

The Binding of Echinomycin to Deoxyribonucleic Acid

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Echinomycin is a peptide antibiotic which binds strongly to double-helical DNA up to a limit of approximately one molecule per five base-pairs. There is no detectable interaction with rRNA and only extremely feeble non-specific interaction with poly(rA)·poly(rU). Heat denaturation of DNA greatly decreases the binding, and similarly limited interaction is observed with naturally occurring single-stranded DNA. Association constants for binding to nine double-helical DNA species from different sources are presented; they vary by a factor of approximately 10, but are not simply related to the gross base composition. The interaction with DNA is ionic-strength-dependent, the binding constant falling by a factor of 4 when the ionic strength is raised from 0.01 to 0.10 mol/litre. From the effect of temperature on the association constant for calf thymus DNA, the enthalpy of interaction is calculated to be about -13 kJ/mol (-3 kcal/mol). Binding of echinomycin persists in CsCl gradients and the buoyant density of nicked bacteriophage PM2 DNA is decreased by 25 mg/ml. Echinomycin interacts strongly with certain synthetic poly-deoxynucleotides, the binding constant decreasing in the order poly(dG)·poly(dC) > poly(dG-dC) > poly(dA-dT). For the latter two polymers the number of base-pairs occluded per bound antibiotic molecule is calculated to be three, whereas for poly(dG)·poly(dC) it is estimated to be four to five. Poly(dA)·poly(dT) and poly(dI)·poly(dC) interact only very weakly with the antibiotic. Poly(dI-dC) interacts to a slightly greater extent, but the binding curve is quite unlike that seen with the three strongly binding synthetic polynucleotides. Echinomycin affects the supercoiling of closed circular duplex bacteriophage PM2 DNA in the characteristic fashion of intercalating drugs. At low ionic strength the unwinding angle is almost twice that of ethidium. Likewise the extension of the helix, determined from changes in the viscosity of rod-like sonicated DNA fragments, is nearly double that expected for a simple (monofunctional) intercalation process. On this basis the interaction process is characterized as bifunctional intercalation. At higher ionic strength the unwinding angle relative to that of ethidium and the helix extension per bound echinomycin molecule fall, indicating a smooth progression towards more nearly monofunctional intercalation. Two simpler compounds which act as analogues of the quinoxaline chromophores of echinomycin, quinoxaline-2-carboxamide and the trypanocidal drug Bayer 7602, interact with DNA very much more weakly than does echinomycin, showing that the peptide portion of the antibiotic plays an essential role in determining the strength and specificity of the interaction.

Echinomycin was originally discovered as an antibiotic present in culture filtrates of *Streptomyces echinatus* (Corbaz *et al.*, 1957). Early structural studies (Keller-Schierlein *et al.*, 1959) established that it contained two quinoxaline-2-carboxylic acid moieties linked to a cyclic octapeptide ring; the peptide portion was shown to be a dilactone consisting of two D-seryl-L-N-methylvalyl-L-N-methylcysteinyl-L-alanyl sequences condensed together, with some sort of cross-bridge connecting the side chains of the cysteine residues, and the chromophores attached via amide linkages to the serine amino groups. It was originally surmised that the cross-bridge was constituted by a dithian ring involving the α -carbon

atoms of the cysteine residues (Keller-Schierlein *et al.*, 1959), but studies using nuclear-magnetic-resonance and mass-spectroscopic techniques (Dell *et al.*, 1975; Martin *et al.*, 1975) have revealed that this suggestion requires revision. The molecule contains one more carbon atom and four more hydrogen atoms than originally thought, leading to a revised mol.wt. of 1100 and the structural formula shown in Fig. 1. The dithioacetal cross-bridge is rather more complicated than the arrangement first proposed (Keller-Schierlein *et al.*, 1959), but a plausible biosynthetic origin involving rearrangement from a simple disulphide precursor can be envisaged (Dell *et al.*, 1975).

Quinoxaline antibiotics from other sources which

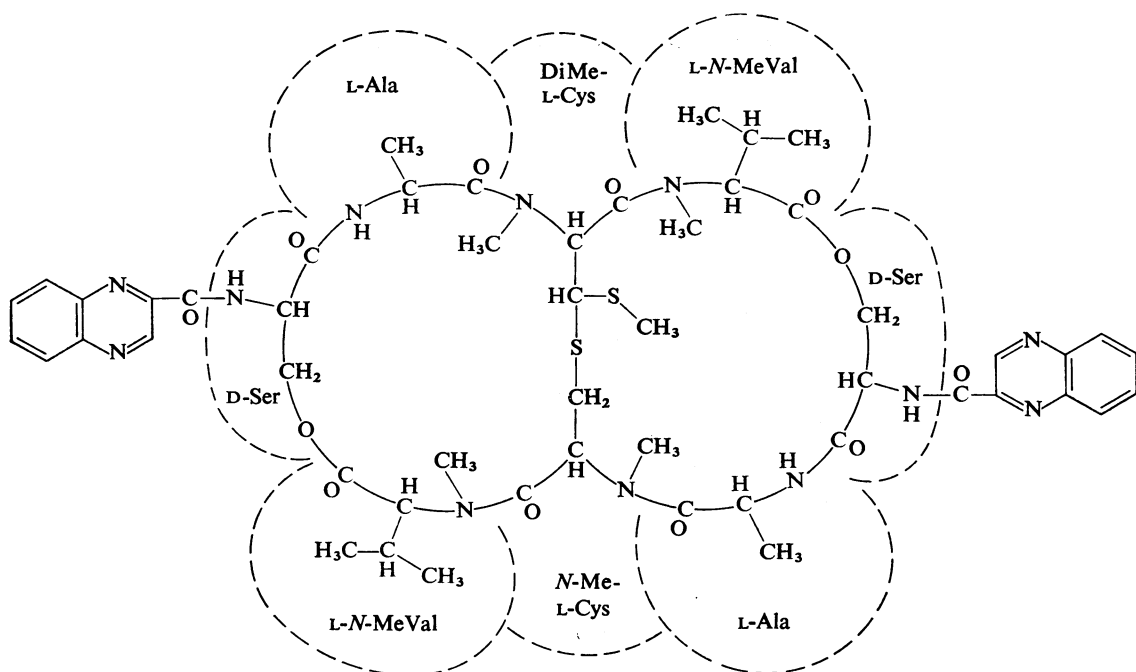


Fig. 1. Structure of echinomycin according to Dell *et al.* (1975)

appear to be chemically identical with echinomycin are compound X-948, actinoleukin, levomycin and quinomycin A (Berger *et al.*, 1957; Ishihara *et al.*, 1958; Katagiri *et al.*, 1962; Martin *et al.*, 1975). They are characteristically highly active against Gram-positive, anaerobic and acid-fast bacteria, whereas Gram-negative bacteria and fungi are generally resistant (Yoshida *et al.*, 1961; Shoji & Katagiri, 1961; Katagiri *et al.*, 1966), most probably for reasons of relative impermeability, since quinomycin A inhibits the incorporation of labelled uridine into RNA by spheroplasts of *Escherichia coli*, whereas intact cells are insensitive to the drug (Sato *et al.*, 1967a). Moderate activity against protozoa and viruses has also been reported (Ueda *et al.*, 1954; Corbaz *et al.*, 1957; Katagiri *et al.*, 1957; Tsunoda, 1962; Sato *et al.*, 1969), but most interest in quinoxaline antibiotics has been focused on their cytotoxicity and promise as antitumour agents (Katagiri & Sugiura, 1961; Matsuura, 1965; Harada *et al.*, 1968; Katagiri *et al.*, 1975).

Their mode of action involves interference with nucleic acid synthesis, attributable to binding to DNA in susceptible cells (Ward *et al.*, 1965; Sato *et al.*, 1967a,b; Waring & Makoff, 1974; Katagiri *et al.*, 1975). Echinomycin is an extremely powerful inhibitor of RNA synthesis in *Bacillus megaterium*, some 4–5 times more potent than actinomycin D,

and is at least as selective as actinomycin in inhibiting DNA-directed RNA synthesis (Waring & Makoff, 1974). It is generally agreed that its antitumour and other biological activities result directly, perhaps solely, from its interaction with DNA (Ward *et al.*, 1965; Sato *et al.*, 1967b; Kageyama *et al.*, 1970; Katagiri *et al.*, 1975).

Our interest in echinomycin stems from its structural and functional analogy with actinomycin, and in particular from the perfect twofold symmetry in its structure as first suggested by Keller-Schierlein *et al.* (1959). In their intercalation model for the binding of actinomycin to DNA, Sobell *et al.* (1971) took care to point out the role of twofold symmetry as an important element in the recognition of the DNA helix by actinomycin. Consequently it seemed possible that the apparent twofold symmetry of echinomycin might play a similar role and endow the molecule with a capacity for bifunctional reaction, i.e. intercalation of both quinoxaline chromophores in a symmetry-related fashion at the same time. Such appears to be the case.

After most of the results reported here had been accumulated, preliminary proton-magnetic-resonance studies (G. C. K. Roberts, J. Feeney & M. J. Waring, unpublished work) indicated that the presumed structure of echinomycin might be in error. As a result the studies leading to the new structure

shown in Fig. 1 were initiated. Thus it now appears that the molecule possesses an imperfect twofold axis of rotational symmetry passing through the central sulphur atom of the cross-bridge linking the two halves of the peptide ring. However, apart from the substituents on the cysteine β -carbon atoms adjacent to the central sulphur atom the symmetry in the structural formula remains exact. A correction for the revised molecular weight has been applied throughout the results reported here.

Evidence for bifunctional intercalation of echinomycin into DNA has been briefly summarized (Waring & Wakelin, 1974). The significance of this novel mode of binding extends beyond its consequences for the high binding constant and substantial distortion of the structure of the DNA helix because it raises the possibility of achieving true sequence-specificity via interaction at discrete loci spaced sequentially along the helix. In the present paper we report details of the interaction between echinomycin and a variety of natural and synthetic polynucleotides, first with a view to determining what selectivity the antibiotic may display with respect to its capacity to bind to different types of nucleotide sequence and secondly to define the general character of the interaction as fully as possible in terms of effects on the macromolecular properties of DNA. Finally we address the question of the role of the quinoxaline chromophores as indicated by the effects of two simpler compounds which act as analogues of these structures.

Materials and Methods

Echinomycin is a product of CIBA-Geigy Ltd., Basel, Switzerland; samples were kindly provided by Dr. H. Bickel, Dr. J. Müller, Dr. J. Nüesch and Dr. K. Scheibli. Bayer 7602 (mol.wt. 494.7) was a gift from Dr. W. E. Gutteridge of the Biological Laboratories, University of Kent, Canterbury, U.K. Quinoxaline-2-carboxamide (mol.wt. 173.2) was synthesized by Dr. A. Dell at the University Chemical Laboratory, Cambridge; we are very grateful to her for a sample of the material and for analytical evidence of its purity. All the drugs were stored dry in a desiccator in the dark at 0–4°C; solutions were freshly prepared as required. There is no evidence that any of these substances are particularly susceptible to photodecomposition, but routine precautions were taken to minimize exposure to intense illumination. Except where otherwise indicated the buffer used throughout (designated 0.01 SHE) contained 2mM-Hepes,* 10 μ M-EDTA and 9.4mM-NaCl dissolved in glass-distilled water or reagent-grade water from a Millipore Milli-Q2 system. It was adjusted with NaOH to

pH 7.0 at 20°C; the resultant ionic strength was 0.01 mol/litre. SHE buffers of ionic strength 0.1 and 0.5 mol/litre were prepared by appropriate supplementation with NaCl.

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 μ g of Pronase (Calbiochem, London W.1, U.K.)/ml at 37°C, an incubation with T1 RNAase (5 μ g/ml) as well as pancreatic RNAase, and additional final purification by one or more extractions with buffer-saturated redistilled phenol. For the measurement of binding curves, all these DNA species were sheared to a standard molecular weight by drawing a solution (1 mg/ml in 2.5M-NaCl) 20 times into a 1 ml syringe through a no. 28 needle (a gift from Dr. C. A. Thomas, Department of Biological Chemistry, Harvard University Medical School, Boston, MA, U.S.A.) at 0°C. This procedure produces fragments having 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 and used immediately or, for calf thymus DNA, stored frozen at –22°C. Bacteriophage T2 DNA was prepared by phenol extraction of purified virus as previously described (Waring, 1965a). Bacteriophage PM2 DNA, consisting of over 95% closed circular duplex molecules, was prepared by the method of Espejo *et al.* (1969), by using strains of virus and host bacteria kindly provided by Dr. R. T. Espejo, Department of Virology, School of Medicine, Santiago, Chile. Samples containing approx. 15% nicked circular molecules (for analytical-ultracentrifugation experiments) or completely converted into the nicked circular species (for measurement of binding curves) were generated by repeated freezing and thawing. DNA concentrations were based on an assumed value for $\epsilon_{(P)260}$ (molar extinction coefficient with respect to nucleotides) of 6600, except for bacteriophage T2 DNA (5974; Rubenstein *et al.*, 1961), *Serratia marcescens* DNA (6400) and *E. coli* and *Micrococcus lysodeikticus* DNA (6300) (Tubbs *et al.*, 1964).

Poly(dA-dT), poly(dG-dC) and poly(dA)·poly(dT) were purchased from Boehringer Corp. Ltd., London W5 2TZ, U.K. Poly(dI-dC), poly(dI)·poly(dC) and poly(dG)·poly(dC) were products of P-L Biochemicals, Milwaukee, WI, U.S.A. Poly(rA) and poly(rU) were obtained from Miles Chemical Co., Elkhart, IN, U.S.A. All were used as supplied without further purification; they were dissolved in 0.01 SHE buffer and dialysed for several hours against the buffer before use. Concentrations were based on the values of $\epsilon_{(P)260}$ listed by

* Abbreviations: Hepes, 2-(N-2-hydroxymethyl-piperazin-N'-yl)ethanesulphonic acid; RNAase, ribonuclease.

Wells & Wartell (1974). Poly(rA)·poly(rU) was formed by mixing equimolar amounts of the polymers and checking the extinction/temperature profile as previously described (Waring, 1974); the T_m (mid-point of the hyperchromic transition) in 0.01 SHE buffer was found to be 41°C. According to the manufacturers' specifications all the deoxy polymers contained equimolar amounts of the specified nucleotide pairs and were 100% double-helical material. Thermal-denaturation profiles were determined for poly(dA)·poly(dT), poly(dA-dT) and poly(dI-dC); the T_m values in 0.01 SHE buffer were 49°C, 40°C and 31°C respectively, in reasonable agreement with the expected values at this ionic strength (Inman & Baldwin, 1962; Riley *et al.*, 1966; Wells *et al.*, 1970). Values of T_m for the G+C-containing polymers (Inman & Baldwin, 1964; Wells *et al.*, 1970) could not be checked because they would be expected to exceed the range accessible to our equipment (Waring, 1974) in 0.01 SHE buffer.

Analytical ultracentrifugation

Sedimentation coefficients were determined by boundary sedimentation in the Beckman model E analytical ultracentrifuge equipped with u.v. optics as previously described (Waring, 1970). They are presented in the form s_{20} , determined directly at 20°C and uncorrected for viscosity, buoyancy or DNA concentration. Echinomycin-bacteriophage PM2 DNA complexes were formed by the method of Kohn *et al.* (1975). The buoyant density in CsCl of complexes between echinomycin and nicked bacteriophage PM2 DNA was determined by filling a standard and 1° positive-wedge cell each with 0.600 ml of CsCl solution in 10 mm-sodium EDTA (pH 7.0) of accurately known density in the region of 1.70 g/ml; to each cell was added 0.3 μg (91.2 nmol) of bacteriophage PM2 DNA dissolved in 5 μl of 0.01 SHE buffer. The contents of the cells were mixed, and they were centrifuged to equilibrium for 20–24 h in an An-D rotor at 20°C and 44000 rev./min. U.v.-absorption photographs were then taken and scanned with a Joyce-Loebl recording micro-densitometer (total resultant magnification factor 9.437). In this initial control run without antibiotic, the apparent buoyant-density difference between the DNA bands in the two cells never exceeded 1.4 mg/ml. Portions of a saturated solution of echinomycin (4.82 μM) in 10 mm-sodium EDTA were then added to the standard cell by using a 10 μl Hamilton syringe, and an identical volume of EDTA solution was added to the wedge cell. The cells were shaken and re-run, and the process was repeated up to four times unless at any stage the meniscus levels were observed to change as a result of leakage, in which case the whole process was started afresh. The buoyant densities

of the complexes (ρ) were evaluated from the equation of Sueoka (1961)

$$\rho = \rho^0 + 0.00892(x^2 - x_0^2) \quad (1)$$

for values of ρ in the range 1.65–1.75 g/ml, where x is the distance from the axis of rotation of the echinomycin-DNA complex band and x_0 is the corresponding distance for the reference DNA having $\rho^0 = 1.693$ g/ml (Espejo *et al.*, 1969). The density of the CsCl medium was chosen such that the two bands formed near the centres of the cells so as to minimize differences due to non-linearity of the gradients. The scaling factor 0.00892 is related to the optical magnification factor and rotor speed (Sueoka, 1961).

Thermal-denaturation profiles

These were determined by using apparatus as previously described with a programmed temperature rise of 0.6°C/min (Waring, 1974). Where appropriate a correction for thermal expansion of the cuvette contents was applied.

Binding curves

These were measured by the solvent-partition method of Waring *et al.* (1975), by using isopentyl acetate as solvent for the antibiotic. The volume of the organic phase containing echinomycin was 4 ml; it was shaken together with 3 ml of buffer containing the nucleic acid at a concentration of 182 μM with respect to nucleotides for 2 h in a water bath maintained at 20 ± 0.1°C (unless otherwise specified). The phases were then separated by centrifugation for 30 min at 2000 rev./min in an MSE Super Minor bench centrifuge in a swing-out rotor. The antibiotic concentration in the upper (organic) phase was determined from the E_{315} measured in a 40 mm light-path semi-micro quartz cuvette by using the molar extinction coefficient in isopentyl acetate of 11 500; for samples having an absorbance exceeding 1.0, a 10 mm light-path cuvette was used. Given the appropriate partition coefficient, this enabled estimation of c , the free echinomycin concentration in the aqueous phase. The total antibiotic concentration in the aqueous phase was determined by measuring the E_{325} in a 40 mm-light-path semi-micro cuvette after dissociating the complex by addition of an equal volume of dimethyl sulphoxide; the molar extinction coefficient under these circumstances is 12 400. For all spectrophotometric measurements the optical reference consisted of the corresponding phase from a 'blank' tube subjected to identical treatment; this tube contained the same polynucleotide concentration in the aqueous phase but no antibiotic in the organic phase. The concentration of bound antibiotic

in the aqueous phase was determined by difference, and divided by the polynucleotide concentration to yield r (mol of antibiotic bound/mol of nucleotides). The relevant partition coefficients for the various SHE-buffer systems and temperature used are summarized in Table 2 of Waring *et al.* (1975); they lie within the range 106 ± 7 to 159 ± 9 . The partition coefficient of echinomycin between isopentyl acetate and 0.05 M-Tris/HCl buffer (pH 7.9 at 20°C), required for the estimation of binding ratios in Table 2, was determined to be 113 ± 5 .

Viscometry

Measurements were made essentially by the method of Cohen & Eisenberg (1966, 1969) by using a simple viscometer having a 10 cm capillary of 0.4 mm bore and a bulb volume of 2.5 ml, maintained at $20.0 \pm 0.01^\circ\text{C}$ by a Techne Accurostat heater/water pump operating in a water bath of 53 litres capacity. The viscometer was filled by weighing-in 2.70 g of the relevant solution. The flow time for water was 125.9 ± 0.3 s and the estimated average shear gradient of the order 1000s^{-1} . Flow times were measured at least in triplicate to an accuracy of 0.1 s; if any readings differed by more than 0.5 s, further measurements were made with a fresh solution and the results averaged accordingly. The DNA used was calf thymus DNA which had been sonicated for 10 min at 0°C under nitrogen by using the exponential probe of an MSE 150 W ultrasonic disintegrator at the highest available power. To prevent excessive heating of the DNA solution (2 mg/ml), sonication was performed in 30 s bursts, allowing 1 min for cooling between bursts. The molecular weight of the fragments produced by this treatment was estimated to be 5.3×10^5 by sedimentation analysis and 5.6×10^5 from the reduced viscosity by using the equations of Crothers & Zimm (1965). A smaller batch of more heavily sonicated DNA fragments was also prepared which had a mol.wt. of 3.8×10^5 .

Echinomycin-DNA complexes of known binding ratio were prepared by the standard solvent-partition method in isopentyl acetate-saturated SHE buffer (Waring *et al.*, 1975). At $I = 0.01$ mol/litre the DNA concentration used was $200 \mu\text{g/ml}$ (0.02 g/dl). At $I = 0.1$ and 0.5 mol/litre the concentration was raised to $300 \mu\text{g/ml}$ (0.03 g/dl). Reduced viscosities were calculated by established methods and were always related to flow times for the relevant isopentyl acetate-saturated SHE buffer.

Since echinomycin is a peptide antibiotic the possibility was considered that it might be surface-active, and therefore free antibiotic in equilibrium with the complexes might cause a systematic error in the estimation of flow times. This was shown not to be the case by measuring flow times for isopentyl acetate-saturated 0.01 SHE buffer containing echino-

mycin at a range of concentrations up to approx. $1 \mu\text{M}$. No significant variation in the flow time could be detected.

Results

Interaction with naturally occurring nucleic acids

Evidence has been presented elsewhere (Waring & Wakelin, 1974; Waring *et al.*, 1975) to show that echinomycin binds specifically to DNA, not RNA, and that it requires the presence of helical structure in the DNA. Interaction with rRNA from *E. coli* was essentially undetectable within the limits of experimental error. A marked preference for ordered helical structure in the polynucleotide was evident in the interaction with heat-denatured calf thymus DNA and single-stranded circular DNA from bacteriophage fd. The binding constants (included in Table 1) are 4–5-fold lower than those for native calf thymus DNA or other double-helical DNA species of equivalent base composition, and the number of nucleotides per binding site is substantially higher. More persuasive evidence that echinomycin has a practically absolute requirement for helical structure in natural DNA was provided by the demonstration that heat denaturation of calf thymus DNA in the presence of 1% (w/v) formaldehyde abolishes the interaction almost completely (Waring & Wakelin, 1974). The presence of formaldehyde has little, if any, effect on the antibiotic itself or the binding reaction with native calf thymus DNA (Wakelin, 1974).

In Fig. 2 are shown Scatchard plots (Scatchard, 1949) for the interaction between echinomycin and eight naturally occurring DNA species of different gross base composition. It is immediately apparent that the experimental data for several (perhaps all) of the DNA species are non-linear. The interpretation of such data depends on the theoretical model used; if the simplest situation (Scatchard, 1949) is considered and the data are assumed to pertain to simple mass-action interaction between the antibiotic and discrete non-interacting sites spaced along the DNA, straight lines can be fitted to the points (e.g. by the method of least squares) and interaction parameters calculated. This was done in our earlier analysis of some of the data (Waring & Wakelin, 1974), but the results are hardly satisfactory. Various authors have considered the significance of curvature in Scatchard plots and its possible implications in terms of (a) heterogeneity of binding sites characterized by different affinities for the drug, or (b) interference between identical sites such that binding to one site affects the affinity of neighbouring site(s), or (c) both (e.g. Latt & Sober, 1967; Crothers, 1968; Zasedatelev *et al.*, 1971; Gursky *et al.*, 1972; Schellman, 1974;

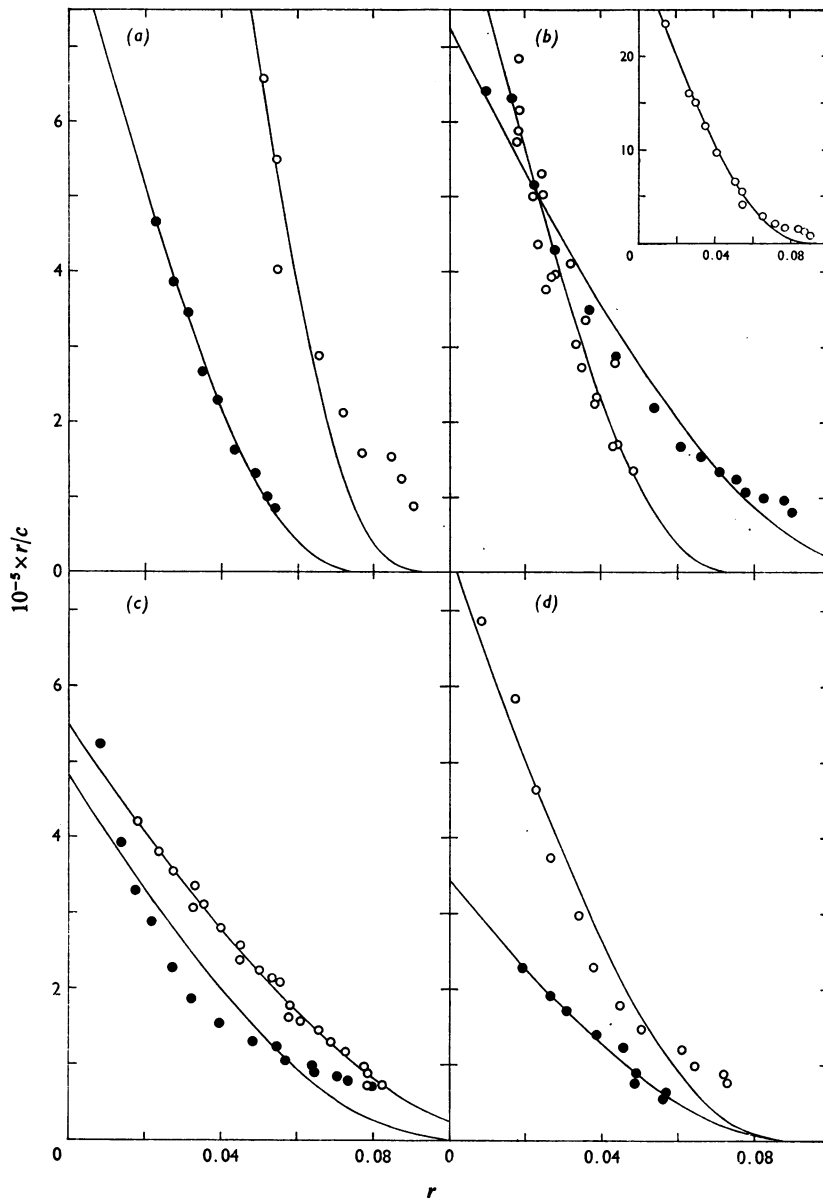


Fig. 2. Effects of base composition on the binding of echinomycin to DNA

The data are presented in the form of the Scatchard (1949) plot, where r is the binding ratio (echinomycin molecules bound per nucleotide) and c is the free antibiotic concentration. The buffer was 0.01 SHE ($I = 0.01$ mol/litre). (a) *M. lysodeikticus* DNA (\circ) and *Ser. marcescens* DNA (\bullet). (b) *E. coli* DNA (\circ ; results derived from experiments with three independent preparations of DNA from *E. coli* B) and *Salmonella typhimurium* DNA (\bullet); inset: data for *M. lysodeikticus* DNA plotted on a different scale in order to show values of r/c at low values of r . (c) calf thymus DNA (\circ) and *Proteus mirabilis* DNA (\bullet). (d) Bacteriophage T2 DNA (\circ) and *Clostridium perfringens* DNA (\bullet). The curves are theoretical, computed to fit eqn. (10) of McGhee & Von Hippel (1974), the values of $K(0)$ and n listed in Table 1 being used.

McGhee & Von Hippel, 1974; J. Bresloff & D. M. Crothers, personal communication). All of these treatments have advantages and limitations related to the underlying assumptions as to whether the lattice may be considered as a linear array of homogeneous or heterogeneous potential binding sites, and the extent of ligand–ligand interaction, whether co-operative or anti-co-operative (or both) in character. Broadly speaking, the more general the treatment the more complex become the equations, with consequent demand for larger amounts of accurate experimental data if all the relevant parameters are to be evaluated. For practical purposes, though necessarily involving some compromise from the ideal, we have found the treatment of McGhee & Von Hippel (1974) most convenient. These authors have developed exact and relatively simple equations describing the binding of both interacting and non-interacting ligands to a homogeneous one-dimensional lattice. Their mathematical approach is based on simple conditional probabilities and the equations are derived in terms of ligand site size n (number of nucleotides occluded per bound ligand molecule), intrinsic association constant to an isolated site $K(0)$, and ligand–ligand co-operativity. This treatment has shown that a curved Scatchard plot is inevitable if the bound drug molecule occupies more than one base pair on the DNA.

The theoretical curves drawn in Fig. 2 were calculated according to eqn. (10) of McGhee & Von Hippel (1974), by using values for the parameters $K(0)$ and n as listed in Table 1. In several instances the fit is quite good (*Ser. marcescens*, *E. coli*, calf thymus and *Cl. perfringens* DNA), whereas for the remainder it varies from moderate to poor. The discrepancy appears most marked for *P. mirabilis* DNA. It is not clear why this should be so; there is no reason to believe that this particular DNA preparation differed markedly from the others with respect to its purity or native condition; thus it may be that in *P. mirabilis* DNA there is a substantial sequence-related heterogeneity of binding sites (the classical explanation for systematic deviation from theoretical expectation in Scatchard plots). It is also evident that there is no simple correlation between the gross base composition of the DNA and its parameters for binding echinomycin. There seems to be a trend for the DNA species richer in G+C to bind the antibiotic more tightly than those rich in A+T; the binding constant for *M. lysodeikticus* DNA is much the highest, and exceeds that for the A+T-rich DNA of *Cl. perfringens* by almost an order of magnitude. However, although the association constants for the other natural DNA species lie between these limits, they do not reveal any monotonic dependence on the base composition. Moreover, there is a clear difference between the results for *E. coli* DNA and those for *Sal. typhimurium* DNA, yet these two DNA

species share the same overall base composition of 50% G+C. On the other hand, the parameters for interaction with calf thymus and bacteriophage PM2 nicked circular DNA (both 42% G+C) are almost the same, notwithstanding the extreme difference in their phylogenetic origins. Comparing the values of n for the different natural DNA species, it is evident that the number of binding sites available to the antibiotic does not vary in any systematic way with the G+C content, but lies within the range 9.5 ± 2.4 nucleotides per binding site. Important in this respect is the result for coliphage T2 DNA, which contains glucosylated 5-hydroxymethylcytosine residues. The substantial occlusion of the major groove of the helix in this DNA caused by the presence of the sugar substituents evidently does not affect the frequency of binding sites, since the observed value of n is close to the average; in fact the frequency of sites in bacteriophage T2 DNA is significantly higher than in the DNA of its host *E. coli* (Table 1).

Effect of ionic strength

It is a common observation that the binding of small molecules to DNA is diminished by raising the ionic strength of the medium (e.g. Waring, 1965*b*; Le Pecq & Paoletti, 1967; Müller & Crothers, 1968). The same is true of echinomycin, though the effect is not entirely straightforward. Scatchard plots for the interaction with calf thymus DNA at ionic strengths 10- and 50-fold higher than that of the standard 0.01 SHE buffer are presented in Fig. 3, and the resultant binding parameters are included in Table 1. The association constant falls to about one-quarter when I is raised to 0.1 mol/litre, but thereafter it changes much less with a further fivefold increase in I . The corresponding changes in the apparent frequency of binding sites are peculiar; the value of n seems first to fall (indicating an almost exactly 50% increase in the frequency of binding sites) and then to return to much the same value as seen at the lowest ionic strength. The Scatchard plot at $I = 0.1$ mol/litre appears much more nearly linear than the others presented thus far. In a further series of experiments, binding curves were determined for bacteriophage PM2 closed circular DNA at ionic strengths 0.01, 0.1 and 0.5 mol/litre (results not shown). Again there was evidence of a decrease in the association constant with increasing ionic strength, but, because of the additional complications introduced as a result of the associated variations in superhelix density, the Scatchard plots were highly curved (cf. Fig. 8) and no meaningful binding parameters could be determined.

The fact that the binding parameters vary at all with salt concentration may appear surprising, since echinomycin itself is uncharged at pH 7. However, there is a precedent in the findings by Müller & Crothers (1968) for the DNA-binding of actinomycin,

Table 1. *Parameters of interaction between echinomycin and nucleic acids*

The experimental data used for calculation of parameters for bacterial DNA species, calf thymus DNA at $I = 0.01$ mol/litre and 20°C , and bacteriophage T2 DNA are shown in Fig. 2. Those for calf thymus DNA at $I = 0.1$ and 0.5 mol/litre at 20°C are shown in Fig. 3. Those for the three synthetic polynucleotides are shown in Fig. 6, and those for nicked circular DNA from bacteriophage PM2 in Fig. 8. The parameters recorded for binding to heat-denatured calf thymus DNA and bacteriophage fd DNA (single-stranded circles) were calculated from data presented previously (Waring & Wakelin, 1974). The method for computation involved an iterative procedure designed to satisfy eqn. (10) of McGhee & Von Hippel (1974):

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

given the experimentally determined values of r and c and an initial guess of n , the number of nucleotides occupied by a bound antibiotic molecule. The program (devised by Dr. J. D. McGhee and operated by Dr. G. Ughetto) was made to recycle until $K(0)$ and n changed by less than 1%, and then to print out the final values of $K(0)$ and n together with a calculated binding isotherm at 5%-saturation increments. The calculated isotherm was then checked for 'goodness of fit' to the original data; examples are shown in Figs. 2, 3, 6 and 8. It should be noted that the binding (association) constant $K(0)$ calculated by this procedure is the intrinsic binding constant to an isolated site, given by the intercept of the calculated curve on the axis of r/c (McGhee & Von Hippel, 1974). In the corresponding treatment of Scatchard (1949) this intercept would be interpreted as K/n . In addition, n as defined here is the reciprocal of the binding-site parameter which results from Scatchard (1949) analysis.

DNA	G + C (%)	I (mol/litre)	Temperature ($^\circ\text{C}$)	$K(0)$ ($10^{-5} \times \text{M}^{-1}$)	n
Calf thymus	42	0.01	20	5.48	7.17
Calf thymus	42	0.01	30	4.62	6.60
Calf thymus	42	0.01	40	3.96	6.68
Calf thymus	42	0.10	20	1.47	4.77
Calf thymus	42	0.10	30	1.14	5.56
Calf thymus	42	0.10	40	1.01	2.76
Calf thymus	42	0.50	20	1.04	6.57
Calf thymus (heat-denatured)	42	0.01	20	1.40	19.49
Bacteriophage fd	42	0.01	20	1.21	11.37
<i>M. lysodeikticus</i>	72	0.01	20	31.0	9.85
<i>Ser. marcescens</i>	58	0.01	20	8.71	11.61
<i>E. coli</i>	50	0.01	20	9.80	11.88
<i>Sal. typhimurium</i>	50	0.01	20	7.38	7.58
Bacteriophage PM2 (nicked)	42	0.01	20	5.07	7.75
<i>P. mirabilis</i>	40	0.01	20	4.79	8.69
Bacteriophage T2	34	0.01	20	7.78	9.97
<i>Cl. perfringens</i>	30	0.01	20	3.44	9.46
Poly(dG)·poly(dC)	100	0.01	20	15.1	9.09
Poly(dG-dC)	100	0.01	20	5.53	5.48
Poly(dA-dT)	0	0.01	20	3.12	5.65

also uncharged at neutral pH. The situation is obviously somewhat complex, but is probably attributable to a combination of several factors related to the influence of electrostatic interactions between negatively charged phosphate groups along the DNA backbone. These interactions must be influenced by conformational alterations induced in the helix by the binding process, and would be sensitive to qualitative changes in the character of the interaction at moderate to high ionic strength, as is discussed below.

Binding of echinomycin to DNA persists at very high ionic strength, as indicated by buoyant-density shifts in CsCl gradients (Fig. 4). The interaction must

be severely weakened in concentrated salt solution, however, since the buoyant density does not reach a saturation plateau until antibiotic/nucleotide ratios above about 0.3 are used, whereas binding curves at lower ionic strengths reach effective saturation at antibiotic/nucleotide ratios of 0.1 or less. The shift in buoyant density at saturation (-25 mg/ml) may be compared with the values reported for several antibiotics and drugs by Kersten *et al.* (1966); it is roughly comparable in magnitude with that exhibited by olivomycin and daunomycin, but 2-3-fold less than was found for nogalamycin, cinerubin, mithramycin and chromomycin. It is much smaller than the large decrease (-160 mg/ml) in SV-40-virus nicked

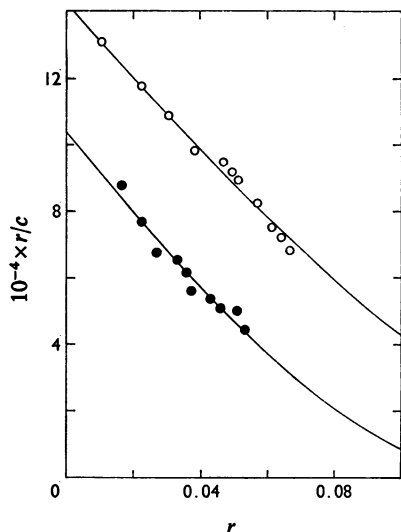


Fig. 3. Scatchard plots for the interaction between echinomycin and calf thymus DNA at $I = 0.1$ (○) and 0.5 (●) mol/litre

circular DNA caused by binding of ethidium reported by Bauer & Vinograd (1968). These comparisons do not enable conclusions to be drawn about the mechanism of interaction, however, because the antibiotics studied by Kersten *et al.* (1966) include non-intercalators as well as intercalators (Waring, 1970), whose relative effects on the buoyant density do not correlate simply with the mode of binding.

In the experiment illustrated in Fig. 4 considerable efforts were made to compare the buoyant-density shifts for bacteriophage PM2 closed and nicked circular DNA, as was done for ethidium and SV-40-virus DNA by Bauer & Vinograd (1968), but, despite repeated attempts with different preparations of closed circles, the presence of echinomycin [D/P (see Fig. 4 for definition) 0.02–0.05] invariably resulted in the appearance of two bands in the CsCl gradients containing roughly equal amounts of DNA. It seems most likely that this splitting resulted from nuclease action in the ultracentrifuge cells during the long equilibrium runs. Consistent with this interpretation, the buoyant-density shift of the heavier band was always about the same as was found for nicked circular DNA examined alone (Fig. 4), suggesting that the lighter band consisted of residual intact closed circles that had bound more antibiotic. However, the uncertainty as to the proportion of the added antibiotic bound to the presumed closed circular species precluded exact evaluation of the true shift for this DNA.

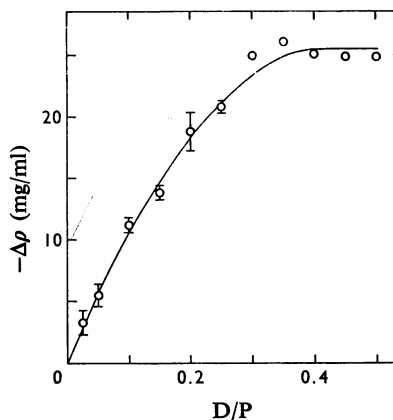


Fig. 4. Effect of echinomycin on the buoyant density of bacteriophage PM2 nicked circular DNA

The solvent was CsCl solution of average density 1.70 g/ml, containing 10 mM-EDTA, pH 7.0. Each band contained $0.3 \mu\text{g}$ of DNA. The abscissa (D/P) represents the ratio of added echinomycin to DNA nucleotides; the ordinate shows the decrease in buoyant density. Three experiments were performed at D/P ratios up to 0.25; the points plotted are averaged over the three determinations, with bars indicating s.e.m.

Effect of temperature

To investigate the thermodynamics of echinomycin–DNA interaction, binding curves were determined for calf thymus DNA at 30° and 40°C (Table 1). At $I = 0.01$ mol/litre there was a steady decrease in $K(0)$ with increasing temperature, but the frequency of binding sites remained practically unaffected. When the experiments were repeated at $I = 0.1$ mol/litre a more complicated pattern emerged: again there was a decrease in the association constant with rise in temperature, but the value of n appeared to decrease substantially between 30° and 40°C to reach a value much lower than seen under any other condition, ostensibly indicating one binding site per $1\frac{1}{2}$ base-pairs in the DNA. It should, however, be emphasized that the parameter n strictly refers to the number of nucleotides occluded per bound ligand molecule as defined by the theory of McGhee & Von Hippel (1974). It need not necessarily provide an accurate assessment of the number of binding sites as represented by the amount of antibiotic bound at saturation. Indeed, the experimental values of r in the experiment in question did not exceed 0.076. Thus the value of n given in Table 1 should be interpreted with caution, depending as it does on a theoretical treatment which has its limitations (see the Discussion section), but nonetheless there seems to be a significant change in the number and/or character of

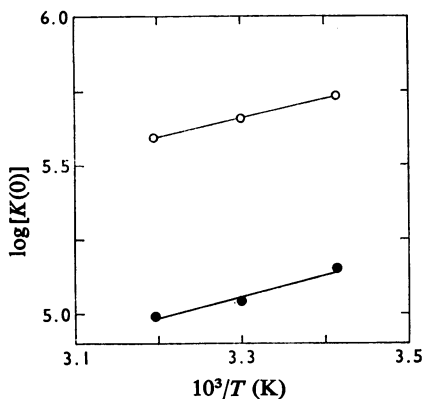


Fig. 5. *van't Hoff plot for binding of echinomycin to calf thymus DNA*

The ordinate shows the log of the association constant (Table 1) and the abscissa the reciprocal of the absolute temperature. The buffers were SHE of $I=0.01$ (O) or 0.1 (●) mol/litre. The enthalpy of interaction was calculated from the relation

$$-\Delta H = 2.303 Rm$$

where R is the gas constant and m is the gradient of the least-squares line drawn through the data points.

binding sites between 30° and 40°C at $I=0.1$ mol/litre which we cannot at present explain.

A *van't Hoff* plot derived from the binding constants is shown in Fig. 5. It yields the following enthalpies of interaction: at $I=0.01$ mol/litre, $\Delta H = -12.6$ kJ/mol (-3.0 kcal/mol); at $I=0.1$ mol/litre, $\Delta H = -13.0$ kJ/mol (-3.1 kcal/mol). The corresponding entropy changes are calculated to be $\Delta S = +16$ e.u. and $+13$ e.u. at $I=0.01$ and 0.1 mol/litre respectively. These estimates are substantially different from those that we reported previously on the basis of least-squares straight lines fitted to the Scatchard-plot data (Waring & Wakelin, 1974); in particular the negative enthalpy at $I=0.01$ mol/litre contrasts sharply with the previous estimate that ΔH was close to zero at low ionic strength. This discrepancy highlights the extreme sensitivity to the precise treatment of Scatchard plots when thermodynamic parameters are derived. In future work it is likely that reliable estimates of ΔH and ΔS will have to depend on direct determinations of ΔH by microcalorimetric methods, as used for other DNA-binding drugs by Quadrifoglio *et al.* (1974), though with echinomycin its very low water-solubility poses a severe experimental limitation which cannot readily be circumvented with existing techniques. For the present it is noteworthy that the calculated ΔH for echinomycin-DNA interaction is moderately negative, as is the measured ΔH for binding of a

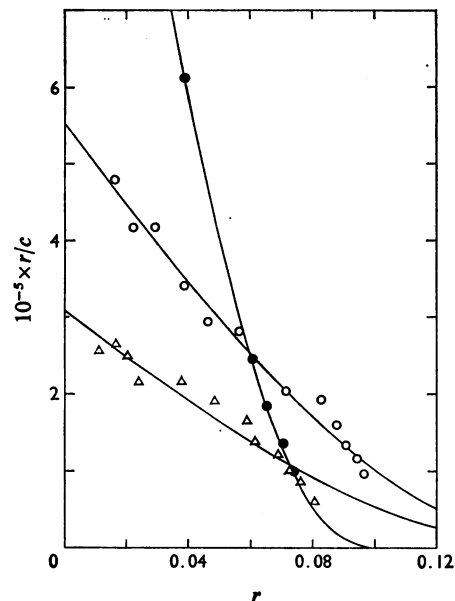


Fig. 6. *Scatchard plots for the interaction between echinomycin and synthetic polydeoxynucleotides*

The buffer was 0.01 SHE ($I=0.01$ mol/litre). As in Figs. 2 and 3 the curves are theoretical, based on the treatment of McGhee & Von Hippel (1974) using the parameters listed in Table 1. Symbols: ●, poly(dG)·poly(dC); O, poly(dG-dC); Δ, poly(dA-dT).

number of acknowledged intercalating agents, such as ethidium bromide, proflavine (Quadrifoglio *et al.*, 1974) and daunomycin (Quadrifoglio & Crescenzi, 1974), though not quite so large. The ΔH values determined for these three intercalators averaged -27 kJ/mol (-6.5 ± 0.5 kcal/mol) (Quadrifoglio & Crescenzi, 1974). On the other hand it is clearly different from the ΔH for binding of actinomycin, determined to be $+8.4$ kJ/mol ($+2$ kcal/mol) at low values of r (Quadrifoglio & Crescenzi, 1974). It may well be that the calculated ΔH for echinomycin binding, lying as it does between the limiting values for the other drugs, reflects its unique mode of interaction (bifunctional intercalation) combined with its peptide constitution (cf. actinomycin).

Interaction with synthetic polynucleotides

Of the seven helical synthetic polynucleotides tested for interaction with echinomycin, three bound the antibiotic strongly (Fig. 6), whereas the remaining four displayed only weak interaction (Fig. 7). The tightest binding was observed with poly(dG)·poly(dC), whose binding constant was exceeded only by that of *M. lysodeikticus* DNA (Table 1). Poly-

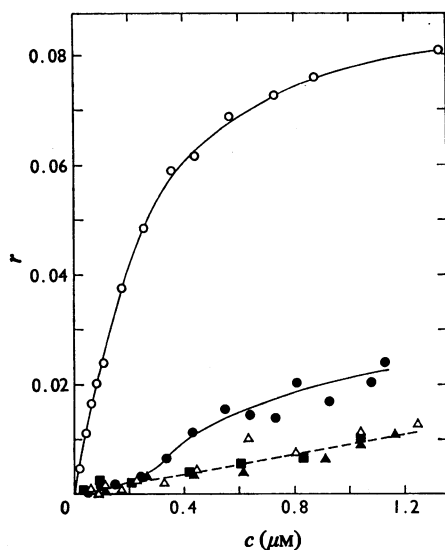


Fig. 7. Interaction between echinomycin and helical polynucleotides composed of A·T, I·C or A·U nucleotide pairs in 0.01 SHE buffer ($I = 0.01$ mol/litre)

Symbols: ○, poly(dA-dT); △, poly(dA)·poly(dT); ●, poly(dI-dC); ▲, poly(dI)·poly(dC); ■, poly(rA)·poly(rU). The curves fitted to the experimental points are not based on any theoretical analysis.

(dG-dC) yielded a binding constant of the same order as found for several of the natural DNA species, and that of poly(dA-dT) was below the range found for all the naturally occurring DNA species in the native state at $I = 0.01$ mol/litre. Surprisingly high extents of binding were attained with the two alternating polydeoxynucleotides [up to r values of 0.081 and 0.097 for poly(dA-dT) and poly(dG-dC) respectively; Fig. 6], reflected in the computed values of n (Table 1), suggesting that the binding capacity of these two polymers is substantially greater than with the natural DNA species. The frequency of sites in poly(dG)·poly(dC), however, is close to the average for natural DNA species.

The weakness of interaction between echinomycin and the I·C polymers poly(dA)·poly(dT) and poly(rA)·poly(rU) is obvious in comparison with the binding curve for poly(dA-dT), itself the least strong of the strongly binding polynucleotides (Fig. 7). It is noteworthy that the slight but significant extents of binding to poly(dA)·poly(dT), poly(dI)·poly(dC) and poly(rA)·poly(rU) are essentially indistinguishable (broken line in Fig. 7), but whether it is significant that all are polypurine-pyrimidine structures we cannot tell. In any event it seems reasonable to ascribe the very limited interaction with these polymers to an essentially non-specific process,

particularly since one of them is a ribopolymer yet no significant binding to a natural RNA could be detected (Waring & Wakelin, 1974).

The binding curve for poly(dI-dC) presents a puzzle. Up to antibiotic concentrations about 250 nM there is very little interaction, and the experimental points lie close to those determined for the other three weakly interacting polymers. Above this concentration, however, more significant extents of binding are observed, eventually reaching r values at least double those for the other polymers at the same free echinomycin concentration. As a consequence the curve has a distinctly sigmoid appearance, even allowing for the scatter in the experimental points at higher values of r and c . This anomalous result may well be correlated with the unusual physical properties of this helical polymer, as discussed below.

Interaction with circular DNA

The capacity of antibiotics and drugs to remove and reverse the supercoiling of closed circular duplex DNA is now well established as a diagnostic criterion for binding by intercalation (Waring, 1970; Bauer & Vinograd, 1971; Gale *et al.*, 1972; Wakelin & Waring, 1974), though not necessarily exclusively so (Waring & Henley, 1975). The effect of echinomycin on the supercoiling of bacteriophage PM2 DNA at $I = 0.01$ mol/litre has already been reported (Waring & Wakelin, 1974); the antibiotic causes the typical fall and rise in the sedimentation coefficient of the closed circles, together with the usual small, essentially monotonic, decrease in s_{20} of nicked circles, with an equivalence region (complete relaxation of the supercoiling) centred round 0.028 ± 0.004 echinomycin molecule bound per nucleotide. With ethidium bromide under the same conditions, equivalence occurs at 0.051 ± 0.007 drug molecule bound per nucleotide (Waring & Wakelin, 1974). Thus in this solvent the helix-unwinding angle of echinomycin is 1.82 ± 0.30 times that of ethidium. Comparable experiments were performed in buffers of ionic strengths 0.036, 0.1 and 0.5 mol/litre; the results are recorded in Table 2. As the ionic strength is raised the helix-unwinding angle relative to that of ethidium (ϕ_{rel}) changes smoothly from a value close to 2, indicating bifunctional intercalation, to a value nearer 1, indicating monofunctional intercalation, at $I = 0.5$ mol/litre. The high value of ϕ_{rel} at $I = 0.01$ mol/litre was checked by comparison of binding curves for bacteriophage PM2 closed and nicked circular DNA, as was done for ethidium bromide and SV-40 virus DNA by Bauer & Vinograd (1968). The curves were found to cross at $r = 0.027$, confirming the equivalence binding ratio determined in the sedimentation experiment and corresponding to an unwinding angle 1.89 times greater than that of ethidium. The cross-over is best determined from a

Table 2. *Apparent intercalation events per DNA-bound echinomycin molecule*

The data summarize results from experiments illustrated in Figs. 8 and 10, together with others reported previously (Waring & Wakelin, 1974). The buffer of $I = 0.036$ mol/litre was $0.05 M$ -Tris/HCl, pH 7.9 at 20°C . $\phi_{rel.}$ is the helix-unwinding angle per bound echinomycin molecule relative to that of ethidium bromide under the same conditions. The column on the extreme right shows the extension of the helix per bound antibiotic molecule determined in viscosity experiments (Fig. 10) divided by 0.34 nm, i.e. the theoretical extension for a single intercalated aromatic chromophore. Values are means \pm S.E.M.

I (mol/litre)	PM2 DNA				Sonicated calf thymus DNA	
	Equivalence binding ratio from s_{20} plot	$\phi_{rel.}$ from s_{20} plot	Equivalence binding ratio from cross-over	$\phi_{rel.}$ from cross-over	Helix extension (nm)	Extension/ 0.34 nm
0.01	0.028 ± 0.004	1.82 ± 0.30	0.027	1.89	0.634 ± 0.015	1.87 ± 0.05
0.036	0.029 ± 0.005	1.74 ± 0.30	—	—	—	—
0.10	0.036 ± 0.006	1.54 ± 0.25	—	—	0.508 ± 0.016	1.50 ± 0.05
0.50	0.041 ± 0.007	1.18 ± 0.19	—	—	0.394 ± 0.011	1.16 ± 0.03

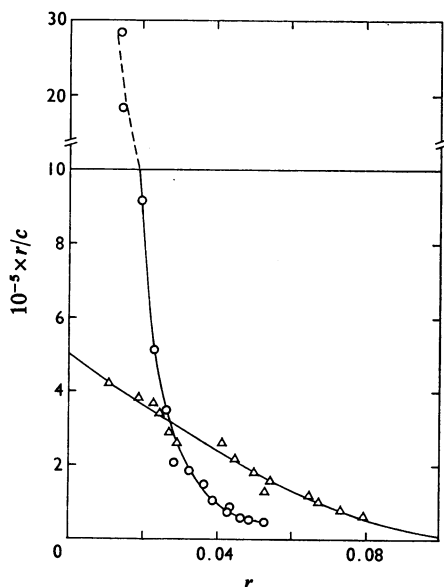


Fig. 8. Scatchard plots for the interaction between echinomycin and closed circular duplex (O) or nicked circular (Δ) bacteriophage PM2 DNA

The solvent was 0.01 SHE buffer, $I = 0.01$ mol/litre. The curve for the nicked circular species is derived from the treatment of McGhee & Von Hippel (1974), based on the parameters listed in Table 1. That drawn through the points for the closed circular species was fitted by eye, not based on any theoretical analysis.

Scatchard plot (Fig. 8), which also illustrates the large enhancement of echinomycin binding to the closed circular species at low values of r owing to its uniquely high helix-unwinding angle, as predicted by the theory of Bauer & Vinograd (1968) and Davidson (1972).

Effects on the viscosity of sonicated DNA fragments

Binding of echinomycin causes a considerable increase in the viscosity of DNA, effectively doubling the reduced viscosity ($\eta_{red.}$) at saturating values of r at low ionic strength (Fig. 9). The intrinsic viscosity of DNA fragments of mol.wt. 5.4×10^5 , determined from the intercept on the ordinate in Fig. 9, is 3.90 dl/g; in the presence of echinomycin at $r = 0.0778$ it is increased to 8.40 dl/g. For both the free DNA and the echinomycin complex the concentration-dependence of $\eta_{red.}$ is small; at DNA concentrations used in subsequent experiments (0.02 and 0.03 g/dl) the difference between the extrapolated intrinsic viscosity and the measured reduced viscosity does not exceed 2.5% . Thus, although the theory developed by Cohen & Eisenberg (1966, 1969) relating changes in viscosity to changes in molecular length strictly speaking requires knowledge of the intrinsic viscosity, the error introduced by substituting reduced viscosity under our conditions should be tolerably small. Because the determination of true intrinsic viscosities at many different values of r would be extremely time-consuming as well as demanding large quantities of echinomycin (not available in unlimited supply), this approximation was judged acceptable.

Fig. 10 shows the calculated changes in molecular length of DNA fragments as a function of echinomycin binding at $I = 0.01$, 0.1 and 0.5 mol/litre. There is evidently no significant dependence on the actual size of the fragments, as shown by the good correspondence between results for fragments of different size in Fig. 10(b). Moreover, the use of reduced viscosities as a basis for calculation is vindicated by the excellent agreement between the filled symbol in Fig. 10(a), derived from true intrinsic viscosities, and the other data points. The slopes of the experimental lines, divided by 2, lead directly to an estimate of the number of intercalation events per bound antibiotic molecule if it is assumed that each intercalated

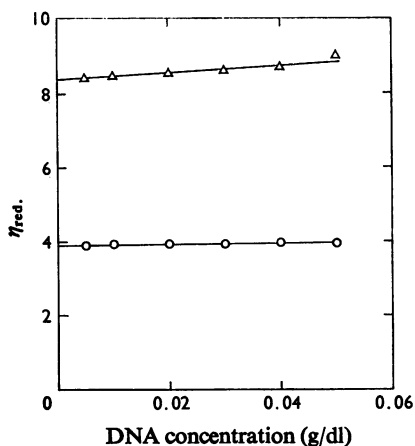


Fig. 9. Determination of intrinsic viscosity for sonicated calf thymus DNA fragments alone (○) or in the presence of echinomycin at $r = 0.0778$ (Δ)

The buffer was 0.01 SHE, $I = 0.01$ mol/litre. The ordinate represents the reduced viscosity. The echinomycin complex was prepared at the highest concentration shown and serially diluted with buffer containing the antibiotic at the free concentration in equilibrium with the complex at $r = 0.0778$ (787 nm, estimated from the binding curve in Fig. 2), to prevent dissociation of the complex caused by simple dilution (Drummond *et al.*, 1966). The lines were drawn by the method of least squares.

aromatic ring system increases the contour length by the thickness of a base-pair; likewise the apparent molecular extension per bound echinomycin may be calculated (Table 2).

Comparison of the results in Table 2 shows that the binding of echinomycin to DNA at low ionic strength proceeds by an essentially bifunctional intercalation mechanism, as judged by the quite independent criteria of helix extension and helix unwinding, the latter estimated by two separate methods (Lerman, 1961; Fuller & Waring, 1964; Gale *et al.*, 1972). In addition, the bifunctional mode of reaction observed at low ionic strength is smoothly converted towards more nearly monofunctional interaction by increasing the salt concentration.

Analogues of the echinomycin chromophores

The assertion that the two chromophores of echinomycin are capable of simultaneous intercalation, at least at low ionic strength, prompts the question as to whether the capacity to intercalate is a general property of quinoxaline compounds, bifunctional or otherwise. It is known that the single quinoline ring system of chloroquine, a well-established antimalarial drug, is an effective inter-

calator (O'Brien *et al.*, 1966; Waring, 1970, 1975). The simplest analogue of the echinomycin chromophores is quinoxaline-2-carboxamide. When tested for possible effects on the sedimentation of bacteriophage PM2 DNA, this compound failed to affect the s_{20} of either closed or nicked circles at molar ratios up to 1:1. Two further tests were applied to verify that quinoxaline-2-carboxamide does not interact detectably with DNA in 0.01 SHE buffer at 20°C. There was no detectable perturbation of the u.v.-absorption peaks of the compound at 245 and 320 nm even in the presence of a 75-fold molar excess of calf thymus DNA. Equilibrium dialysis performed by the method of Müller *et al.* (1973) also failed to reveal perceptible interaction with calf thymus DNA; in these experiments the DNA concentration was held constant at 91 μ M and the concentration of quinoxaline-2-carboxamide was varied to yield ligand/nucleotide ratios in the range 0.12–1.07 after equilibrium (20 h stirring at 20°C). The compound crossed the dialysis membrane readily, without significant adsorption, to become equally distributed between the cell compartments within the limits of sensitivity imposed by spectrophotometric detection. These experiments effectively eliminated any interaction characterized by a binding constant greater than $2 \times 10^3 \text{ M}^{-1}$.

A second compound tested as a potential analogue for the chromophore moieties of echinomycin was Bayer 7602, a drug which displays trypanocidal activity (Gutteridge, 1976). It contains two substituted quinoline chromophores, presumably not dissimilar in size to those of echinomycin, linked by a relatively simple chain such that the whole molecule has a central twofold axis of rotational symmetry (Fig. 11). This drug is certainly capable of binding to DNA: its absorption spectrum undergoes a bathochromic and hypochromic shift in the presence of calf thymus DNA and the thermal-denaturation profile of the DNA is shifted to substantially higher temperatures in 0.01 SHE buffer ($\Delta T_m = 15^\circ\text{C}$ at D/P 0.25; L. P. G. Wakelin, unpublished work). Its effects on the sedimentation behaviour of bacteriophage PM2 DNA are, however, much weaker than those of echinomycin. The supercoiling of the closed circles is progressively diminished, but apparently complete relaxation is not attained until D/P ratios in excess of 0.2 are used (Fig. 11). Addition of further amounts of drug does not lead to reversal of the supercoiling; instead, a gradual precipitation takes place, which is substantially complete at D/P ratios of 0.5 or more. This precipitation no doubt reflects the formation of an electrostatically neutral complex due to protonation of the amino-quinoline ring nitrogen atoms at neutral pH. In any event it is clear that this drug is far from analogous to echinomycin with respect to its interaction with circular DNA, and therefore the strong bifunctional effect of echinomycin under the same conditions cannot simply be attributed to the

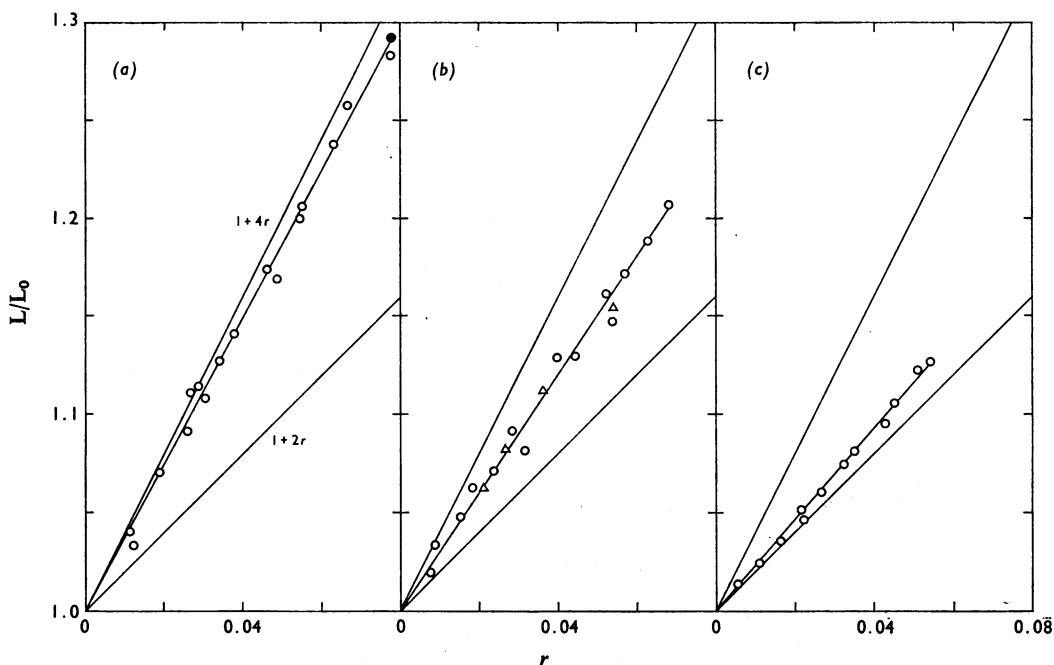


Fig. 10. Effect of echinomycin on the relative contour length of sonicated calf thymus DNA fragments in SHE buffers of 1 0.01 mol/litre (a), 0.1 mol/litre (b) and 0.5 mol/litre (c)

The ordinate represents the calculated contour length in the presence of echinomycin L as a ratio of the length in the absence of antibiotic L_0 (Cohen & Eisenberg, 1966, 1969). \circ , \bullet , Results with fragments of average M_r 5.4×10^5 ; \triangle , results determined with fragments of average M_r 3.8×10^5 . Each panel contains reference lines corresponding to the relations $L/L_0 = 1 + 4r$ or $1 + 2r$. 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 correspond to $1 + (3.73 \pm 0.09)r$ (a), $1 + (2.99 \pm 0.09)r$ (b) and $1 + (2.32 \pm 0.05)r$ (c). \bullet was evaluated from the change in true intrinsic viscosity at $r = 0.0778$ (Fig. 9).

presence of a pair of two-ring chromophores linked within one molecule, regardless of the nature and stereochemical properties of that linkage.

Discussion

The first objective of the present experiments was to investigate possible selectivity in the interaction between echinomycin and polydeoxynucleotides. Selectivity undoubtedly exists: it is most striking with respect to the results for the synthetic polymers, three of which bound the antibiotic well, whereas the other four showed little more than weak, probably non-specific, interaction (Figs. 6 and 7). However, among the nine natural double-helical DNA species tested there were also significant differences which point to the existence of distinct preferences in the binding of echinomycin. The interpretation of these differences is to a large extent dependent on the confidence that can be placed in the reliability of the McGhee & Von Hippel (1974) treatment of the Scatchard plots. The

principal objection to this treatment is that it considers the DNA molecule as a homogeneous one-dimensional lattice of potential binding sites, each of which is characterized by the same intrinsic binding constant $K(0)$. This condition is adequately satisfied in the experiments with the synthetic polydeoxynucleotides, but is probably not the case where natural DNA species are used, as the experimental data in Fig. 2 clearly imply. However, this same objection applies to the other available methods of treatment, at least in their simplest forms, and an equation formally equivalent to eqn. (10) of McGhee & Von Hippel (1974) is the common denominator of most if not all of them. In effect, by applying this treatment to natural DNA species one attempts to determine mean binding parameters, averaged over the microscopic binding constants for all possible types of site. The resulting values of $K(0)$ and n then reflect, albeit crudely, the relative abundances of potential binding sites of different affinity for the ligand. Some justification for this approach may be

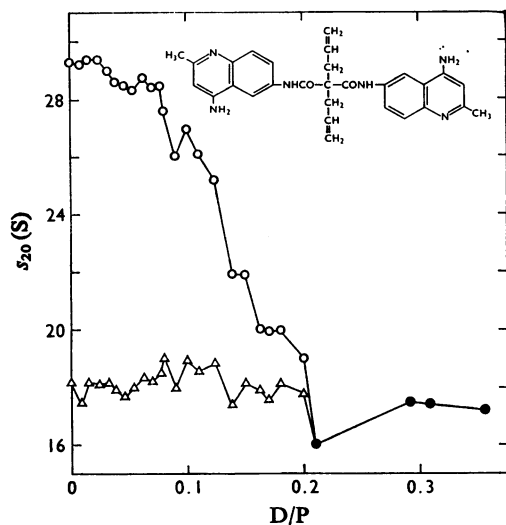


Fig. 11. Effect of Bayer 7602 on the sedimentation coefficient of bacteriophage PM2 DNA

The solvent was 0.01 SHE buffer ($I = 0.01$ mol/litre). The DNA preparation contained 85–90% closed circular duplex molecules (s_{20} represented by \circ); the s_{20} of the nicked circles is represented by Δ , and when the two components co-sedimented as a single unresolved boundary the symbol \bullet is plotted. Bayer 7602, dissolved in 0.01 SHE buffer, was added to 0.6 ml of the DNA ($91 \mu\text{M}$ with respect to nucleotides) by method 2 of Waring (1970) to generate the ligand/nucleotide ratios shown on the abscissa. Sedimentation coefficients are uncorrected values determined directly at 20°C . The formula of Bayer 7602 is shown.

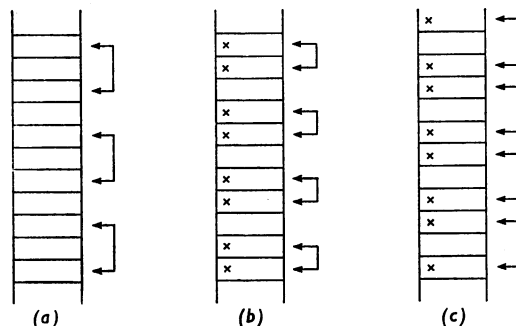


Fig. 12. Schematic illustration of bifunctional interaction between echinomycin and a helical polydeoxynucleotide

It is assumed that the antibiotic occludes four (a) or three (b, c) nucleotide pairs per bound molecule. The ladder represents the DNA helix and the antibiotic molecule is shown as a vertical line with its chromophores as horizontal arrowed lines. (a) represents the situation in which two base-pairs are sandwiched between the intercalated chromophores and four successive nucleotide pairs constitute a binding site. In (b) and (c) the binding site contains only three nucleotide pairs and the number of base-pairs sandwiched between the chromophores is either one (b) or two (c). Either of cases (b) and (c) necessitates violation of the neighbour-exclusion principle, and the offending lattice positions are marked with a cross. If the number of base-pairs sandwiched between the chromophores is three, the three-nucleotide-pair binding site could not exist (unless two chromophores on different antibiotic molecules could intercalate at the same place, which is assumed to be impossible) and violations similar to those shown in (c) would occur for a four-nucleotide-pair binding site.

gained from the good fit of the computed curves to the experimental points for most of the DNA species in Fig. 2; in any event the treatment is far superior to the estimation of binding parameters from straight lines fitted to the data from low r up to some arbitrarily chosen point of unacceptable curvature.

In general it would appear that the antibiotic has highest affinity for polymers rich in guanine and cytosine nucleotides. This is certainly so among the synthetic polydeoxynucleotides tested, where $K(0)$ decreases in the order $\text{poly}(\text{dG}) \cdot \text{poly}(\text{dC}) > \text{poly}(\text{dG-dC}) > \text{poly}(\text{dA-dT})$. However, it should be noted that the value of $K(0)$ estimated for *M. lysodeikticus* DNA (72% G+C) is twice that for $\text{poly}(\text{dG}) \cdot \text{poly}(\text{dC})$, suggesting that the base sequence (or sequences) generating the optimum conditions for binding of echinomycin may contain all four nucleotides. Further experiments with more complicated polymers containing all four common nucleotides in defined sequence would be required to clarify this point. With other DNA-binding drugs, i.e. actino-

mycin D and ethidium bromide, spectral changes in the presence of simple dinucleotides have thrown light on sequence preferences (Krugh, 1972; Krugh *et al.*, 1975), but thus far all attempts to detect perturbation of the near-u.v. absorption of echinomycin in the presence of such compounds have failed.

Whatever specificity exists in the interaction between echinomycin and nucleic acids, it must ultimately depend on the capacity to recognize distinct nucleotide sequences, either by direct interaction between functional groupings on the base-pairs and the antibiotic molecule or indirectly via recognition of a local conformational peculiarity of the DNA associated with a particular sequence. In this respect, a major difficulty in interpreting synthetic polynucleotide-binding data lies in evaluating and differentiating consequences genuinely arising from different sequential arrangements of nucleotide pairs from those that reflect gross differences in the helical structures of the polymers. The best-defined structures of synthetic polynucleotides used in this

work have come from X-ray-diffraction studies of oriented fibres. Arnott *et al.* (1974) have shown that DNA polymers containing alternating purine and pyrimidine sequences [i.e. poly(dI-dC), poly(dA-dT) and poly(dG-dC)] can assume a right-handed eight-fold helical form with an axial rise per residue of 0.303 nm. The furanose rings have the standard C-3'-*exo* conformation, but the bases are positioned unusually with respect to the helix axis such that they have a backward tilt. In addition, the duplex is narrower than is typical for B-type DNA, which results in the phosphate groups being about 0.1 nm closer to the helix axis. Similar results for these alternating co-polymers have been obtained by Mitsui *et al.* (1970) and Grant *et al.* (1972). It may be concluded that the structures adopted by these polymers are related to the B-type helix. In addition, Davies & Baldwin (1963) have shown that poly(dA-dT) can give rise to X-ray patterns that are virtually identical with those observed for natural DNA.

The echinomycin-binding capacity of these three polynucleotides is strikingly different, in contrast with the similarity of their proposed fibre structures. However, the c.d. (circular-dichroism) spectra of these polymers do in fact reveal differences in their solution configurations. The c.d. spectrum of poly(dA-dT) appears to be typical of natural DNA (Wells *et al.*, 1970), whereas those of poly(dI-dC) and poly(dG-dC) are not (Mitsui *et al.*, 1970; Pohl & Jovin, 1972). At low ionic strength, poly(dI-dC) has an inverted spectrum, with the longest-wavelength band being negative; a similar anomalous spectrum has been obtained for poly(dG-dC) in 2.5M-NaCl (Mitsui *et al.*, 1970; Pohl & Jovin, 1972). Arnott *et al.* (1974) have postulated that these inverted c.d. spectra are related to the backward tilt of the bases as described in their molecular model. The low-ionic-strength conditions of our binding experiments may result in poly(dA-dT) and poly(dG-dC) having similar solution configurations which permit strong interaction with echinomycin. In contrast, the configuration of poly(dI-dC) under these conditions is evidently different (Mitsui *et al.*, 1970), resulting in only weak and peculiar binding of the antibiotic.

Arnott & Selsing (1974a) have proposed a structure for poly(dA)·poly(dT) based on X-ray fibre-diffraction patterns in which the molecule exists as a 10-fold right-handed helix with an axial rise of approx. 0.33 nm per nucleotide. The structure is very similar to B-type DNA, but the molecule differs from natural DNA in that it will not undergo the B → A transition. Langridge (1969) found identical diffraction patterns for poly(dA)·poly(dT) and poly(dI)·poly(dC), which led to the same conclusion that these polymers adopt the 10-fold B-type helix. The c.d. spectra of these two polymers are not identical but are typical of natural DNA (Mitsui *et al.*, 1970; Wells

et al., 1970). Thus, although these polymers adopt helical structures closely related to that of natural DNA, they exhibit extremely feeble interaction with echinomycin.

The association of echinomycin with poly(rA)·poly(rU) is likewise barely detectable, which is in accord with the inability of the antibiotic to bind to rRNA (Waring & Wakelin, 1974). In marked contrast, the polymer with the strongest affinity for echinomycin is poly(dG)·poly(dC), which has been shown by Arnott & Selsing (1974b) to give rise to fibre-diffraction patterns typical of A-type DNA. Further, the polynucleotide would not readily adopt the B-type configuration when the humidity and salt concentration were raised. The c.d. spectrum of this polymer is inconsistent with the theoretical spectrum derived for similar sequences in natural DNA and suggests that the solution structure is not identical with that of naturally occurring DNA (Wells *et al.* 1970; Allen *et al.*, 1972). In view of the tendency of poly(dG)·poly(dC) to form the A-type helix, and the similarity of this structure to the RNA helix, its ability to form such a tight complex with echinomycin is quite striking.

It is apparent from these considerations that the pattern of echinomycin binding to synthetic polynucleotides does not correlate with their gross helical structures, so far as they are understood. Notwithstanding the remaining uncertainty about solution structures of these polydeoxynucleotides, it is difficult to see how sites in natural DNA species having differential affinities for binding echinomycin could arise due to local peculiarities of conformation originating from clustering of nucleotide pairs of a given type, though there is evidence that such peculiarities may exist (Bram, 1971a,b; Bram & Tougaard, 1972). It would appear more likely that sequence-dependence of the binding constant for echinomycin may be related to the nature and order of functional groupings on the DNA helix, especially hydrogen-bonding groups (such as the 2-amino group of guanine), which would be placed in close proximity to the octapeptide ring of the antibiotic molecule.

The variation in apparent ligand site size n between different polymers (Table 1) presents a much more confusing picture. For the natural DNA molecules and for poly(dG)·poly(dC) at $I=0.01$ mol/litre, n averages 9.5 nucleotides per binding site, indicating that each bound echinomycin molecule occludes approx. five base-pairs along the DNA helix. This may be compared with the estimate of about six base-pairs per binding site for actinomycin D (Müller & Crothers, 1968; Sobell *et al.*, 1971). However, there is substantial variation in the calculated values of n and, for three DNA species having G+C contents in the range 42–50% (calf thymus, *Sal. typhimurium* and bacteriophage PM2 nicked DNA), it indicates a site size of somewhat less than four nucleotide pairs

(Table 1). Still lower values result when the temperature and/or ionic strength are raised, and also in the interaction with the alternating polymers poly(dG-dC) and poly(dA-dT). In view of the evidence that the mechanism of binding changes to what may be called a sesquifunctional ($1\frac{1}{2}$ -fold) mode of intercalation at $I = 0.1$ mol/litre (Fig. 10 and Table 2), it is tempting to ascribe the fall in n at this ionic strength to the altered type of binding: if half of the bound antibiotic molecules are only monofunctionally intercalated they might be able to pack, on average, more closely together along the helix. But this would not explain why the trend in n is not continued when the ionic strength is raised to 0.5 mol/litre and the binding becomes more nearly monofunctional in character (cf. Tables 1 and 2), and the anomalously low value of n at $I = 0.1$ mol/litre at 40°C remains quite inexplicable.

It should, in principle, be easier to place confidence in the calculated values of n for poly(dG-dC) and poly(dA-dT), since these polymers correspond better to the homogeneous one-dimensional array of potential sites required by the model of McGhee & Von Hippel (1974). As a check on the values of n for these polymers and for poly(dG)·poly(dC), the original data were recalculated according to the statistical-mechanical treatment of Zasedatelev *et al.* (1971). Again, the best agreement between theory and experimental points occurred with three base-pairs per binding site for the alternating polymers and four to five base-pairs per site for poly(dG)·poly(dC) (Wakelin, 1974). These results highlight an important issue concerning the neighbour-exclusion hypothesis of intercalative binding to DNA. If it can be assumed that the interaction between echinomycin and these polymers occurs by the same bifunctional mechanism as it does with calf thymus or bacteriophage PM2 DNA under these experimental conditions, then a serious conflict with the principle of neighbour exclusion arises for the alternating polydeoxynucleotides (Fig. 12). Despite uncertainty as to the number of base-pairs sandwiched between the intercalated chromophores of an echinomycin molecule, the minimum site size required to be compatible with neighbour exclusion is four nucleotide pairs, not three. This dilemma is unlikely to be resolved until more concrete information is available on three points. (1) The bifunctional character of echinomycin binding to poly(dG-dC) and poly(dA-dT) needs to be supported by direct evidence; as yet it is only assumed to be so, on the basis of results with natural DNA molecules in the same solvent. (2) The relation between calculated estimates of site size, based on theoretical models, and true extents of antibiotic binding at saturation needs to be clarified. It was noted above that for the polymers in question the observed extents of binding did not exceed one echinomycin molecule per five base-pairs. It may be that, as high

extents of binding are approached, additional anti-cooperativity effects begin to dominate the interaction parameters; in this respect it is noteworthy that the experimental points for poly(dA-dT) at high r seem to deviate downwards from the theoretical line (Fig. 6), and the same could well be true for the measurements on poly(dG-dC). (3) The number of base-pairs sandwiched between bifunctionally intercalated chromophores will depend on the precise conformation and rigidity of the peptide ring, and there is no reason *a priori* why it need be fixed at any particular value, nor why it need remain constant for interaction with different sites on the same or different polymers. An extensive study of conformational possibilities for the echinomycin molecule, based on van der Waals interaction energies and geometrical conditions for closure of the peptide ring, suggests that there is remarkably little flexibility in the structure of the antibiotic and that the spacing between the roughly parallel chromophores is close to 1 nm, sufficient to accommodate two base-pairs in between (G. Ughetto & M. J. Waring, unpublished work). However, until alternative possibilities are rigorously eliminated or a crystal structure becomes available, conclusions on this point must be held in abeyance.

The second objective of this work, to characterize the principal features of the binding mechanism, rests largely on the data summarized in Table 2. The excellent agreement between the estimates of helix unwinding and increase in DNA contour length on binding echinomycin leaves little room for doubt that the antibiotic intercalates in almost ideal bifunctional fashion at low ionic strength and that a gradual transition towards more nearly monofunctional reaction occurs as the ionic strength is raised. It only remains to attempt to explain this salt-dependence of the binding mechanism. Various authors have considered the effect of salts on ligand-DNA interaction in terms of electrostatic forces, paying particular attention to the role of charged phosphate groups in the polynucleotide backbone (Peacocke & Skerrett, 1956; Waring, 1965*b*; Le Pecq & Paoletti, 1967; Müller & Crothers, 1968). For the binding of cationic ligands, competition with salts as counterions for the phosphate groups may be expected to play a dominant part in dictating the equilibrium and thermodynamic aspects of the interaction (Le Pecq & Paoletti, 1967). However, since echinomycin is uncharged it is most likely that salt effects must originate largely from influences on phosphate-phosphate interactions within the structure of the DNA, as Müller & Crothers (1968) discussed in relation to the binding of actinomycin. Wang (1969) has shown that the winding of the DNA helix itself is subtly influenced by the nature and concentration of salts present, but the magnitude of these alterations seems too small to account for the considerable

quantitative and qualitative changes in echinomycin binding. A more obvious source of explanation for the ionic-strength-dependence of the binding parameters, as well as the transition from bifunctional towards monofunctional reaction, lies in the perturbation of the DNA structure induced by the binding process itself, especially the local unwinding which must necessarily involve relative movement of certain charged phosphate groups with respect to others in the polynucleotide backbones. At present it is impossible to formulate a more precise hypothesis to account for the salt-dependence, but if and when a detailed molecular model (or models) for the various possible intercalated states of echinomycin can be built the issue should be clarified.

The stereochemical relationship which exists between the intercalated chromophores remains an intriguing problem. It is conceivable that the two quinoxaline ring systems interact with DNA completely independently of each other; on the other hand they may bind in some concerted fashion. The ionic-strength-dependence of the unwinding angle and helix extension suggests that the former postulate is certainly feasible. If the mechanism is concerted, as might be expected at low ionic strength, what factors govern the relative orientation and positioning of the chromophores? The key to this question may lie in the (imperfect) twofold rotational symmetry of the antibiotic molecule. If echinomycin interacts with DNA in such a fashion that the determinants of binding are symmetrically related, then one would predict that whatever constraints apply to the intercalation site for one chromophore should apply in antiparallel fashion to the site for the other. This might offer a ready explanation for the origins of its sequence selectivity. The experiments with the synthetic DNA molecules are consistent with this notion, for in every case more antibiotic is bound to the polynucleotide isomer having the base-pairs arranged in a rotationally symmetrical fashion, i.e. the alternating co-polymer. Within the structure of the antibiotic the determinants of binding are clearly elsewhere than on the chromophores, as shown by the failure of quinoxaline-2-carboxamide and Bayer 7602 to mimic the binding of echinomycin to any significant extent. Nevertheless, the role played by the chromophores is far from irrelevant to an understanding of specificity determinants, since their intercalation causes the helix to unwind and extend, and it is in this distorted configuration that interactions between the peptide portion of the antibiotic and substituents in the DNA must be optimized. Such interactions with the undistorted helix may be unfavourable or indeed impossible.

Preliminary experiments with molecular models show that it is possible, at least in principle, to fit an echinomycin molecule into the narrow groove of the helix such that specific hydrogen-bonding contacts

can be made between functional groups on the antibiotic and the nucleotide pairs. The narrow groove appears better suited than the wide groove to provide an acceptable binding site, since it can just neatly accommodate the bulk of the octapeptide ring with numerous contacts between the peptide portion and the deoxyribose phosphate backbones. Some experimental support for choosing the narrow groove to provide binding sites is provided by the strong typical interaction between echinomycin and bacteriophage T2 DNA, whose wide groove is substantially occluded by sugar substituents on the 5-hydroxymethylcytosine residues (Erikson & Szybalski, 1964). More detailed proposals on the exact nature of intermolecular contacts and specificity determinants must await the results of theoretical as well as experimental studies on the conformational properties of echinomycin, together with calculations of geometrical and other constraints imposed by the structure of receptor polynucleotides.

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