Bifunctional Intercalation and Sequence Specificity in the Binding of Quinomycin and Triostin Antibiotics to Deoxyribonucleic Acid

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Quinomycin C, triostin A and triostin C are peptide antibiotics of the quinoxaline family, of which echinomycin (quinomycin A) is also a member. They all remove and reverse the supercoiling of closed circular duplex DNA from bacteriophage PM2 in the fashion characteristic of intercalating drugs, and the unwinding angle at I 0.01 is, in all cases, almost twice that of ethidium. Thus, as with echinomycin, they can be characterized as bifunctional intercalating agents. For the triostins this conclusion has been confirmed by measurements of changes in the viscosity of sonicated rod-like DNA fragments; the helix extension was found to be almost double that expected for a simple monofunctional intercalation process. For triostin A, further evidence for bifunctionality was derived from the cross-over point of binding isotherms to nicked circular and closed circular bacteriophage-PM2 DNA. Binding curves for the interaction of quinomycin C and triostin A with a variety of synthetic and naturally occurring nucleic acids were determined by solvent-partition analysis, but triostin C was too insoluble in aqueous solution to make this method applicable. For quinomycin C the highest binding constant was found with *Micrococcus lysodeikticus* DNA, and its pattern of specificity among natural DNA species was broadly similar to that of echinomycin, although the binding constants were 2–6 times as large. For triostin A the highest binding constant was again found for *M. lysodeikticus* DNA, but the specificity pattern was quite different from that of the quinomycins. In particular, triostin A bound better to poly(dA-dT) than to poly(dG-dC), whereas this order was reversed for quinomycin C. There was also evidence that the binding to poly(dA-dT) might be co-operative in nature. No significant interaction could be detected with poly(dA)·poly(dT) or with RNA from *Escherichia coli*. Poly(dG)·poly(dC) gave variable results, depending on the source of the polymer. The different patterns of specificity displayed by the quinomycins and triostins are tentatively ascribed to differences in their conformations in solution.

The quinoxaline group of antibiotics is characterized by a cross-bridged cyclic octapeptide dilactone containing both D- and L-amino acids to which two quinoxaline-2-carboxylic acid chromophores are attached. Some ten or more members have been identified which form two families, the quinomycins and the triostins (Katagiri *et al.*, 1975). The families differ only in the nature of the cross-bridge formed between two *N*-methylcysteine residues in the peptide portion. The structure of the quinomycins has been revised (Dell *et al.*, 1975; Martin *et al.*, 1975), and the representative member echinomycin (quinomycin A) is shown in Fig. 1. The triostin group has a simple disulphide cross-bridge in place of the thioacetal cross-bridge of the quinomycins. The representative member triostin A is also shown in Fig. 1. Further members of the quinoxaline group contain amino acid replacements in one or both of the *N*-

methylvaline sites of the peptide ring. In particular, quinomycin C and triostin C contain *N*, γ -dimethyl-alloisoleucine instead of both *N*-methylvaline residues (Otsuka & Shoji, 1965; Martin *et al.*, 1975). Thus triostin A and triostin C possess a perfect twofold rotational axis of symmetry, whereas in quinomycin C (and in echinomycin) the exact symmetry is destroyed by the sulphur-containing cross-bridge. There seems little doubt that the anti-tumour and other biological activities of these molecules result directly from their interaction with DNA (Katagiri *et al.*, 1975; Waring & Makoff, 1974).

In general, molecules of this size (mol.wt. 1100 or more) would be expected to be able to adopt many different conformations having similar energies. However, the cross-bridged cyclic structure, together with the twofold (or nearly so) rotational axis of symmetry, imposes severe restrictions on the number of possible conformations. For this reason the quinoxaline antibiotics have recently attracted atten-

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Echinomycin (quinomycin A)

Triostin A

Fig. 1. Structural formulae of echinomycin (Dell et al., 1975; Martin et al., 1975) and triostin A (Otsuka et al., 1976)

tion as model compounds for conformational studies, and indeed relatively precise solution conformations have been proposed on the basis of empirical potential-energy calculations and n.m.r. experiments (Ughetto & Waring, 1977; Cheung *et al.*, 1978; Blake *et al.*, 1977; J. R. Kalman, T. J. Blake, D. H. Williams, J. Feeney & G. C. K. Roberts, unpublished work). Consequently an investigation of the DNA-binding characteristics of quinoxaline compounds presents a uniquely propitious opportunity to study, at the molecular level, the effect of small structural changes on the interaction of a peptide molecule with DNA.

The interaction between echinomycin and DNA has been thoroughly investigated (Ward *et al.*, 1965; Waring & Wakelin, 1974; Waring *et al.*, 1975; Wakelin & Waring, 1976). This work led to two main conclusions: firstly, at low ionic strength (0.01 mol/litre) echinomycin behaved as a bifunctional intercalating agent, the first such substance ever to be characterized. Secondly, not only was the molecule specific for double-helical DNA, but it also exhibited considerable base-sequence specificity. Preliminary studies with triostin A revealed a similar requirement for double-stranded DNA (Waring *et al.*, 1975). In the present paper, detailed results are reported on three naturally occurring quinoxaline antibiotics, quinomycin C, triostin A and triostin C, with particular regard to the mechanism and specificity of their interaction with DNA. In the following paper, the study is extended to chemically synthesized analogues of echinomycin and triostin A, and some attempt is made to correlate the binding characteristics of all the quinoxalines, both naturally occurring and synthetic, with their solution configurations as far as they are understood (Lee & Waring, 1978).

Materials and Methods

Triostin A and triostin C, products of Shionogi and Co., Osaka, Japan, were gifts of Dr. H. Otsuka and Dr. T. Yoshida. Quinomycin C was a gift from Dr. D. G. Martin, The Upjohn Co., Kalamazoo, MI, U.S.A. All the antibiotics were used as supplied without further purification. The purity of triostin A and quinomycin C has been estimated by liquid/liquid chromatography on a coil planet centrifuge to be better than 98% (I. A. Sutherland, J. S. Lee & D. J. Gauvreau, unpublished work). All experiments were conducted at 20°C in a Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.0, I 0.01, designated 0.01 SHE buffer (Waring & Wakelin, 1974). A stock solution containing 0.2M-Hepes, 1mM-EDTA and 0.94M-NaCl was adjusted to pH 7.0 at 20°C with NaOH. This was diluted 100-fold with no significant change in the pH to give $I = 0.01$. For use in solvent-partition analysis isopentyl acetate was purified as previously described (Waring *et al.*, 1975). In addition, the organic solvent

isopentyl acetate/n-heptane (7:3, v/v) was prepared. Mixing n-heptane with isopentyl acetate in the above proportions caused a slight haze because of small amounts of water coming out of solution. This was removed by low-speed centrifugation and the resulting solvent was designated '70/30 IPA/heptane'.

Calf thymus DNA (highly polymerized sodium salt, type 1) was obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Bacterial DNA species were prepared by standard procedures based on the method of Marmur (1961), the principal modifications being an overnight incubation of the crude lysate with 50 µg of Pronase/ml at 37°C and incubation with T1 ribonuclease (5 µg/ml) as well as pancreatic ribonuclease, followed by final purification by two extractions with buffer-saturated redistilled phenol. For measurement of binding curves all DNA samples of high molecular weight were sheared to a standard molecular weight by drawing a solution (2mg/ml in 2.5M-NaCl) 20 times into a 5ml syringe through a no. 28 needle at 0°C. This procedure produces fragments with a sedimentation coefficient of approx. 18S with a minimal content of single-stranded ends (Pyeritz *et al.*, 1972). After 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). A sample of the completely nicked circular species was prepared by maintaining a preparation of closed circular DNA in 0.01 SHE buffer at room temperature (20-25°C) until analytical ultracentrifugation showed that it had been completely converted into the nicked species. This was then repurified to recover the high-molecular-weight DNA by ethanol precipitation. rRNA was a gift from Dr. L. P. G. Wakelin, Department of Pharmacology, University of Cambridge. It had been extracted from ribosomes of *Escherichia coli* B by two successive treatments with phenol followed by ethanol precipitation. Nucleic acid concentrations were based on an assumed value for $\epsilon_{(P)260}$ (molar absorption coefficient with respect to nucleotides) of 6600, except for *Micrococcus lysodeikticus* DNA (6300) (Tubbs *et al.*, 1964) and rRNA from *E. coli* B (8500) (Waring *et al.*, 1975).

Poly(dA-dT), poly(dG-dC) and poly(dA)·poly(dT) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., and poly(dI-dC) was a product of P-L Biochemicals, Milwaukee, WI, U.S.A. Samples of poly(dG)·poly(dC) were purchased from Boehringer and from Miles Chemical Co., Elkhart, IN, U.S.A. All these synthetic polymers were found to be readily soluble in 0.01 SHE buffer and were used as supplied without further purification. Concentrations were based on the values of $\epsilon_{(P)}$ given by Wells & Wartell (1974).

Table 1. *Molar absorption coefficients of quinoxaline antibiotics*

Experimental procedures for measurements were as described by Waring *et al.* (1975). The wavelength of measurement, corresponding to the peak maximum, is shown in parentheses after each value.

Solvent	Triostin A	Triostin C	Quinomycin C
Isopentyl acetate	12100 (317 nm)	—	—
'70/30 IPA/heptane'	—	—	12870 (315 nm)
50% (v/v) Dimethyl sulphoxide/isopentyl acetate-saturated buffer	12000 (325 nm)	—	—
50% (v/v) Dimethyl sulphoxide/'70/30 IPA/heptane'-saturated buffer	—	—	11860 (323 nm)
50% (v/v) Dimethyl sulphoxide/buffer	—	13800 (325 nm)	—
Isopentyl acetate-saturated buffer	10900 (325 nm)	—	—
	53100 (243 nm)	—	—
'70/30 IPA/heptane'-saturated buffer	—	—	11800 (325 nm)
	—	—	50800 (243 nm)

Absorption coefficients of quinoxaline antibiotics

These were measured by the methods of Waring *et al.* (1975) and are listed in Table 1.

Partition coefficients

For triostin A the partition coefficient between isopentyl acetate and 0.01 SHE buffer was measured to be 943 ± 23 , as described by Waring *et al.* (1975). For quinomycin C preliminary experiments with isopentyl acetate as the organic phase showed that the partition coefficient was probably higher than 1500. This was considered to be too high for practical purposes, since it would have necessitated the use of very high concentrations of quinomycin C in the organic phase to obtain a reasonable concentration of free antibiotic in the aqueous phase, consequently demanding large quantities of quinomycin C, whereas the total available amounted to only 40 mg. However, addition of n-heptane to isopentyl acetate steadily decreased the partition coefficient, since quinomycin C is completely insoluble in n-heptane. Isopentyl acetate/n-heptane (7:3, v/v) ('70/30 IPA/heptane') was eventually chosen, since this decreased the partition coefficient to below 1000. The addition of n-heptane also has the effect of lowering the saturation concentration of isopentyl acetate in the aqueous phase, since in effect the isopentyl acetate is now partitioning between n-heptane (which has a negligible water solubility) and the aqueous phase. Without n-heptane, isopentyl acetate is soluble to approx. 0.17% in water (Doolittle, 1935), whereas the saturation concentration, assessed on the basis of the A_{270} (see below) after partitioning from '70/30 IPA/heptane' solvent, is approximately half this value. Consequently small changes in ambient temperature do not cause isopentyl acetate to come out of solution from the aqueous phase, which tends to be a problem when 100% isopentyl acetate is used as

the organic phase. Thus the addition of n-heptane presents three advantages: firstly, it promotes greater flexibility in solvent-partition analysis by allowing the experimenter to choose the partition coefficient; secondly, it decreases the total amount of antibiotic required; and thirdly, it lowers the concentration of organic solvents in the aqueous phase.

As mentioned previously (Waring *et al.*, 1975) isopentyl acetate develops u.v. absorption with a peak at approx. 270 nm when shaken with aqueous solutions; this interferes with the measurement of the antibiotic concentration at 243 nm unless stringent blanking precautions are taken. Although this problem was alleviated by the use of '70/30 IPA/heptane' solvent as the organic phase, a general method was developed for measuring aqueous antibiotic concentrations in the presence of variable absorption from isopentyl acetate or its degradation products. The ratio of A_{243} to the A_{270} due to dissolved isopentyl acetate remained constant even though the absolute concentration might vary. Thus $A(\text{IPA})_{243} = pA(\text{IPA})_{270}$ where $A(\text{IPA})_{\lambda}$ refers to the absorption due to isopentyl acetate at wavelength λ with 0.01 SHE buffer as the reference, and p is a constant. Similarly, $A(\text{Q})_{243} = mA(\text{Q})_{270}$ where $A(\text{Q})_{\lambda}$ refers to the absorption of a quinoxaline antibiotic at wavelength λ with 0.01 SHE buffer as the reference and m is a constant. For quinomycin C, m was 5.08 when the ratio was measured for a range of antibiotic concentrations. Consequently, for solutions containing both antibiotic and isopentyl acetate an estimate of the antibiotic concentration can be made by measuring both the A_{243} and A_{270} with 0.01 SHE buffer as the reference:

$$A_{243} = A(\text{Q})_{243} + A(\text{IPA})_{243}$$

$$A_{270} = A(\text{Q})_{270} + A(\text{IPA})_{270}$$

where A_{λ} is the total absorption at wavelength λ . Substituting for $A(\text{Q})_{270}$ and $A(\text{IPA})_{270}$ and elimin-

ating $A(\text{IPA})_{243}$ from the simultaneous equations gives:

$$A_{243} - pA_{270} = \left(1 - \frac{p}{m}\right)A(Q)_{243}$$

This formula only assumes that p and m are independent variables, which is surely valid since the absolute concentrations of both antibiotic and organic solvent in the aqueous phase are small. Thus by knowing p and m , $A(Q)_{243}$ can be calculated, from which the antibiotic concentration can be found by using the absorption coefficient listed in Table 1. This 'internal blanking' procedure effectively eliminates errors due to differences in concentrations of organic solvents in the reference and sample solutions.

The partition coefficient for quinomycin C was measured essentially as described for triostin A (Waring *et al.*, 1975), except that to conserve the antibiotic only 2ml of '70/30 IPA/heptane' solvent was shaken with 45ml of 0.01 SHE buffer. One of the eight flasks contained no antibiotic and was used as the reference for subsequent absorption measurements as well as for the determination of p for this experiment. The concentration of antibiotic in the organic phase was determined from the A_{315} in a 10mm-light-path cuvette. The concentration of quinomycin C in the aqueous phase was determined from the A_{243} and A_{270} in 100mm-light-path

cuvettes by using the 'internal blanking' method described above. The partition coefficient was determined to be 928 ± 35 (see Fig. 2).

For triostin C, its extremely low water solubility (approx. $0.8 \mu\text{M}$, compared with $1.7 \mu\text{M}$ for both triostin A and quinomycin C) and short supply precluded its study by solvent-partition analysis. When a saturated solution of triostin C in isopentyl acetate was shaken with calf thymus DNA in 0.01 SHE buffer, the binding reached only about 0.01 drug molecule bound per nucleotide, too low to enable detailed study, and there is no reason to believe that this degree of binding could have been increased by the addition of heptane.

Binding curves and binding parameters

The experimental procedure was as previously described (Waring *et al.*, 1975), except that for experiments with triostin A, 2ml each of both phases were used, whereas for quinomycin C the organic phase (70/30 IPA/heptane) was decreased to 0.5ml to conserve materials. After shaking for 2h in a water bath maintained at $20 \pm 0.1^\circ\text{C}$, the phases were separated by centrifugation at 2000 rev./min for 30min in an MSE Super Minor bench centrifuge. The antibiotic concentration in the organic phase was determined (after appropriate dilution) from absorption measurements in 10mm-light-path semi-micro quartz cuvettes by using the absorption coefficients listed in Table 1. Given the partition coefficient, this yielded an estimate of the free quinoxaline concentration in the aqueous phase, c . The total quinoxaline concentration in the aqueous phase was determined from the absorption at the appropriate wavelength (by using the absorption coefficients listed in Table 1), in 40mm semi-micro cuvettes after dissociation of the complex by addition of an equal volume of dimethyl sulphoxide. For all spectrophotometric measurements the optical reference consisted of the appropriate phase from a quinoxaline-free blank, treated in an identical fashion. The concentration of bound antibiotic in the aqueous phase was determined by difference and divided by the nucleotide concentration to give r (mol of quinoxaline bound/mol of nucleotides). These values of r and c were routinely analysed in terms of eqn. (10) of McGhee & Von Hippel (1974) by using a program written by Dr. J. D. McGhee and installed in the University of Cambridge computer by Dr. G. Ughetto. The programme used an iterative procedure to estimate $K(0)$, the intrinsic association 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.

Fig. 2. Partition of quinomycin C between '70/30 IPA/heptane' solvent and 0.01 SHE buffer

The data were determined by using the 'internal blanking' method as described in the text. The line fitted to the points is a least-squares line constrained to pass through the origin, of slope 928 ± 35 [concentration in organic phase ($c_{\text{org.}}$)/concentration in aqueous phase ($c_{\text{aq.}}$)].

Analytical ultracentrifugation

Sedimentation coefficients were measured by boundary sedimentation at $20 \pm 0.1^\circ\text{C}$ in a Beckman model E ultracentrifuge equipped with u.v.-absorption optics as previously described (Waring, 1970). They are presented in the form s_{20} and are uncorrected for viscosity, buoyancy or DNA concentration. Complexes of bacteriophage-PM2 DNA with the quinoxaline antibiotics were prepared as follows.

For triostin A and quinomycin C, two tubes were prepared as if for solvent-partition analysis, except that the DNA concentration was $91\ \mu\text{M}$ with respect to nucleotides and the volume of the aqueous phase was increased to 4 ml or more. One tube was a quinoxaline-free blank and the other contained sufficient quinoxaline in the organic phase to produce a complex having a high r value. After shaking, the aqueous phases were separated and the total concentration of quinoxaline in the aqueous phase was estimated from the absorption of a small portion after addition of an equal volume of dimethyl sulphoxide. The blank was treated identically and used as the optical reference. Thus two aqueous solutions of bacteriophage-PM2 DNA were produced, one having a known quinoxaline/nucleotide ratio (designated D/P) and an identical solution containing no quinoxaline. Because of its insolubility a 'solid shake' procedure had to be adopted to generate high D/P values for triostin C. Approx. 0.2 mg of antibiotic was shaken with 4 ml of 0.01 SHE buffer containing $91\ \mu\text{M}$ -bacteriophage-PM2 DNA for $\frac{1}{2}$ h at 20°C . The solution was filtered through two GF/C glass-fibre filters to remove the undissolved triostin C, and the total antibiotic concentration was again measured from the absorbance of a portion of the solution to which an equal volume of dimethyl sulphoxide had been added. This yielded a solution with a D/P value of approx. 0.04.

Solutions thus prepared were used to generate the desired D/P values by direct weighing into the ultracentrifuge cells. Typically 0.6 ml of one of the solutions was introduced into one of the cells and the cell was weighed. After the sedimentation run, the cell was vigorously shaken and again weighed. Then a small volume was removed and replaced by an equal volume of the other solution. The cell was weighed after every step in the procedure so that the amount removed and added was accurately checked. This procedure was continued up to five times with the same solutions. As indicated by the weighing, leakage from the cells and cumulative errors in the serial dilutions did not exceed 3%. Intermediate D/P values were thereby generated without having to prepare a fresh complex for each one. Apart from its obvious advantages in the economical use of bacteriophage-PM2 DNA this procedure provides presumptive evidence that the antibiotic-DNA complexes are

freely reversible; if the antibiotic did not dissociate properly from the 'high- r ' DNA molecules to equilibrate with the drug-free DNA molecules added, multiple or blurred boundaries would be expected in the ultracentrifuge photographs, and/or a curved plot of $\log x$ versus time would result (where x is the distance from the axis of rotation). These were never seen. Evidently the time that elapsed before the first exposure (at least 30 min) was more than adequate for attainment of equilibrium. Where binding isotherms to bacteriophage-PM2 DNA were available, the D/P values were corrected for the degree of binding to yield r , the number of antibiotic molecules bound per nucleotide.

Viscometry

Measurements were made essentially by the method of Cohen & Eisenberg (1966, 1969) in a simple viscometer with a 10 cm capillary of 0.4 mm bore and a bulb of volume 2 ml. It was securely clamped in a water bath containing 53 litres of deionized water maintained at $20 \pm 0.02^\circ\text{C}$ by a Techne Accurostat heater opposing the action of a cooling coil. The viscometer was filled by weighing-in 1.80 g of the relevant solution. Flow times were measured in triplicate to an accuracy of 0.1 s. If readings differed by more than 0.5 s then further measurements were made and the results averaged accordingly. The viscometer had a flow time for buffer of 186.6 ± 0.3 s and an estimated average shear gradient of the order of 1000 s^{-1} .

Reduced viscosities were calculated by established procedures and used directly in the equations of Cohen & Eisenberg (1966, 1969). Wakelin & Waring (1976) have shown that the approximation involved in using reduced rather than intrinsic viscosities does not exceed 3% even for echinomycin-DNA complexes of high r value.

The sonicated DNA used in these experiments was prepared as previously described (Wakelin & Waring, 1976) and had an estimated mol.wt. of approx. 5×10^5 . Complexes with triostin A and triostin C, with high D/P values, and blank solutions containing no antibiotic were prepared as described in the 'Analytical ultracentrifugation' section, except that the DNA concentration was approx. $600\ \mu\text{M}$. Complexes with intermediate D/P values were generated as before by direct weighing into the viscometer. However, because of the larger volumes involved and the open nature of the viscometer the same solutions were only re-used up to three times, to keep the error as assessed from the weighing procedures below 5%.

Since the triostins are peptide antibiotics, the possibility was considered that they might be surface-active, and thus the free uncomplexed antibiotic in solution might cause a systematic error in the estima-

tion of flow times. However, no significant variation in flow times could be detected even when saturated solutions of the antibiotics were used. A similar conclusion was drawn for echinomycin (Wakelin & Waring, 1976).

Results

Mechanism of the interaction between DNA and quinomycin C, triostin A and triostin C

The effect of all three antibiotics on the sedimentation coefficient of closed circular duplex DNA is shown in Fig. 3. For triostin A the abscissa is in terms of drug molecules bound per nucleotide (r), whereas for quinomycin C and triostin C it shows the antibiotic/nucleotide input ratio (D/P). The latter could not be corrected for the extent of binding because of the lack of suitable binding data to bacteriophage-PM2 DNA. However, the correction of D/P to r for triostin A at $r = 0.03$ was only approx. 0.001, and it would be expected to be even smaller for quinomycin C and triostin C because their much more hydrophobic character is indicative of even stronger binding to DNA. In all cases the s_{20} of the

closed circular molecules shows the characteristic fall and rise attributable to removal and reversal of the supercoiling, whereas the nicked circular DNA shows a small overall decrease in s_{20} which is typical of intercalating agents (Waring, 1970). For triostin A the equivalence point (corresponding to exact relaxation of the supercoiling) occurs at 0.028 ± 0.005 molecules bound per nucleotide, whereas for quinomycin C and triostin C it occurs at antibiotic/nucleotide ratios of 0.026 ± 0.003 and 0.028 ± 0.006 respectively. Ethidium, under identical conditions, gives a value of 0.051 ± 0.007 molecules bound per nucleotide (Waring & Wakelin, 1974). In all cases the quoted estimate of error is derived from the total span of the equivalence region where closed and nicked circular molecules co-sedimented in the ultracentrifuge and thus should be regarded as limits rather than statistical errors. On this basis for triostin A the unwinding angle is 1.82 ± 0.40 times that for ethidium, and for quinomycin C and triostin C respectively it is not less than 1.92 ± 0.20 and 1.81 ± 0.40 times that for ethidium. These ratios are to be compared with the value of 1.82 ± 0.30 found for echinomycin (Waring & Wakelin, 1974) and thus, except for the unwinding angle of quinomycin

Fig. 3. Effect of quinomycin C (a), triostin A (b) and triostin C (c) on the sedimentation coefficient of bacteriophage-PM2 DNA. The s_{20} of closed DNA circles is shown by $\circ$, that of nicked DNA circles by Δ , and that of unresolved boundaries by $\bullet$. For triostin A the abscissa shows r , the average amount of triostin A bound (molecule/nucleotide) determined by the solvent-partition method. For quinomycin C and triostin C the abscissa shows the drug/nucleotide ratio (D/P). In all cases complexes were prepared by direct weighing into the ultracentrifuge cells.

Fig. 4. Interaction between triostin A and bacteriophage PM2 DNA

The binding ratio r (triostin A molecules bound per nucleotide) is plotted as a function of the free antibiotic concentration in the aqueous phase, c . $\circ$, Closed circular DNA; Δ , nicked DNA.

C, which seems to be a little larger, the values are indistinguishable.

For triostin A another estimate of the unwinding angle could be derived from the cross-over point of binding isotherms to closed and nicked circular bacteriophage-PM2 DNA. This is seen in Fig. 4, which clearly shows the enhanced binding to closed circular species at low values of r , with decreased binding beyond the equivalence point. The cross-over occurs at an r value of 0.0275 antibiotic molecule bound per nucleotide, which is indistinguishable from that determined in the sedimentation experiment.

Further evidence for the bifunctional intercalation of triostins A and C was gained from viscometric studies on sonicated calf thymus DNA. Plots of the relative increase in contour length as a function of the r or D/P value are shown in Fig. 5. For triostin A at this high DNA concentration ($600\mu\text{M}$) the values of r and D/P differ by less than $\frac{1}{2}\%$, so that the use of D/P values in the experiments with triostin C, for which the correction of D/P to r values may well be smaller, is not expected to invalidate the analysis

Fig. 5. Effect of triostin A (a) and triostin C (b) on the relative contour length of sonicated calf thymus DNA fragments. The ordinate represents the calculated contour length in the presence of antibiotic (L) as a ratio of the length in the absence of antibiotic (L_0) (Cohen & Eisenberg, 1966, 1969). The abscissae show the binding ratio, r , for triostin A and the drug/nucleotide ratio, D/P, for triostin C. Also shown are theoretical lines corresponding to the lengthening expected for ideal monofunctional ($1 + 2r$) and ideal bifunctional ($1 + 4r$) intercalation. In both cases complexes were prepared by direct weighing into the viscometer.

in any way. The lines labelled $1 + 2r$ and $1 + 4r$ describe the ideal results to be expected for perfect mono- and bi-functional intercalation respectively. The line fitted to the experimental points was constrained to pass through the origin and was fitted by the method of least squares. For triostins A and C the lines have slopes of 3.57 ± 0.17 and 3.89 ± 0.14 respectively, which correspond to 1.79 ± 0.09 and 1.95 ± 0.07 intercalation events per bound antibiotic molecule. Values for echinomycin and ethidium under similar conditions were reported to be respectively 1.87 ± 0.05 and 0.81 ± 0.1 intercalation events per bound molecule (Wakelin, 1974; Reinert, 1973). Thus, although in every instance the values are slightly below the theoretical for bifunctional or monofunctional intercalation as the case may be, those for the quinoxaline antibiotics are all at least twice that found for ethidium.

Specificity of the interaction between DNA and quinomycin C and triostin A

Scatchard plots for the binding of quinomycin C and triostin A to naturally occurring double-stranded DNA species are shown in Fig. 6. The lines drawn are calculated isotherms based on eqn. (10) of McGhee & Von Hippel (1974). In general, they give a good fit to the experimental points, which lends credence to the applicability of this treatment. It is also immediately apparent from the difference in the curves that

these antibiotics, like echinomycin, show some preference for particular bases or base-sequences. Three further conclusions emerge without recourse to the calculated binding parameters. Firstly, the binding of quinomycin C is much tighter than that of triostin A (note the change in the ordinate scale). Secondly, the quinomycin C curves cover a much wider range than do those for triostin A, which suggests that the specificity patterns are different. Thirdly, the binding of triostin A to calf thymus and nicked bacteriophage-PM2 DNA, both containing 42% (G + C), is significantly different, whereas for echinomycin it was virtually the same (Wakelin & Waring, 1976). Thus the specificity patterns shown by triostin A and echinomycin also appear to be different.

For triostin A the low degree of binding to denatured calf thymus and bacteriophage-fd DNA has already been reported (Waring *et al.*, 1975), showing that, as with echinomycin (Waring & Wakelin, 1974), there is a requirement for ordered helical structure. The results of binding studies with triostin A and quinomycin C and some further nucleic acids are shown in Fig. 7. The low or insignificant interaction with rRNA from *E. coli* again shows that binding occurs only to DNA. Also in agreement with the data for echinomycin (Wakelin & Waring, 1976) are the low degrees of binding to poly(dA)·poly(dT) and poly(dI-dC). Not only are the curves shown for interaction with alternating poly(dA-dT) significantly different, but also that for quinomycin C has a rather

Fig. 6. Effect of base composition on the binding of quinomycin C (a) and triostin A (b) to DNA

The data are presented in the form of Scatchard plots, where r is the binding ratio (quinomycin C or triostin A molecules bound per nucleotide) and c is the free antibiotic concentration. Curves describing eqn. (10) of McGhee & Von Hippel (1974) were fitted to the experimental points by computer. ●, *M. lysodeikticus* DNA; ▲, nicked bacteriophage-PM2 DNA; ○, *Clostridium perfringens* DNA.

Fig. 7. Interaction of quinomycin C (a) and triostin A (b) with polynucleotides

The binding ratio r (quinomycin C or triostin A molecules bound per nucleotide) is plotted as a function of the free antibiotic concentration, c . ●, Poly(dA-dT); ○, poly(dA)·poly(dT); △, rRNA from *E. coli* B; ▲, poly(dI-dC).

Fig. 8. Interaction of quinomycin C (a) and triostin A (b) with poly(dA-dT) and poly(dG-dC)

The data are presented in the form of a Scatchard plot, where r is the binding ratio (quinomycin C or triostin A molecules bound per nucleotide) and c is the free antibiotic concentration. A curve describing eqn. (10) of McGhee & Von Hippel (1974) was fitted to each set of experimental points by computer (—). Also shown (---) are curves describing eqn. (15) of McGhee & Von Hippel (1974), which includes a co-operativity parameter. See the text for details. ○, Poly(dA-dT); △, poly(dG-dC).

unusual shape. This is considerably more obvious in Scatchard plots of the data (Fig. 8), where results for the binding of quinomycin C and triostin A to poly(dG-dC) are also represented. In all cases lines are drawn representing the McGhee & Von Hippel (1974) treatment of the experimental points, but also shown for poly(dA-dT) are 'humped' co-operative binding curves. These are drawn according to eqn. (15) of McGhee & Von Hippel (1974), where a co-operativity parameter, ω , is introduced such that the binding constant to singly and doubly contiguous sites is $\omega K(0)$ and $\omega^2 K(0)$ respectively. Trial curves were plotted out by a PDP 8/E computer, the parameters $K(0)$, n and ω being varied by trial and error. The best fit to the experimental points was chosen by eye. The quality of the data for the binding of triostin A to poly(dA-dT) makes the choice of parameters rather arbitrary, but the fit is at least as good if not better than that from the usual non-co-operative binding curve. In this case the values of the parameters $K(0)$, n and ω were 8.0×10^5 , 7.5 and 6.2 respectively, demonstrating a fair degree of co-operativity. These are to be compared with values of $K(0)$ and n of 9.7×10^5 and 4.8 from the non-co-operative treatment, the most important difference being the much smaller value of n in the latter case. For quinomycin C the quality of the data is such that the first part of the plot is much better represented by a 'humped' co-operative binding curve, though no amount of parameter variation allowed the 'tail' to be fitted simultaneously. This suggests two binding processes, the primary one being co-operative. The 'humped' curve shown has parameters $K(0)$, n and ω of 4.1×10^5 , 19 and 31, whereas the non-co-operative treatment gives $K(0)$ and n values of 5.7×10^5 and 7.0 respectively. Again the non-co-operative value of n is much smaller, and the higher ratio of ω to n for the co-operative binding curve in this case gives a distinct maximum to the 'hump'.

Perhaps the most important conclusion to be drawn from Fig. 8 concerns the very large differences

in binding to these synthetic DNA molecules. Moreover, the order of preference shown by quinomycin C (which is the same as that of echinomycin) is reversed for triostin A. Thus again the specificity pattern shown by triostin A is clearly different from that displayed by the quinomycin antibiotics.

In preliminary binding experiments with quinomycin C and triostin A, only very low degrees of binding to poly(dG)·poly(dC) were detected ($r_{\max.} = 0.015$), whereas this polymer was reported to bind echinomycin well (Wakelin & Waring, 1976). Another experiment was therefore performed with echinomycin and the same batch of polymer by the method of Waring *et al.* (1975). This, too, revealed rather low binding. Further studies on the interaction of echinomycin with two other batches of poly(dG)·poly(dC) again displayed different degrees of binding, which in all cases were less than that originally reported. Consequently it appeared impossible to derive reliable information about specificity from binding studies on this polymer, no doubt owing to the known difficulty of obtaining material containing only molecules with defined stoichiometry and secondary structure (see the Discussion section). Evidently the result published for echinomycin (Wakelin & Waring, 1976) should be interpreted with care.

The complete list of binding parameters derived from analysis in terms of eqn. (10) of McGhee & Von Hippel (1974) for the various DNA species that bound the antibiotics well is shown in Table 2. These can be compared with the values for echinomycin given in Table 1 of Wakelin & Waring (1976). The values for the parameter n , the number of nucleotides occluded by the binding of one molecule, do not vary greatly, though, as with echinomycin, there seems to be some tendency for the alternating synthetic polymers to yield the smallest values. Indeed the treatment of McGhee & Von Hippel (1974) determines some sort of averaged binding parameters, so that for DNA species with multiple binding sites having different

Table 2. Binding parameters for quinomycin C and triostin A

$K(0)$, the intrinsic association constant, and n , the number of nucleotides occluded by the binding of one molecule, were evaluated from an analysis of the binding data in terms of eqn. (10) of McGhee & Von Hippel (1974) as described in the text.

DNA	Quinomycin C		Triostin A	
	$10^{-6} \times K(0)$ (M^{-1})	n	$10^{-6} \times K(0)$ (M^{-1})	n
<i>M. lysodeikticus</i>	8.26	8.34	1.13	7.73
Calf thymus	2.60	8.30	0.70	7.29
Bacteriophage PM2 (nicked circular molecules)	—	—	0.42	9.42
<i>Cl. perfringens</i>	0.91	9.56	0.49	8.52
Poly(dG-dC)	3.36	6.12	0.43	7.41
Poly(dA-dT)	0.57	7.02	0.97	4.76

affinities, the value of n would be expected to increase with increasing variation in the affinities of various sites. For quinomycin C the binding constants fall in the same ranking order as those for echinomycin, though they are approximately two to six times as large. Thus the specificity patterns for echinomycin and quinomycin C appear to be broadly similar. For triostin A, on the other hand, the variations in binding constants are much smaller and the specificity pattern is very different from that of echinomycin.

Discussion

The first objective of the present experiments was to investigate the mechanism of interaction between quinoxaline antibiotics and DNA. The results leave no room for doubt that the four naturally occurring quinoxalines so far studied are bifunctional intercalators at low ionic strength. It is likely therefore that the whole family of quinoxaline antibiotics will be characterized by a common capacity for bifunctional intercalation. It would be surprising if other members of the group, which are intermediate between the A and C forms in the number of methyl groups on the valine residues, showed different functionality.

The lack of consistency in the binding of echinomycin to poly(dG)·poly(dC) is unfortunate, since Wakelin & Waring (1976) reported a binding constant for this polymer that was larger than all others except for that to *M. lysodeikticus* DNA. However, several authors (Inman, 1964; Wells *et al.*, 1970; R. D. Wells, personal communication) have reported non-reproducible results with authentic samples of the homopolymer DNA species poly(dG)·poly(dC) and poly(dI)·poly(dC). In particular, Wells *et al.* (1970) found six different buoyant-density values for six different preparations of poly(dG)·poly(dC). It was suggested that this was due to the ability of the polymer to exist in several metastable forms. These are of a type involving self-association of individual strands, such as two poly(dG) strands, and triple-stranded helices, such as 2 poly(dG)·poly(dC). Consequently if the preparations used in the present work contained different proportions of the various metastable forms, then the degree of binding of echinomycin (with its known requirement for ordered double-helical structure in natural DNA) would indeed be expected to vary.

The second objective was to investigate the pattern of specificity shown by quinomycin C and triostin A in their interaction with DNA. The specificity pattern found for quinomycin C is broadly similar to that of echinomycin (Wakelin & Waring, 1976), whereas that found for triostin A is very different. These similarities and differences are most probably reflected in the conformations that the molecules are capable of adopting. Some conformational information can be

gained without a detailed study by simple reference to the n.m.r. spectra. Triostins A and C in chloroform both show two conformations in approximately the same proportions (Blake *et al.*, 1977); likewise the spectra of echinomycin and quinomycin C are also very similar, allowing for the additional signals of the latter (J. Kalman, personal communication). Thus in each case the replacement of *N*-methylvaline by *N*, γ -dimethylalloisoleucine does not appear to affect the conformation adopted by the peptide ring. Consequently the similarity in the specificity patterns shown by echinomycin and quinomycin C suggests that these amino acid side chains are not involved in the specificity-determining interactions with the DNA helix. Thus the main effect of the additional methyl groups present in quinomycin C is to increase the binding constants, presumably attributable to increased hydrophobic character.

The binding of echinomycin to natural DNA species suggested a broad preference for those rich in (G+C) (Wakelin & Waring, 1976). This is also true for the more limited data on quinomycin C. For triostin A, the highest binding constant was again found for *M. lysodeikticus* DNA, but in this case the lowest was for nicked bacteriophage-PM2 DNA and not *Clostridium perfringens* DNA. Furthermore triostin A displays a much smaller overall variation in binding constants to natural DNA, and thus appears to show less preference for (G+C)-rich DNA species than do the quinomycins. Extension of these considerations to the interaction with synthetic polymers is hindered by the problem of differentiating between consequences arising from the presence of different base-pairs or those arising from gross structural differences. This was discussed at length by Wakelin & Waring (1976), who concluded that the binding of echinomycin to synthetic polynucleotides did not correlate with their helical structures so far as they were understood. Suffice it to say here, therefore, that the degree of binding to poly(dG-dC) and poly(dA-dT) [and perhaps some preparations of poly(dG)·poly(dC)] would in itself suggest that these polymers adopt helical structures under the experimental conditions similar to those of natural DNA species. However, the possibility of co-operative binding to poly(dA-dT), which has also been suggested for echinomycin (L. P. G. Wakelin, personal communication), would reinforce the view that structural differences also play a part even with these polymers. Consequently, if synthetic polynucleotides are included in considerations of specificity, then the fact that for the quinomycins the highest binding constants are found for *M. lysodeikticus* DNA would suggest that the preferred binding sites contain all four bases. The same may also be true for triostin A, although the sequence of the bases in the preferred sites is undoubtedly different; this view is unequivocally confirmed by the reversal of preference among

the synthetic polynucleotides poly(dA-dT) and poly(dG-dC) compared with the quinomycins.

Characterization of preferred binding sites, an essential preliminary to the development of molecular models of complex-formation, is inevitably difficult for large molecules which occlude many base-pairs on the helix. One method of quantifying preferences among potential binding sites is the calculation of α parameters as described by Müller & Crothers (1975), Müller *et al.* (1975) and Müller & Gautier (1975). The value of α is simply the ratio of binding constants for selected DNA species of different base composition. It can be compared with theoretical values calculated by assuming a particular degree of specificity with the DNA having an effectively random nucleotide sequence. For the quinoxaline antibiotics it is convenient to calculate α values from binding constants to *M. lysodeikticus* and *Cl. perfringens* DNA, which have (G+C) contents of 72 and 30% respectively. Thus a ligand showing preference for one (G·C) base-pair in the binding site would be expected to yield an α value of 2.4, the ratio of the (G+C) contents in these DNA species. Similarly, an α value of 5.76 would suggest preference for two (G·C) base-pairs and so on. Preference for (A·T) is shown by α values less than 1.0; a value of 0.4 characterizes a clear preference for one (A·T) base-pair, a value of 0.16 for two (A·T) base-pairs, and so on. The α value for quinomycin C is 9.12, which is between 2.4^2 and 2.4^3 , suggesting moderate preference for sites containing three (G·C) base-pairs. For echinomycin an α value of 9.01 can be calculated from the data of Wakelin & Waring (1976), which stresses the similarity in the specificity shown by these two quinomycins. Indeed, these very large values of α would suggest that the quinomycins are the most (G·C)-specific ligands yet investigated. For triostin A the α value is only 2.32, indicating preference for only one (G·C) base-pair in the binding site.

The origins of base preferences in the quinoxaline group of antibiotics must lie in either the chromophores, the peptide ring or possibly both. The chromophores are unlikely candidates as the sole determinants of specificity, since triostin A and the quinomycins show different patterns. However, they undoubtedly determine to some extent the position of functional groups in the intercalated complex. The peptide rings, on the other hand, can potentially form many hydrogen bonds and specific contacts with nucleotides in the helix, and the peptide ring conformations in triostin A and the quinomycins are undoubtedly different (Cheung *et al.*, 1978; J. R. Kalman, T. J. Blake, D. H. Williams, J. Feeney & G. C. K. Roberts, unpublished work). Nevertheless there must also remain considerable elements of similarity to allow for bifunctional intercalation in both groups of antibiotics. These considerations are

developed more fully when the probable conformations of the molecules are discussed in the following paper (Lee & Waring, 1978).

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*As originally published in *J. Mol. Biol.* **86**, 469–489 (1974), eqn. (15) of McGhee & Von Hippel contains a misprinted sign. The correct form of the equation is given in *J. Mol. Biol.* **103**, 679 (1976) and is the equation referred to in the text and Fig. 8 of the present paper.

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