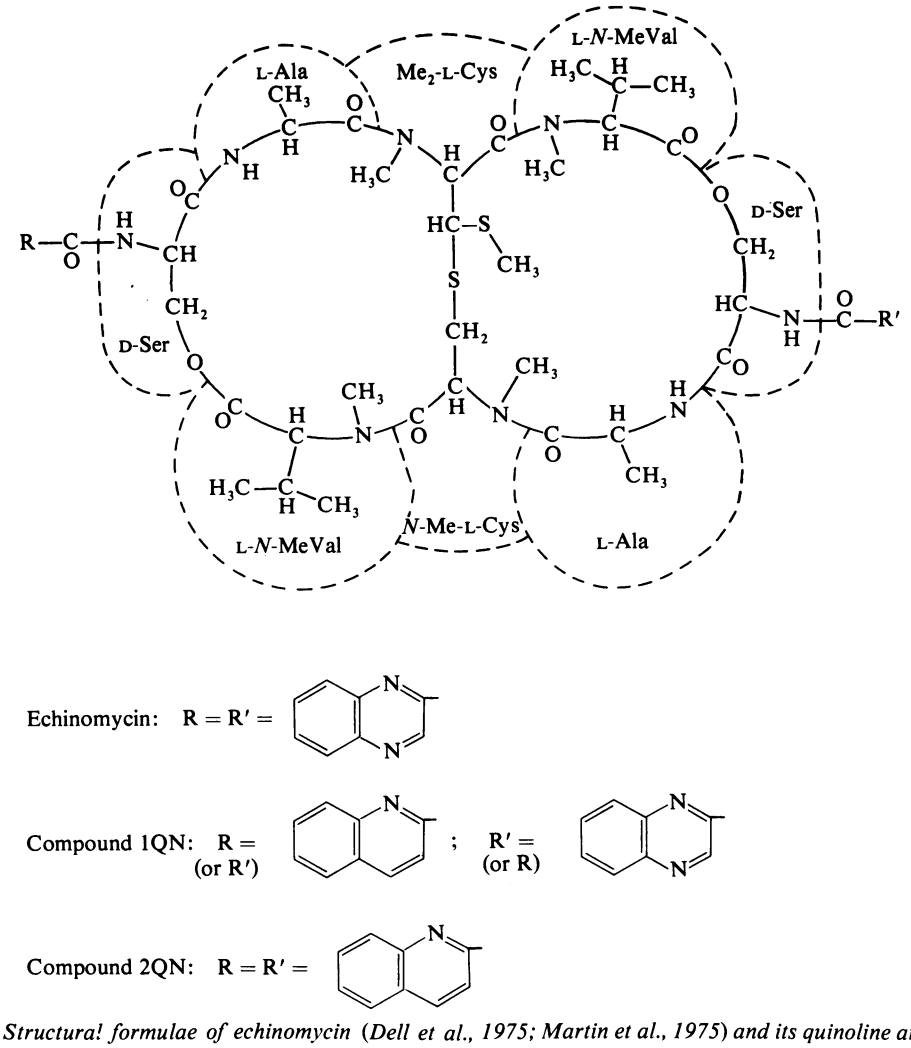

Fig. 1. *Structural formulae of echinomycin (Dell et al., 1975; Martin et al., 1975) and its quinoline analogues*

hepatane'-saturated 0.01 SHE buffer were prepared by shaking the solid material with buffer. Excess of antibiotic was removed by centrifugation and filtering through two Whatman GF/C glass-fibre filters. The absorbance was then measured in 100mm-light-path cuvettes at the wavelengths corresponding to peak maxima. The solutions were then diluted with an equal volume of dimethyl sulphoxide and the absorbance of each was measured at the appropriate wavelength. The absorption coefficient in 50% dimethyl sulphoxide having previously been determined, the absorption coefficient in aqueous buffer could then be calculated. The absorption coefficients thus determined are listed in Table 1.

Partition coefficients

The partition coefficients of the two antibiotics between 0.01 SHE buffer and '70/30 IPA/heptane' were measured as described by Waring *et al.* (1975). The values determined for compounds 1QN and 2QN were 198 ± 5 and 885 ± 60 respectively. The relevant plots are shown in Fig. 2.

Solvent-partition analysis

Binding curves were determined by the solvent-partition technique of Waring *et al.* (1975) as described in Lee & Waring (1978*a*). The measured values of r (mol of antibiotic bound/mol of nucleotides) and c (the free ligand concentration)

Table 1. *Molar absorption coefficients for compounds 1QN and 2QN*

The experimental procedures for measurements are described in the text. The wavelength of measurement corresponding to the peak maximum is shown in parenthesis after each value.

Solvent	Compound 1QN	Compound 2QN
'70/30 IPA/heptane'	8660 (315 nm)	6000 (315 nm)
50%-(v/v)-Dimethyl sulphoxide + '70/30 IPA/heptane'-saturated 0.01 SHE buffer	8180 (318 nm)	8020 (300 nm)
'70/30 IPA/heptane'-saturated 0.01 SHE buffer	8770 (317 nm)	8780 (300 nm)
	38000 (241 nm)	49800 (240 nm)

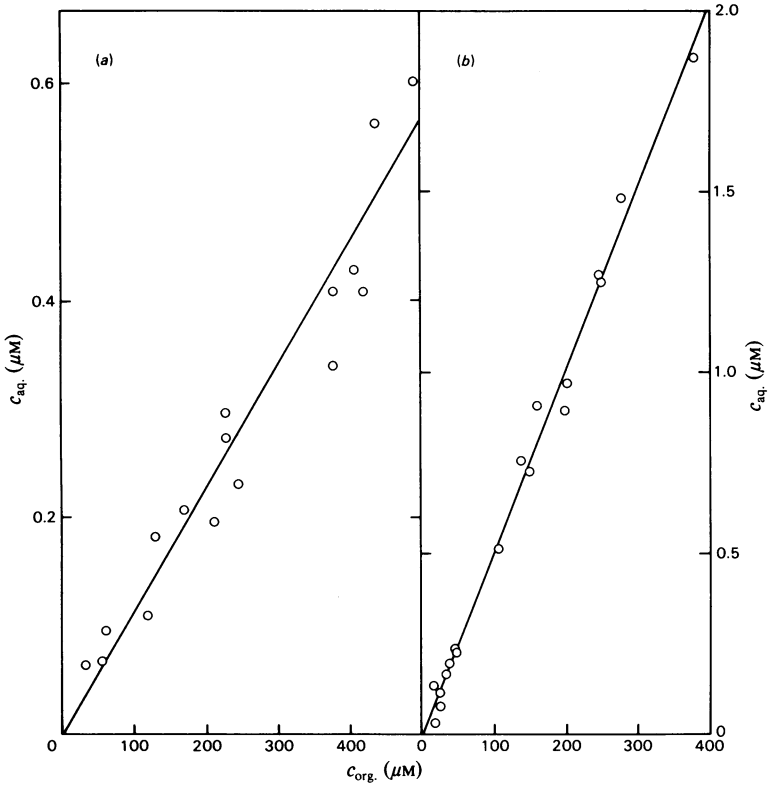

Fig. 2. *Partition of compound 1QN (a) and compound 2QN (b) between '70/30 IPA/heptane' solvent and 0.01 SHE buffer*

The lines fitted to the points are least-squares lines constrained to pass through the origin, of slope 885 ± 60 for compound 2QN and 198 ± 5 for compound 1QN [concentration in organic phase ($c_{org.}$)/concentration in aqueous phase ($c_{aq.}$)].

were used to construct Scatchard (1949) plots and analysed as described below.

Fluorescence quenching

An important bonus arising from the substitution of quinoline for the quinoxaline rings in echinomycin is the appearance of fluorescence; this is too weak to be of value in compound 1QN but is sufficiently strong in compound 2QN to permit the estimation of DNA-binding by using this property.

Fluorescence measurements were made with a Perkin–Elmer MPF-4 spectrofluorimeter operated in the ratio mode. In this apparatus excitation light is provided by a xenon lamp and monochromator. Emitted light is analysed by a second monochromator and photomultiplier tube. An emission filter is available to exclude emitted radiation below a specified frequency. The wavelength of excitation was 310 nm and the wavelength of the emission 410 nm. The emission filter was set to exclude radia-

tion of frequency less than 390nm. Binding to DNA caused an approximately 10-fold quenching of the fluorescence of compound 2QN with no change in the wavelength of peak emission. The excitation slit was routinely set at a spectral width of 4nm and the emission slit at 18nm. The 0.01 SHE buffer was found to produce a larger background emission than a K^+-based buffer, so all fluorescence experiments were performed in 0.01 PHE buffer.

To perform binding experiments a solution of the antibiotic was prepared by shaking some solid in 0.01 PHE buffer overnight to obtain a final drug concentration between 2 and 3μM. This solution was filtered through two Whatman GF/C glass-fibre filters, and its concentration was checked by measuring the absorbance at 240nm. The solution was then placed in the spectrofluorimeter and the fluorescence intensity measured. Complexes of different input ratios were generated by the addition of 50μl portions of nucleic acid solution of appropriate concentration. After each addition the solution was stirred with a fine glass rod, left to stand for 5min, and its fluorescent emission measured again. This procedure was continued until there was no further change in the intrinsic fluorescence of the antibiotic. To calculate the fluorescent emission of compound 2QN a correction was applied to account for the background from the buffer and the DNA solution, a sample of each being placed in the spectrofluorimeter during the experiment. This final value of the fluorescence intensity, when corrected, was divided by the final compound 2QN concentration to give the intrinsic fluorescence of the bound antibiotic.

The concentration of bound ligand at intermediate input ratios was calculated from the equations:

$$I_0 = I_B \cdot C_B + I_F \cdot C_F$$

and

$$C_T = C_B + C_F$$

where I_0 is the measured fluorescence intensity, I_F and I_B represent the intrinsic fluorescence of the free and bound antibiotic respectively (in μM^{-1}), and C_F and C_B represent the concentrations (μM) of free and bound antibiotic respectively. These can be combined to give:

$$C_B = \frac{I_0 - I_F \cdot C_T}{I_B - I_F}$$

whence r and c as defined above, the total concentrations of antiobiotic and DNA being known.

Analysis of binding data

The values of r and c obtained from the binding experiments were routinely analysed in terms of eqn. (10) of McGhee & Von Hippel (1974), which describes a model whereby individual ligand molecules bind to a homogeneous one-dimensional lattice of non-interacting binding sites:

$$\frac{r}{c} = K(0) \cdot (1 - nr) \left[\frac{1 - nr}{1 - (n-1)r} \right]^{n-1}$$

Theoretical curves were computed by using a program written by Dr. J. D. McGhee and installed in the University of Cambridge IBM 370 computer by Dr. G. Ughetto. The program used an iterative procedure to estimate $K(0)$, the intrinsic binding constant, and n, the number of nucleotides occluded by the binding of one molecule, which was recycled until these parameters changed by less than 1%, whereupon it printed out the final values together with a calculated isotherm at 5% saturation increments.

Analytical ultracentrifugation

Sedimentation coefficients were determined by boundary sedimentation with mixtures of bacteriophage-PM2 DNA with the compounds of interest prepared by direct weighing of solutions as described by Lee & Waring (1978*a*).

Viscometry

Experiments were performed by using apparatus and procedures as previously described (Lee & Waring, 1978*a*).

Results

Evidence for intercalation of compounds 1QN and 2QN into DNA

The effects of the two novel antibiotics on the sedimentation coefficient of closed circular duplex DNA are illustrated in Fig. 3. Binding of both ligands elicits the characteristic fall and rise in s_{20} of the closed circles attributable to removal and reversal of the supercoiling and typical of intercalative binding (Waring, 1970). The gentler, steady, decrease in s_{20} of the nicked circular molecules is also readily apparent. For compound 1QN the equivalence point (corresponding to exact relaxation of the supercoiling) occurs at $D/P = 0.030 \pm 0.002$, whereas that for compound 2QN appears at 0.051 ± 0.007. Ethidium under identical conditions yields a value of 0.051 ± 0.007 (Waring & Wakelin, 1974). The quoted estimates of error relate to the span of the range over which closed and nicked circular DNA molecules co-sediment in the analytical ultracentrifuge; they should be regarded as limits rather than statistical standard errors. By simple proportionality we calculate that the un-

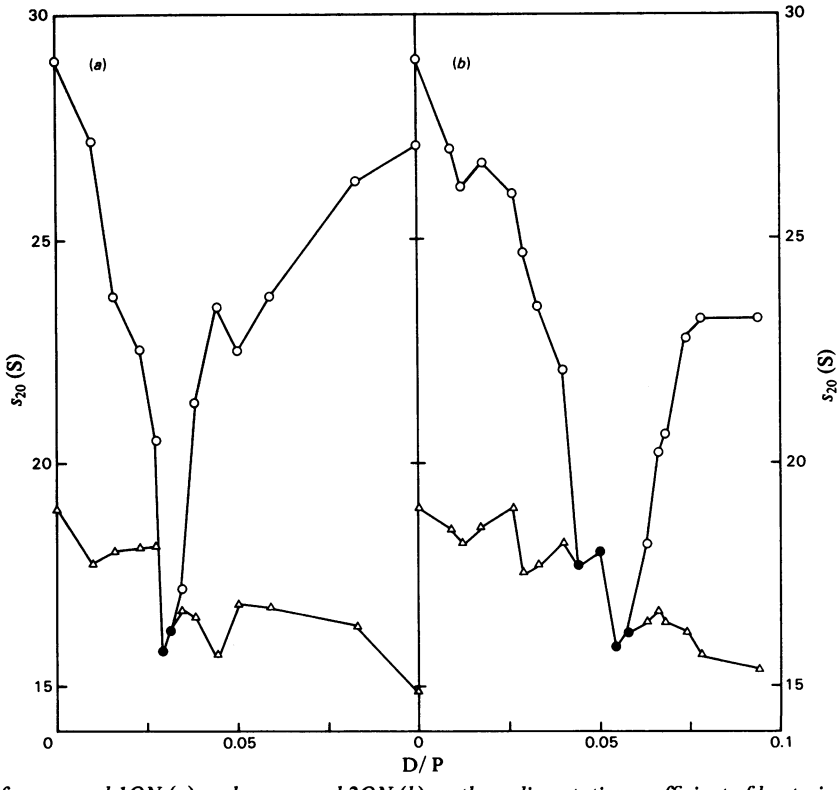

Fig. 3. *Effects of compound 1QN (a) and compound 2QN (b) on the sedimentation coefficient of bacteriophage-PM2 DNA*
The s_{20} of closed DNA circles is shown by O, that of nicked DNA circles by △, and that of unresolved boundaries by ●. The abscissa shows the molar ratio of added antibiotic to total DNA nucleotides (D/P).

winding of the helix associated with the binding of compound 1QN is $\geqslant$1.7 times that seen with ethidium, whereas the unwinding produced by compound 2QN is effectively indistinguishable from that of ethidium. These must be considered minimal estimates, since they refer to the ratio of added ligand to DNA nucleotides (D/P) at equivalence. The proportion of free antibiotic could not be determined directly because of shortage of materials; however, data for other DNA species reported below reveal that even with the weakest-binding combination of drug and polymer the fraction of free drug present at D/P 0.03–0.05 would not exceed a few per cent. Thus, unless the interaction with bacteriophage-PM2 DNA is grossly anomalous (and all available evidence suggests that it is not), the unwinding angles quoted must approximate to those that characterize the bound antibiotic molecules. The unwinding of the helix induced by compound 1QN is of the same order as that reported for the naturally occurring quinoxaline antibiotics (Waring & Wakelin, 1974; Lee & Waring, 1978*a*,*b*; Waring, 1979). However, the unwinding produced by binding of compound 2QN appears to be substantially lower than has previously been observed for the intercalation of any quinoxaline antibiotic into DNA at low ionic strength.

Further insight into the mechanism of the binding reaction can be gained from viscometric studies of complexes with sonicated linear DNA. Plots of the relative increase in contour length of calf thymus DNA as a function of D/P are shown in Fig. 4. The broken lines labelled 1 + 2 (D/P) and 1 + 4 (D/P) indicate theoretical plots that would be expected to result from ideal mono- and bi-functional intercalation respectively. The line drawn through the experimental points is a least-squares best fit to the data with the line constrained to pass through the origin (0.0, 1.0). The slopes of the plots for compounds 1QN and 2QN are 3.33 ± 0.08 and 3.34 ± 0.09 respectively, corresponding to 1.66 ± 0.04 and 1.67 ± 0.05 'idealized' intercalation events per drug molecule.

As with the estimates of helix unwinding, these values should be treated as lower limits, since they are derived in terms of D/P rather than r, though the conditions of the experiment are such that very little drug is expected to be free. Comparable values

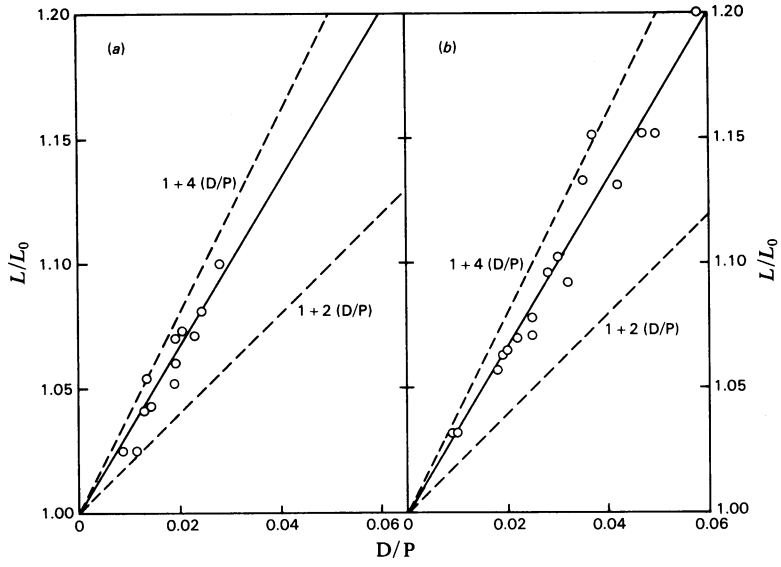

Fig. 4. *Effects of compound 1QN (a) and compound 2QN (b) on the relative contour length of sonicated calf thymus DNA fragments*

The ordinate represents the calculated contour length in the presence of the antibiotic (L) as a ratio of the length in the absence of the antibiotic (L_0) (Cohen & Eisenberg, 1966, 1969). The abscissae show the drug/nucleotide ratio (D/P). Also shown are theoretical lines corresponding to the lengthening expected for ideal monofunctional [1 + 2 (D/P)] and ideal bifunctional [1 + 4 (D/P)] intercalation. The lines passing through the experimental points were fitted by the method of least squares and constrained to pass through the origin (0,1). Their slopes are 3.33 ± 0.09 for compound 1QN and 3.34 ± 0.08 for compound 2QN.

determined for echinomycin and triostin A under identical conditions are 1.87 ± 0.05 and 1.79 ± 0.09 intercalation events per bound drug molecule respectively (Wakelin & Waring, 1976; Lee & Waring, 1978*a*; Waring, 1979). Thus, although the helix extension produced by binding of compounds 1QN and 2QN is probably slightly lower than seen with the natural quinoxaline antibiotics, it is much nearer to that characteristic of the bifunctional quinoxalines (and bis-intercalating diacridines; Wakelin *et al.*, 1978) than to that reported for simple monofunctional intercalators (Hogan *et al.*, 1979; Waring *et al.*, 1979, and references cited therein). Data reported for ethidium, for example, correspond to about 0.8 of the extension predicted by an idealized (monofunctional) intercalation model (Reinert, 1973; Hogan *et al.*, 1979).

Specificity of the interaction between DNA and compounds 1QN and 2QN

Scatchard plots for the binding of compound 1QN to various naturally occurring DNA species determined by the technique of solvent partition are presented in Fig. 5. Similar data for compound 2QN, together with results from fluorescence-quenching experiments, are shown in Figs. 6 and 7. In all instances the lines drawn through the experimental points are computer fits to the data in terms of eqn. (10) of McGhee & Von Hippel (1974). It can be seen that, in general, the quality of the curve-fitting is acceptable. As is frequently the case in quantitative studies of ligand–DNA interaction, the deviations from the idealized model are most apparent at low and high values of r, the former because of inaccuracies inherent in the experimental techniques (and possibly also because of the existence of strong preferences for a small number of highly favourable binding sites, as suggested in the Discussion section), the latter most likely because of the onset of weaker, non-specific, secondary binding. The 'goodness of fit' of the theoretical curves is better if the solvent-partition and fluorescence data for compound 2QN are treated independently, as was done to determine the binding parameters listed in Table 2, but in the Figures the data obtained by the two techniques have been treated together for simplicity.

It is pleasing to note the satisfactory agreement between equivalent sets of data for compound 2QN determined by solvent partition and fluorescence quenching (Figs. 6 and 7). With one exception the estimated binding constants differ by no more than

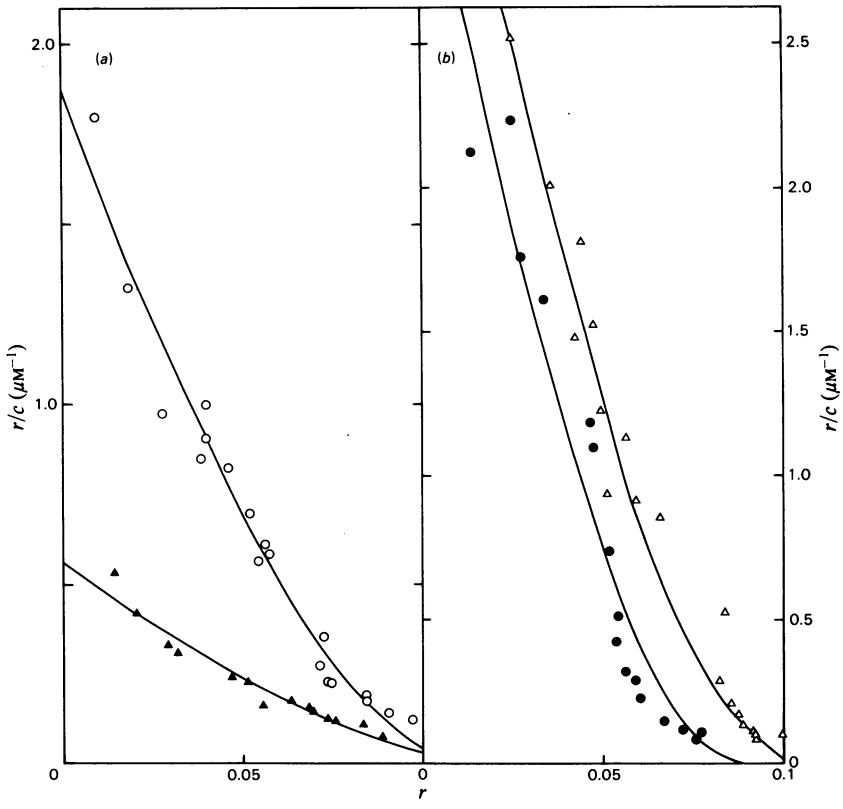

Fig. 5. *Effects of base-composition on the binding of compound 1QN to naturally occurring DNA species*
The data are presented in the form of Scatchard plots where r is the binding ratio (antibiotic molecules bound per nucleotide) and c is the free ligand concentration. △, *Micrococcus lysodeikticus* DNA (72% G + C); ●, *Escherichia coli* DNA (50% G + C); ○, calf thymus DNA (42% G + C); ▲, *Clostridium perfringens* DNA (30% G + C). The curves are theoretical, computed to fit eqn. (10) of McGhee & Von Hippel (1974), the values of $K(0)$ and n listed in Table 2 being used.

±13% from the mean, and there is no consistent tendency for either method to underestimate or overestimate in comparison with the other. The exception occurs in the data for binding to *E. coli* DNA (Fig. 7); here the estimated values of $K(0)$ differ by a factor of nearly 1.6 (Table 2). In view of this discrepancy, several repeat determinations were performed by each method in turn, but, despite the accumulation of a large number of data points, the difference persisted and must be presumed to be real. It can be explained on the basis that the true curve for binding to *E. coli* DNA is significantly more concave upwards than the McGhee & Von Hippel (1974) treatment predicts, i.e. that additional anti-co-operativity and/or heterogeneity of binding sites exists with this DNA. Such a situation has been encountered previously in Scatchard plots for quinoxaline antibiotic–DNA interaction (Wakelin & Waring, 1976; Lee & Waring, 1978*b*). The reason why the two methods yield different values for $K(0)$ is then accounted for by the fact that they furnish points covering different ranges of r; the solvent-partition results span the range from low to moderately high extents of binding, whereas fluorescence quenching provides accurate data at high binding ratios but becomes unreliable at $r < 0.025$. For practical purposes the 'average' association constant of $1.66\,\mu M^{-1}$ obtained by considering only data points in the region of overlap ($0.02 < r < 0.08$) is probably a fair representation of the situation, but it should be remembered that this is something of an approximation and that in this instance there is definite evidence that *E. coli* DNA may contain binding sites with a significantly higher binding constant than the average.

It can be seen from Figs. 5–7 and Table 2 that compound 1QN binds more tightly to all the natural DNA species studied than does compound 2QN or echinomycin. This trend is not, however, followed in the interaction with synthetic polymers (Figs.

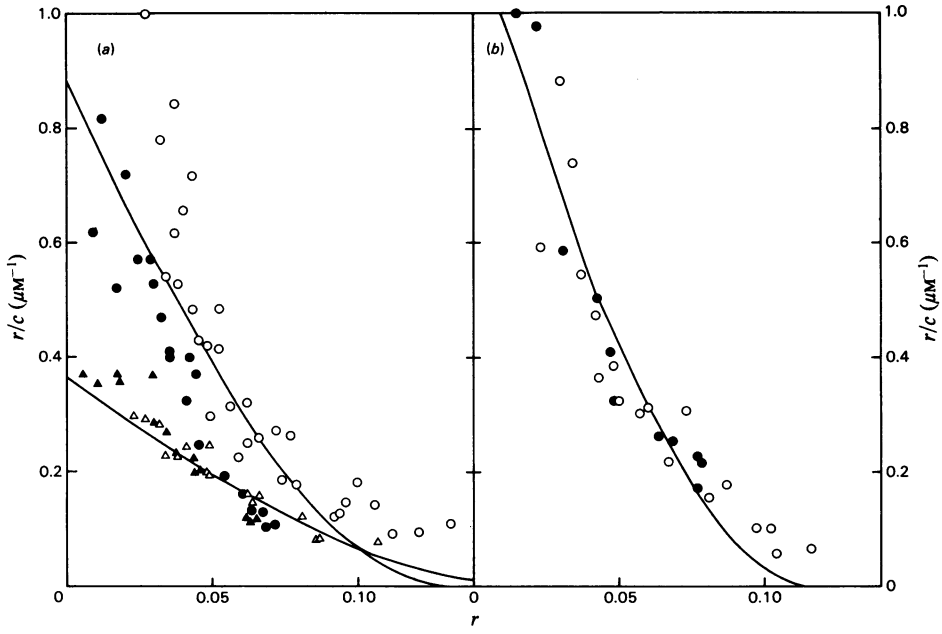

Fig. 6. *Scatchard plots for the interaction of compound 2QN with naturally occurring DNA species*
The data were determined by both solvent-partition analysis and fluorescence quenching. (*a*) Calf thymus DNA: ●, by solvent partition; ○, by fluorescence quenching. *Cl. perfringens* DNA: ▲, by solvent partition; △, by fluorescence quenching. (*b*) *M. lysodeikticus* DNA: ●, by solvent partition; ○, by fluorescence quenching. The curves shown are fitted to the combined data points from solvent partition and fluorescence quenching by using the treatment of McGhee & Von Hippel (1974), and are characterized by the parameters: calf thymus DNA, $K(0) = 0.88\,\mu M^{-1}$, $n = 6.70$; *Cl. perfringens* DNA, $K(0) = 0.36\,\mu M^{-1}$; $n = 5.48$; *M. lysodeikticus* DNA, $K(0) = 1.15\,\mu M^{-1}$, $n = 7.5$.

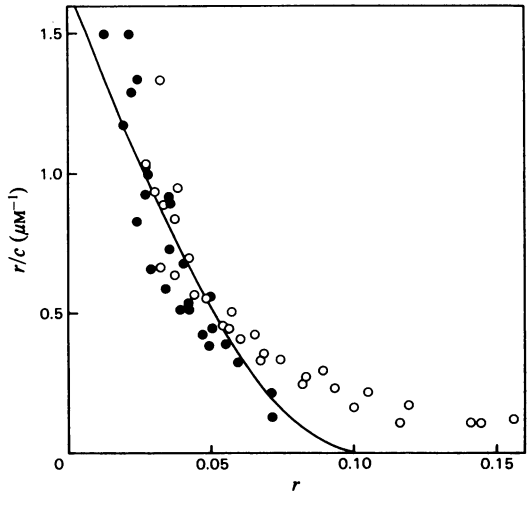

Fig. 7. *Scatchard plot for the interaction of compound 2QN with E. coli DNA*
Binding was determined by solvent-partition analysis (●) and fluorescence quenching (○). The curve shown is fitted to the combined data points in the region where they overlap, by using the treatment of eqn. (10) of McGhee & Von Hippel (1974). It has the parameters $K(0) = 1.66\,\mu M^{-1}$ and $n = 8.40$.

8 and 9). For compound 1QN binding to poly(dA-dT), $K(0)$ is almost identical with that of echinomycin (Wakelin & Waring, 1976), whereas the corresponding value for compound 2QN is the highest observed for the interaction of this antibiotic with any of the nucleic acids studied, binding being approximately 18 times tighter than that of echinomycin to this same polymer. Scatchard plots of data for binding of quinoxaline antibiotics to poly(dA-dT) sometimes show a 'humped' shape (Lee & Waring, 1978*a*,*b*), which is consistent with co-operative binding (McGhee & Von Hippel, 1974). Among the natural antibiotics this phenomenon is most pronounced with quinomycin C (Lee & Waring, 1978*a*). The data points for binding of compounds 1QN and 2QN to poly(dA-dT) obtained from solvent-partition analysis also display a slight negative (concave downwards) curvature. Accordingly, these data were subjected to analysis both in terms of eqn. (10) of McGhee & Von Hippel (1974) and in terms of their eqn. (15). In this latter treatment a co-operativity parameter, ω, is introduced such that the binding constant to singly and doubly contiguous sites is given by $\omega K(0)$ and $\omega^2 K(0)$ respectively. A set of trial curves was plotted out by a PDP8/E computer, the values of the parameters

Table 2. *DNA-binding parameters for echinomycin, compound 1QN and compound 2QN*

$K(0)$, the intrinsic association constant, and n, the number of nucleotides occluded by the binding of one molecule were calculated from an analysis of the binding data in terms of eqn. (10) of McGhee & Von Hippel (1974). Data for poly(dA-dT) were also analysed in terms of their eqn. (15), as described in the text. The results shown for echinomycin are taken from Wakelin & Waring (1976).

	Echinomycin		Compound 1QN		Compound 2QN (by solvent partition)		Compound 2QN (by fluorescence quenching)	
DNA	$K(0)$ (μM^{-1})	n	$K(0)$ (μM^{-1})	n	$K(0)$ (μM^{-1})	n	$K(0)$ (μM^{-1})	n
M. lysodeikticus DNA	3.10	9.85	3.93	8.25	1.24	7.85	0.99	6.83
E. coli DNA	0.98	11.88	3.36	9.76	2.06	10.40	1.36	6.41
Calf thymus DNA	0.55	7.17	1.87	7.59	0.88	8.51	1.11	6.98
Cl. perfringens DNA	0.34	9.46	0.56	6.82	0.44	7.02	0.39	5.63
Poly(dG-dC)	0.55	5.48	3.87	7.42	[3.83]	[3.44]	3.27	5.93
Poly(dA-dT)	0.31	5.65	0.34	4.32	5.29	4.76	5.83	4.69

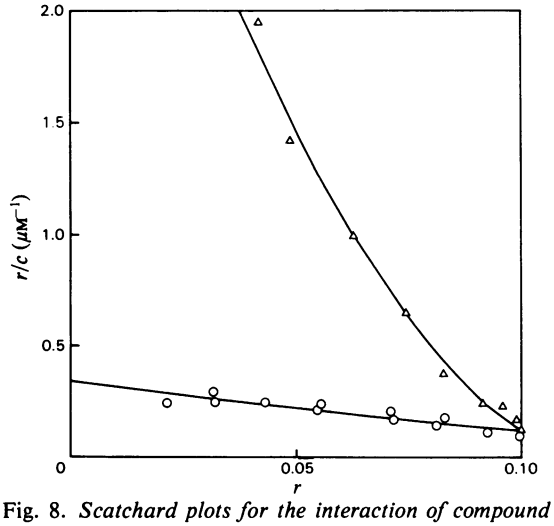

Fig. 8. *Scatchard plots for the interaction of compound 1QN with synthetic DNA species*

O, Poly(dA-dT). △, Poly(dG-dC). The curves shown were fitted to the data by using the treatment of eqn. (10) of McGhee & Von Hippel (1974), with the parameters listed in Table 2.

$K(0)$, n, and ω being varied by trial and error until no further improvement in the fit to the experimental points (judged by eye) could be achieved. The best fits to the solvent-partition data on binding of both compounds 1QN and 2QN to poly(dA-dT) were found with $\omega = 6$, suggesting a lower degree of co-operativity than that observed in the data for interaction between poly(dA-dT) and quinomycin C ($\omega = 31$; Lee & Waring, 1978*a*) or the synthetic quinoxaline analogue des-*N*-tetramethyltriostin A ('TANDEM', for which $\omega = 15$; Lee & Waring, 1978*b*). As noted in the previous analyses, the chief effect of applying the equation for a co-operative-binding model is to decrease the apparent site size (increase the value of n) without much change in the intrinsic association constant. For compound 1QN the optimized parameters were $K(0) = 0.32\,\mu M^{-1}$, $\omega = 6$ and $n = 7.5$. For compound 2QN they were $K(0) = 4.6\,\mu M^{-1}$, $\omega = 6$ and $n = 7.1$ (cf. the parameters for non-co-operative treatment recorded in Table 2). Negative (concave downwards) curvature is not evident in the points on the Scatchard plot for compound 2QN derived from measurements of fluorescence quenching, no doubt owing in part to the different (higher) range of r that this technique covers (Fig. 9).

Binding curves for the interaction between the quinoline analogues and poly(dG-dC) are included in Figs. 8 and 9. They reveal that this polymer binds both antibiotics quite tightly, some 6–7-fold more tightly than echinomycin, and that, in contrast with what was found with poly(dA-dT), the binding constants for compounds 1QN and 2QN are much the same (Table 2). Only data from fluorescence-quenching experiments are shown for compound 2QN. It proved impossible to measure the interaction of compound 2QN with this polymer by the standard solvent-partition procedure because poly(dG-dC) absorbs significantly in dimethyl sulphoxide at 300 nm, the maximum in the absorption spectrum of compound 2QN at which spectrophotometric measurements must be made. A rough estimate of the strength of binding could be gained by monitoring the disappearance of antibiotic from the organic phase in the solvent-partition system, leading to the bracketed values included in Table 2, but, because the actual total concentration of

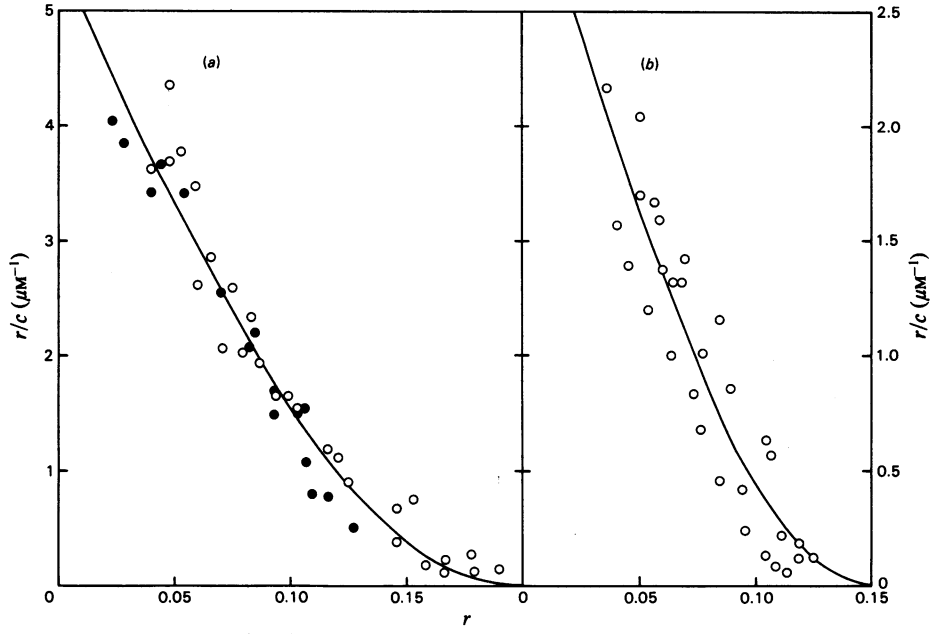

Fig. 9. *Scatchard plots for the interaction of compound 2QN with synthetic DNA species*
(*a*) Poly(dA-dT): ●, by solvent partition; ○, by fluorescence quenching. (*b*) Poly(dG-dC): from fluorescence quenching only. The curves were fitted to the combined data points by using the treatment of eqn. (10) of McGhee & Von Hippel (1974) with the parameters: poly(dA-dT), $K(0) = 5.46\mu M^{-1}$, $n = 4.65$; poly(dG-dC), $K(0) = 3.27\mu M^{-1}$, $n = 5.93$.

compound 2QN in the aqueous phase was not determined directly, these values cannot be regarded with the same confidence as the others in the Table. Since there is no evidence of negative curvature, suggestive of co-operativity in the curves for binding to poly(dG-dC) (Figs. 8 and 9), no attempt was made to analyse them in terms of the co-operative-binding model.

Discussion

The principal aim of the present experiments was to investigate the role, if any, played by the chromophores of quinoxaline antibiotics in determining the mechanism and specificity of their interaction with DNA. The results leave no room for doubt that replacement of one or both chromophores by quinoline moieties can be tolerated without loss of the capacity to intercalate into DNA, and indeed the substitution can lead to greatly enhanced affinity as well as altered properties of recognition of DNA, which must surely presage changes in biological effect. Such changes are not examined in detail here.

The evidence for intercalation rests on the finding that both compound 1QN and compound 2QN produce the characteristic increase in contour length of sonicated rod-like DNA fragments and share the ability to remove and reverse the supercoiling of closed circular duplex DNA. Moreover, the results for compound 1QN are in accord with those established in previous work as being characteristic of bifunctional intercalation by quinoxaline antibiotics (Waring & Wakelin, 1974; Wakelin & Waring, 1976; Lee & Waring, 1978*a*,*b*). Compound 1QN, in fact, provides the first fully characterized example of a bifunctional intercalator bearing two different chromophores. The results for compound 2QN are not, however, of the classical type. Strong evidence for the bifunctional character of its binding to DNA is provided by the results of the viscometric experiments, which indicate an increase in contour length well up to the values seen with acknowledged bis-intercalating agents. On the other hand, the sedimentation studies indicate an unwinding of the helix per bound drug molecule approximately equal to that produced by monofunctional intercalation of ethidium. The apparent discrepancy could be explained by postulating that intercalation of a quinoline chromophore in compound 2QN induces only half the unwinding seen with the quinoxaline chromophore, or with ethidium, under the same conditions. Intercalating agents that produce only half the unwinding of ethidium have been characterized (Waring, 1970), including closely related phenanthridinium compounds (Wakelin & Waring, 1974).

This suggestion must, at first sight, appear surprising, in view of the slight structural difference

between quinoxaline and quinoline. However, there are other observations that reveal that an altered unwinding angle is not the only difference between compound 2QN and echinomycin, prompting the conclusion that many properties of the two antibiotics, and of their binding to DNA, are distinctly different. We may point to the fact that compound 2QN is fluorescent whereas echinomycin is not. Perhaps more significantly, there is no detectable fluorescence from compound 1QN even at saturation in aqueous solution, and compound 1QN is approximately twice as soluble as compound 2QN. The quantum yield per chromophore in compound 2QN is about 25 times that of quinoline-2-carboxylic acid. Hence it appears that the electronic state of the chromophores in compound 2QN is different from that in the free acid and, significantly, from that in compound 1QN. Of course, the fluorescent properties of these compounds depend largely on π–π^* character in the lowest excited state, irrespective of the electronic structure of the ground state, whereas their potential for intercalation is surely a phenomenon related to the properties of the ground state. Although the two facets of behaviour need not be causally connected, we cannot escape the conclusion that important differences do exist between the behaviour of a quinoline ring system in compounds 2QN and 1QN. These considerations may help to counter the possible objection that the apparent helix-unwinding angle of compound 1QN does not lie mid-way between those of echinomycin and compound 2QN. The objection might also be countered by suggesting that there may exist steric constraints that prevent the DNA double helix accommodating two different unwinding angles at closely adjacent intercalation sites: in this case the resultant unwinding produced by binding of a heterodimeric ligand might well be dominated by the properties of that chromophore which produces the largest perturbation. Lastly, it is worth noting that, although the naturally occurring quinoxaline antibiotics have been shown to cause twice the helix unwinding seen with ethidium, there is no reason *a priori* why any bifunctional intercalating agent not possessing phenanthridinium chromophores should produce an unwinding effect of this magnitude. Indeed, the unwinding angles reported for other bifunctional intercalators such as bis-acridines (Canellakis *et al.*, 1976) are considerably smaller than those found with quinoxalines (Le Pecq *et al.*, 1975; Wakelin *et al.*, 1978; R. G. McR. Wright, L. P. G. Wakelin, A. Fieldes, R. M. Acheson & M. J. Waring, unpublished work).

The second objective of the present work was to investigate patterns of specificity shown by compounds 1QN and 2QN in their binding to DNA. Before discussion of the significance of differences in binding constants, it is worth emphasizing the excellent general agreement between the binding parameters for compound 2QN calculated from fluorescence quenching and from solvent-partition analysis. The low solubility of all quinoxaline antibiotics in aqueous solution presents formidable problems for determining values for binding, and this is the first instance where it has proved possible to compare measurements made by using two fundamentally different techniques, albeit on a bisquinoline analogue of a natural quinoxaline antibiotic.

It can be seen from Table 2 that the patterns of specificity displayed by compounds 1QN and 2QN are distinctly different from each other and from that of echinomycin. In no case are the binding constants related in simple monotonic fashion to the gross base-composition of the DNA species employed. This observation is in accord with previous work on quinoxaline antibiotics, and must be interpreted to mean that binding of these antibiotics is sequence-selective, i.e. dependent on the arrangement of nucleotides rather than the gross content, as discussed previously (Wakelin & Waring, 1976; Lee & Waring, 1978*a*,*b*). Moreover, the details of the sequence preferences must differ significantly among the different antibiotics. With echinomycin a broad preference for (G + C)-rich natural DNA species has been observed, though some DNA species yielded binding constants higher or lower than would be expected on that basis (Wakelin & Waring, 1976). Such preference appears rather less evident for compound 1QN and less still for compound 2QN. Although admittedly an approximation, it is possible to purse the point more quantitatively by calculating values for the parameter α introduced by Müller & Crothers (1975), Müller *et al.* (1975) and Müller & Gautier (1975). It can be defined as the ratio of the binding constants for two selected DNA species of widely different base-compositions. Experimentally determined values can be compared with theoretical values calculated by considering DNA of random base-sequence and assuming a given degree of base-pair specificity in the binding reaction. It is convenient to choose binding constants for *M. lysodeikticus* DNA and *Cl. perfringens* DNA, which have (G + C) contents of 72 and 30% respectively. A ligand having an absolute requirement for one (G + C) base-pair in the binding site would yield a theoretical value for α of 2.4, whereas a preference for two (G + C) base-pairs would be indicated by an α value of 5.76 (i.e. 2.4^2). Calculated values of α for compounds 1QN and 2QN derived from the data in Table 2 are 6.98 and 2.82 respectively. On this basis compound 1QN appears selective for a sequence of two or more (G + C) base-pairs in the binding site, whereas compound 2QN has a preference for only one. How-

ever, this analysis may represent an over-simplification, since it can be seen from Table 2 that compound 2QN binds more tightly to *E. coli* DNA, containing 50% (G + C), than to *M. lysodeikticus* DNA. Values of α calculated for echinomycin and quinomycin C were 9.01 and 9.12 respectively (Lee & Waring, 1978*a*).

The binding constants for compounds 1QN and 2QN measured with the synthetic polymers (Table 2) also reveal patterns of specificity differing from each other as well as from that of echinomycin. Compound 1QN exhibits a marked preference for poly(dG-dC), binding over 11-fold more tightly to it than to poly(dA-dT). The difference from echinomycin is almost entirely attributable to a large increase in the binding constant for poly(dG-dC). By contrast, when the second chromophore is also replaced by quinoline, the trend is reversed: the poly(dG-dC) binding constant for compound 2QN remains much the same as that for compound 1QN but it is overtaken by a massive increase in the affinity for poly(dA-dT).

The significance of these changes may be easier to grasp by comparison with the results for naturally occurring DNA species. Although echinomycin appears to display a broad preference for (G + C)-rich natural DNA species, it binds less tightly to poly(dG-dC) than to *M. lysodeikticus* DNA, which was interpreted as indicating a preference for some combination of all four bases in the optimum intercalation site (Wakelin & Waring, 1976). On the face of it, the results for the quinoline analogues point to the acquisition of additional preference(s) for alternating purine–pyrimidine nucleotide sequences. If the effect of substituting one quinoxaline chromophore by quinoline were to enhance the affinity for such sequences while retaining a distinct preference for (G + C)-rich nucleic acids, the binding properties of compound 1QN might be explained. With the substitution of both chromophores, as in compound 2QN, it would be necessary to postulate that whatever element of the original specificity were retained it becomes swamped by an over-riding preference for alternating sequences, irrespective of their base-composition. This could explain the observation that the binding constants for the alternating deoxyribonucleotide polymers exceed those for any of the natural DNA species, and perhaps also the finding that the binding constant for *E. coli* DNA appears higher than that for *M. lysodeikticus* DNA notwithstanding its lower (G + C) content. Because of the lack of detailed sequence information on the DNA species employed to determine the parameters recorded in Table 2 it is not possible to test this hypothesis with the data available at present, but experiments to investigate the strength of binding to natural DNA species at low values of D/P could be expected to shed light on the nature of the most tightly binding sites. However, it is already clear that the precise nature of the chromophore(s) has a profound influence on the strength and selectivity of DNA binding by these intercalating antibiotics: the chromophores are not merely inert plugs that serve only to help locate the peptide portion of the molecule within one of the grooves of the DNA helix after the fashion of a staple, any more than the octapeptide ring serves merely as a 'convenient handle' (Waring & Wakelin, 1974).

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* As originally published in *J. Mol. Biol.* (1974) **86**, 469–489, eqn. (15) of McGhee & Von Hippel contains a misprinted sign. The correct form of the equation is given in *J. Mol. Biol.* (1976) **103**, 679, and is the equation referred to in the text.

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