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Interaction between Synthetic Analogues of Quinoxaline Antibiotics and Nucleic Acids

CHANGES IN MECHANISM AND SPECIFICITY RELATED TO STRUCTURAL ALTERATIONS

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The interaction with DNA of six chemically synthesized derivatives of the quinoxaline antibiotics was investigated. Five of the compounds bound only weakly to DNA or not at all; for these substances spectrophotometric measurements, sedimentation studies with closed circular duplex bacteriophage-PM2 DNA and thermal-denaturation profiles were used to determine limits for the binding constants. No interaction could be detected with two products of degradation of echinomycin (quinomycin A), one of which, echinomycinic acid dimethyl ester, had the lactone linkages opened, whereas the other retained an intact octapeptide ring but had a broken cross-bridge. The other compounds studied were des-*N*-tetramethyl-triostin A ('TANDEM') and its derivatives. A derivative of 'TANDEM' in which benzyloxycarbonyl moieties replace both quinoxaline chromophores had binding constants to nucleic acids in the range 10^2–10^3 M^{-1}, whereas no interaction could be detected for a benzyloxycarbonyl derivative that, in addition, had the cross-bridge broken. The derivative of 'TANDEM' with L-serine in place of D-serine in both positions showed no detectable interaction with *Clostridium perfringens* DNA, whereas the binding constant to poly(dA-dT) was approx. 2×10^3 M^{-1}. 'TANDEM' itself bound strongly to DNA, and the bathochromic and hypochromic shifts in its u.v.-absorption spectrum in the presence of DNA were similar to those seen with echinomycin. From the effect on the sedimentation coefficient of closed circular duplex bacteriophage-PM2 DNA the mechanism of binding was shown to involve bifunctional intercalation, typical of the naturally occurring quinoxaline antibiotics. Solvent-partition analysis was used to determine binding constants for the interaction between 'TANDEM' and a variety of natural and synthetic DNA species. The pattern of specificity thus revealed differed markedly from that previously found with the naturally occurring quinoxaline antibiotics. Most striking was the evident large preference for (A + T)-rich DNA species, in complete contrast with echinomycin and triostin A. The highest binding constant was found for poly(dA-dT), the interaction with which appeared highly co-operative in character. The conformations adopted by those quinoxaline compounds that bind strongly to DNA were examined with the aid of molecular models on the basis of results derived from n.m.r. and computer studies. It appears that the observed patterns of base-sequence specificity are determined, at least in part, by the structure and conformation of the sulphur-containing cross-bridge.

In the preceding paper (Lee & Waring, 1978), the interaction between DNA and three naturally occurring quinoxaline antibiotics, quinomycin C, triostin A and triostin C, was investigated with a view to elucidating the mechanism and specificity of the DNA-binding reaction. All three antibiotics were found to act as bifunctional intercalating agents, in agreement with the studies on echinomycin at low ionic strength reported earlier (Waring & Wakelin, 1974; Wakelin & Waring, 1976). Furthermore the quinomycin antibiotics were found to share a similar pattern of specificity, which was very different from that shown by triostin A. (The specificity of triostin C was not amenable to investigation because of its very low water-solubility.) In the present paper a series of chemically synthesized quinoxaline compounds have been investigated to elucidate further the relationship between the structure and conformation of a quinoxaline compound and its properties with respect to binding to DNA.

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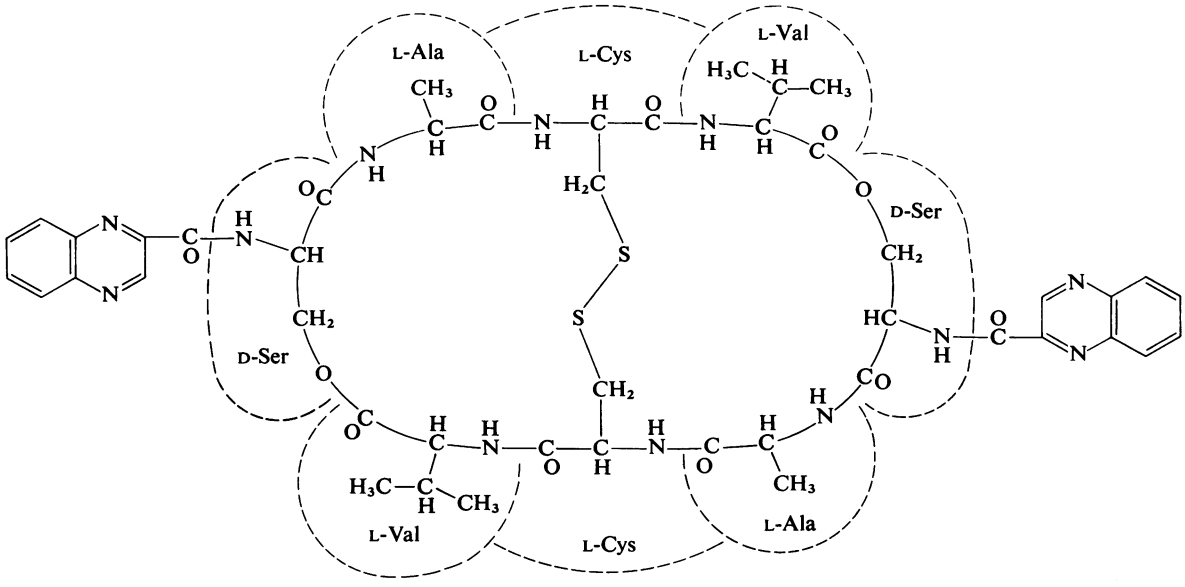
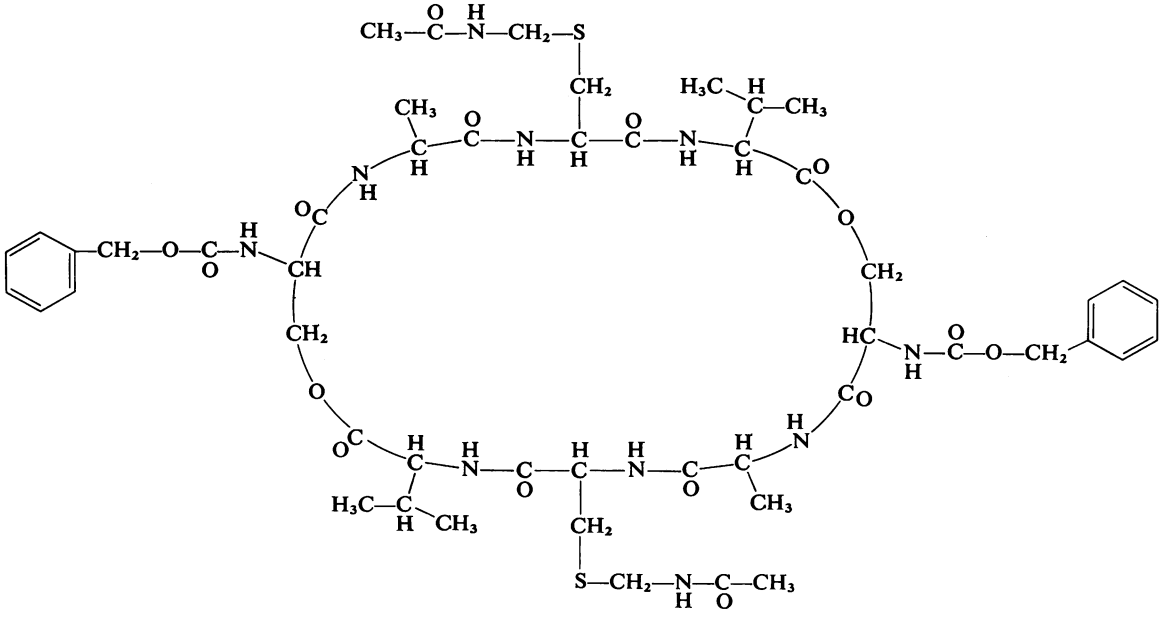


'TANDEM'



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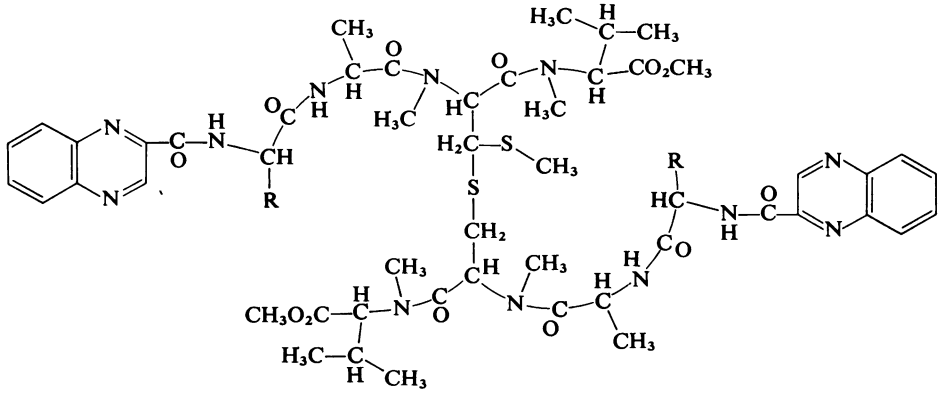
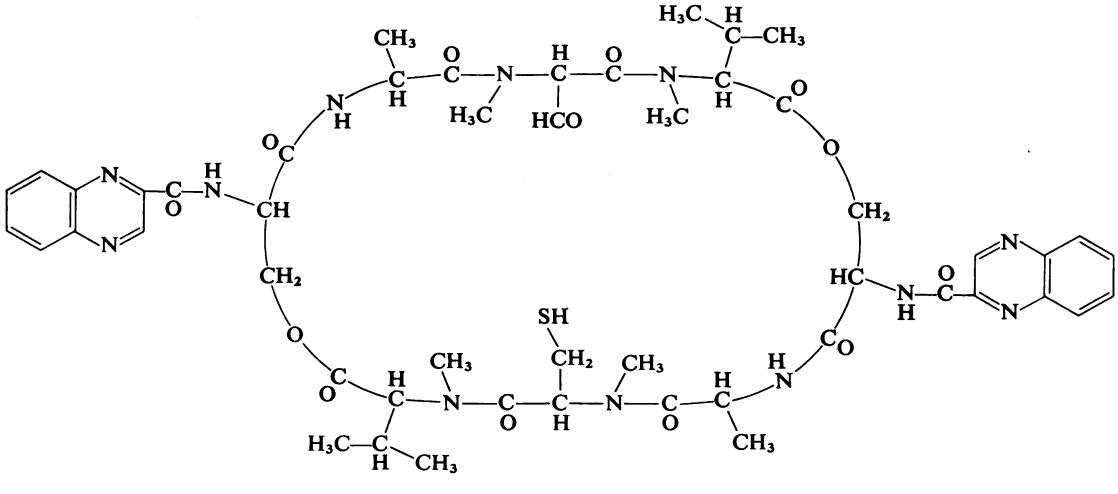


Echinomycinic acid dimethyl ester



Echinomycin split product 1

Fig. 1. *Structural formulae of quinoxaline analogues and derivatives*

In the formula shown for 'TANDEM' the constituent amino acid residues of the peptide ring are indicated; the structure of 'ELSERTA' is identical but for the L-configuration of both serine residues. Not shown is the formula of 'BOCANTA', which has the same cross-bridged octapeptide ring as 'TANDEM', but the serine residues bear benzyloxycarbonyl substituents, as shown for 'ACIMBO'. In the formula of echinomycinic acid dimethyl ester the various possible fates of the exposed serine side-chain are represented by R, which may be $-CH_2OH$, $-CH_2OCH_3$, or a $=CH_2$ moiety (the α-H is missing in this case) representing the $\alpha\beta$-unsaturated amide. The proposed structure of echinomycin split product 1 is not rigorously established (see the text).

Four of the compounds studied here were originally synthesized as part of a programme to investigate the feasibility of achieving the total synthesis of triostin A (Chen *et al.*, 1975; Ciardelli & Olsen, 1977). Des-*N*-tetramethyl-triostin A (designated 'TANDEM') lacks the methylation of four peptide nitrogen atoms in triostin A (see Lee & Waring, 1978), but otherwise is identical in structure. Benzyloxycarbonyl-'TANDEM' (designated 'BOCANTA') has benzyloxycarbonyl substituents in place of both quinoxaline chromophores of 'TANDEM', but its peptide-ring structure remains identical with that of 'TANDEM'. Acetamidomethyl-'BOCANTA' (designated 'ACIMBO') has additional acetamidomethyl protecting groups on the sulphur atoms of the cysteine residues, and thus lacks a bridge across the two halves of the peptide ring (Fig. 1). In the synthesis of 'TANDEM', 'ACIMBO' is formed from peptide fragments. The acetamidomethyl protecting groups are removed to close the cross-bridge and form 'BOCANTA'. The benzyloxycarbonyl protecting groups can then be readily replaced by quinoxaline chromophores to complete the 'TANDEM' molecule (Ciardelli & Olsen, 1977). Finally, an L-serine analogue of 'TANDEM' (designated 'ELSERTA') has L-serine in place of D-serine in both positions of the 'TANDEM' molecule. This modification would be expected to affect the orientation of the chromophores with respect to the peptide ring, since it is to the serine residues that the quinoxaline moieties are attached.

Two further compounds studied were prepared by selective degradation of echinomycin. Echinomycinic acid dimethyl ester has the lactone linkages of the parent antibiotic opened, but the resulting di-acid has been methylated so that the molecule should remain uncharged at neutral pH. The structure is shown in Fig. 1, where R represents the three possible variants resulting from exposure of the serine residues (Dell *et al.*, 1975). These consist of the α,β-unsaturated amide, the derivative with $R = -CH_2OH$ or the derivative with $R = -CH_2OCH_3$. Thus if the asymmetry of the cross-bridge is taken into account there are nine possible components to echinomycinic acid dimethyl ester. The other derivative of echinomycin (designated echinomycin split product 1) has the thioacetal cross-bridge broken, but the lactone linkages remain intact. The proposed structure is also shown in Fig. 1.

It was hoped that investigation of these six compounds would allow a preliminary definition of those structural features of the quinoxaline antibiotics that are necessary for interaction with DNA. The insight thus gained would be important not only from the point of view of a structure/activity study but also as an essential step towards the rational design of antitumour agents based on the structure of quinoxaline antibiotics.

Preliminary experiments with 'TANDEM' suggested that the molecule bound strongly to DNA, and thus solvent-partition analysis (Waring *et al.*, 1975) was used to investigate the specificity of the interaction. For the other five compounds, on the other hand, only weak (if any) interaction could be detected and thus considerable efforts were made to place limits on the magnitude of binding constants to DNA. For the most part this was achieved by a novel method involving the analysis of thermal-denaturation profiles.

Materials and Methods

Echinomycin was a gift from Dr. H. Bickel and Dr. K. Scheibli, CIBA–Geigy Ltd., Basel, Switzerland. 'TANDEM', 'ELSERTA', 'BOCANTA' and 'ACIMBO' were gifts from Dr. R. K. Olsen, Department of Chemistry and Biochemistry, Utah State University, Logan, UT, U.S.A. These compounds were used as supplied, without further purification, and were stored in the dry state in a desiccator in the dark at 0–4°C. All experiments were performed in 0.01 SHE buffer, I 0.01, pH 7.0 at 20°C, as described in the preceding paper (Lee & Waring, 1978), except for experiments with echinomycin split product 1, where the buffer solution was supplemented with 2% (v/v) methanol throughout. General reagents and solutions of naturally occurring and synthetic nucleic acids were prepared as previously described (Lee & Waring, 1978). In addition, *Escherichia coli* B DNA (type VIII) and poly(rA)·poly(rU) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and were used directly as supplied.

Preparation of echinomycinic acid dimethyl ester

This derivative was prepared in two stages essentially by the method of Dell *et al.* (1975). In the first step echinomycin was converted into the dibasic acid by using 0.04M-NaOH in 90% (v/v) methanol and extracted from the reaction mixture as described by Keller-Schierlein *et al.* (1959). In the second step the dibasic acid was esterified with acidic methanol (100mg of the dibasic acid in 4ml of acetyl chloride and 80ml of methanol). The reaction could be followed by t.l.c. on alumina plates with ethanol/conc. NH_3 (sp.gr. 0.880)/water (8:1:1, by vol.) solvent, in which the acid had R_F 0.1 and the product R_F 0.9. After 5h the reaction was stopped by the addition of an excess of $NaHCO_3$. The solution was filtered and evaporated to approx. 20ml. On addition of 180ml of water a precipitate formed, which was extracted with 4 × 25ml of chloroform. The combined chloroform extracts were dried to yield 65mg of impure echinomycinic acid dimethyl ester (an overall yield of 50% from echinomycin).

This product (65mg) was separated into two main components by chromatography with a 20ml volume of neutral alumina in a 10cm column. The eluate was collected in 7ml fractions and the A_{325} monitored in a 10mm-light-path cuvette. The initial solvent was methanol, and the first component was eluted in fractions 2–6. The eluent was then changed to methanol/conc. NH_3 (sp.gr. 0.880) (9:1, v/v) after fraction 11, and most of the second component was collected in fractions 13–20. After evaporation of the solvents the components were dried under vacuum over P_2O_5. T.l.c. on alumina with ethanol/NH_3/water (8:1:1, by vol.) revealed nothing that could be attributed to acidic materials. The yields of components 1 and 2 were 10mg and 40mg respectively (overall yields of 8 and 30% respectively from echinomycin).

The work of Dell *et al.* (1975) suggested that one of these components would have the cross-bridge broken, whereas the desired component, echinomycinic acid dimethyl ester, would not. A critical test of this hypothesis could be provided by sulphur analysis, since breaking the cross-bridge via an acid-catalysed reaction would result in the loss of sulphur as methyl sulphide. The analysis was kindly performed by the University of Cambridge Chemical Laboratories, which found $2.8 \pm 0.4\%$ sulphur for component 1 and $5.1 \pm 0.4\%$ for component 2. Component 1 was thus identified as a compound or compounds having the cross-bridge broken and component 2 as echinomycinic acid dimethyl ester. Use of a mol.wt. assumed to be 1192 (see legend to Table 2) gives an expected sulphur content of 5.37%, which is well within the experimental error. The proposed structure of echinomycinic acid dimethyl ester is shown in Fig. 1.

Preparation of echinomycin split product 1

Methyl iodide is a standard reagent for cleaving thioacetals (Fetizon & Jurion, 1972). Echinomycin (800mg) was gently refluxed together with 10ml of acetone, 10ml of methyl iodide, a trace of water and 50mg of $CaCO_3$ for 48h. The contents of the reaction vessel were filtered and the filtrate was evaporated to dryness to yield a dark-brown product. Echinomycin split product 1 and a related product (designated product 2) were purified from this material by liquid/liquid chromatography on a coil planet centrifuge (I. A. Sutherland, J. S. Lee & D. Gauvreau, unpublished work). At this stage the 100MHz p.m.r. spectra of these two components revealed that only echinomycin split product 1 was a pure compound. The overall yield of product 1 from echinomycin was 11.5mg (approx. 1%).

Because of the very small yield, sulphur analysis could not be performed. However, structural information was deduced from mass-spectrometry and p.m.r. studies (kindly performed by Mr. T. Blake and Dr. J. Kalman, Department of Chemistry, University of Cambridge, and Dr. J. Feeney, National Institute for Medical Research, Mill Hill, London N.W.7, respectively). A satisfactory mass spectrum and molecular ion was obtained by electron-impact techniques, which were also used in the elucidation of the structure of echinomycin (Dell *et al.*, 1975). An internal standard of tris(perfluoroheptyl)triazine was used to provide reference peaks of known mass, so that by interpolation the peaks of the compound could be estimated to within 2 mass units. The molecular ion was found at *m/e* 1071, in excellent agreement with the structure proposed in Fig. 1 (mol.wt. 1070). Additionally, in the high-mass region there was a peak at *m/e* 1038, which corresponds to loss of –SH from the molecular ion. In the low-mass region of the spectrum, where the *m/e* value could be estimated by simple peak counting, high-intensity peaks were found at *m/e* 509, 380, 310, 297, 268, 226, 173, 157, 129 and 102. These are identical with those found for echinomycin under similar conditions (Dell *et al.*, 1975); for example the peak at *m/e* 509 corresponds to the quinoxaline tetrapeptide formed by cleavage at the lactone linkages and at the cross-bridge. Similarly the peaks at *m/e* 380, 297 and 226 are quinoxaline tri-, di- and mono-peptides respectively, and the peaks at *m/e* 157, 129 and 102 can be attributed to fragments of the quinoxaline-2-carboxyl moiety. These peaks in the low-mass region give no information about the cross-bridge, but they confirm the suggestion that the remainder of the structure of echinomycin split product 1 is identical with that of echinomycin.

The p.m.r. spectrum of echinomycin split product 1 was also consistent with it being a reasonably pure compound having the cross-bridge broken and the lactone linkages intact. For echinomycin in chloroform (Dell *et al.*, 1975) there are two signals, at δ 6.1 and 6.4 p.p.m. from hexamethyldisiloxane, attributable to the α-CH protons of the cysteine residues; if the cross-bridge had been broken this region of the spectrum might be expected to be different. Indeed in echinomycin split product 1 there is now a weakly coupled proton at δ 6.3 p.p.m. and the signal of the other α-CH group has been shifted upfield. The small coupling constant (approx. 2.5Hz) of the proton at δ 6.3 p.p.m. is consistent with the presence of a vicinal aldehyde proton. However, the signal of this aldehyde proton could not be assigned, and it is assumed that it is masked by the two 3-H protons of the chromophores, which would occur at a similar δ value. Chemical tests to confirm the structure proposed in Fig. 1 could not be performed because of the very small quantities available.

Absorption coefficients

All u.v.-absorption measurements were performed by using a Unicam SP500 series II spectrophoto-

meter. The molar absorption coefficients of the compounds in organic solvents were measured by direct weighing. Measurements of absorption were made at various concentrations to ensure that the absorption coefficient was not a function of the antibiotic concentration, at least over the working range.

For 'TANDEM', the coefficients in 0.01 SHE buffer saturated with '70/30 IPA/heptane' solvent (isopentyl acetate/n-heptane, 7:3, v/v), prepared as described by Lee & Waring (1978), were determined by extrapolating to zero methanol concentration the absorption coefficients in various methanol/buffer mixtures as in the procedure of Waring *et al.* (1975).

For 'ELSERTA', having previously determined an absorption coefficient in 50% (v/v) dimethyl sulphoxide by direct weighing, we measured the absorption coefficient in buffer as follows. The A_{244} of a series of solutions of 'ELSERTA' in buffer was measured. The solutions were then diluted with an equal volume of dimethyl sulphoxide and the A_{323} was measured. Thus, knowing the absorption coefficient at 323nm in 50% (v/v) dimethyl sulphoxide, the absorption coefficient in buffer was readily calculated.

For 'BOCANTA' the lack of a suitable absorption peak in the u.v. necessitated the use of a different method. A stock solution of known concentration was made up in methanol, and a portion of this was diluted 80-fold with buffer to yield a solution in 1.25% (v/v) methanol of known concentration. The complete spectrum was then taken and used as a standard against which the spectrum of other aqueous solutions could be compared. Thus by taking the ratio of the absorbance for the standard and the solution of interest at several different wavelengths, a good estimate of the concentration could be made.

Because only very small quantities of echinomycin split product 1 were available, only one stock solution could be made up by direct weighing. For this the solvent chosen was methanol. Portions of this solution were then diluted 50-fold with buffer and the A_{243} was measured to yield values for the absorption coefficient in 2% (v/v) methanol.

The absorption coefficients determined by these methods are recorded in Table 1.

Solubilities and molecular weights

The solubilities and molecular weights of the derivatives are listed in Table 2, which also includes triostin A for comparison. The solubilities are all very low, except for echinomycinic acid dimethyl ester and 'ACIMBO'. Aqueous solutions of these latter two could be made up by direct weighing. Solubilities were measured by shaking a small portion of the sample vigorously with buffer for several hours and then removing excess solid by filtering or centrifugation. Knowing the absorption coefficient in water, a measurement of the absorption thus yielded an estimate of the concentration of the saturated solution. The same procedure was used for the preparation of aqueous solutions of known concentration.

Analytical ultracentrifugation

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

Spectrophotometric titrations

For 'BOCANTA' and 'ACIMBO' the lack of a long-wavelength peak in the absorption spectra precluded the use of this technique to detect interaction with nucleic acids. Even for quinoxaline compounds the absorption maximum at approx. 325nm is sufficiently close to that of DNA at 260nm to necessitate efficient blanking procedures. Routinely, fresh solutions were prepared for every measurement at a given

Table 1. *Molar absorption coefficients of quinoxalines and related substances*

Compound	Solvent	Molar absorption coefficient at $\lambda_{max.}$
'TANDEM'	'70/30 IPA/heptane'	12410 (316nm)
	50% (v/v) Dimethyl sulphoxide/'70/30 IPA/heptane'-saturated 0.01 SHE buffer	14010 (325nm)
	'70/30 IPA/heptane'-saturated 0.01 SHE buffer	12130 (323nm)
		57850 (245nm)
'ELSERTA'	50% (v/v) Dimethyl sulphoxide/0.01 SHE buffer	11960 (325nm)
	0.01 SHE buffer	49600 (244nm)
'BOCANTA'	0.01 SHE buffer	800 (250nm)
'ACIMBO'	0.01 SHE buffer	450 (250nm)
Echinomycinic acid dimethyl ester	0.01 SHE buffer	12800 (325nm)
Echinomycin split product 1	2% (v/v) methanol/0.01 SHE buffer	38900 (243nm)

Table 2. *Solubilities and molecular weights of quinoxalines and related compounds*

Aqueous solubility refers to 0.01 SHE buffer, except for echinomycin split product 1, where the buffer was supplemented with 2% (v/v) methanol. Molecular weights are based on the value of 1100 determined for echinomycin (Dell *et al.*, 1975). The values given for echinomycinic acid dimethyl ester and echinomycin split product 1 are calculated for the proposed structures represented in Fig. 1, with $R = -CH_2OCH_3$.

Compound	Approximate water solubility (μM)	Mol.wt.
Echinomycin	5	1100
Triostin A	1.7	1086
Triostin C	0.8	1142
Quinomycin C	1.7	1156
'TANDEM'	6	1030
'ELSERTA'	5	1030
'BOCANTA'	25	986
'ACIMBO'	100	1130
Echinomycinic acid dimethyl ester	150	1192
Echinomycin split product 1	5	1070

drug/nucleotide ratio (D/P), and special care was taken to ensure that all reference solutions contained exactly the same DNA concentration as the test samples. In this way the spectra could be directly compared for maximum accuracy without having to allow for dilution of solutions by the addition of portions of DNA. For every compound at least one test was performed with not less than a 20-fold molar excess of nucleotide.

Thermal-denaturation profiles

'Melting' curves were measured by using a Unicam SP500 series II spectrophotometer coupled to a Servoscribe pen recorder. Up to four Teflon-stoppered quartz cuvettes of 10mm light-path and 3ml capacity were placed in a water-jacketed cell-holder. The spectrophotometer was equipped with an automatic sample changer so that three nucleic acid samples could be examined in a single experiment. The fourth position of the cell holder was occupied by a cuvette containing the appropriate buffer and a thermistor probe, the resistance of which was continuously monitored to provide a direct measurement of the temperature of the liquid inside the cuvettes. The temperature of the cell holder was raised by a Haake circulating water bath, the contact thermometer of which was driven by a synchronous electric motor. The gearing of the motor gave a temperature rise of approx. 0.6°C/min. The A_{260} was monitored for all nucleic acids. The 'melting' temperature, T_m, was taken to be the mid-point of the hyperchromic transition.

Mixtures of the compounds with nucleic acids were prepared by direct pipetting of aqueous solutions, and, once denatured, samples were never re-used. In addition, some 'TANDEM' complexes were produced by shaking nucleic acid solutions with solutions of 'TANDEM' dissolved in '70/30 IPA/heptane' solvent as if for solvent-partition analysis (see below). These necessarily contained some isopentyl acetate, which on heating sometimes came out of solution and obscured the thermal transition. This problem was circumvented by shaking the complex before heating three times with an equal volume of n-heptane. This treatment was not expected to remove any 'TANDEM', because it is completely insoluble in heptane. A blank solution containing no 'TANDEM' was prepared in a similar manner.

For compounds that did not interact strongly with DNA only small changes in the T_m were observed, and so a control sample was always run simultaneously for which D/P was zero. Consequently, even though the absolute T_m values measured may have contained systematic errors, the T_m differences (ΔT_m) were subject to considerably smaller errors. The possibility of a temperature gradient across the water-jacketed cell holder was also considered. It was shown that this and/or other artifacts could not account for apparent differences in T_m of more than 0.5°C by running similar samples in all three cuvette positions simultaneously. Consequently ΔT_m values are considered to be accurate to within 1°C.

The magnitude of the observed change in the 'melting' temperature can be used to estimate binding constants. If two ligands bind only to the helix and not to the coil form of DNA, occlude the same number of base-pairs (i.e. have the same value for n defined below), and at particular input ratios give rise to the same change in 'melting' temperature for the same DNA, then:

$$K_1c_1 = K_2c_2$$

where K is the binding constant and c the free ligand concentration at the 'melting' temperature, and the subscript denotes the appropriate ligand (McGhee, 1976). This very simple relationship is exact even for the McGhee & Von Hippel (1974) treatment of ligand binding. Consequently if binding parameters and 'melting' temperatures are known for one quinoxaline compound, binding constants can be estimated for others. The validity of the estimate (excluding random errors) will largely depend on the assumptions that for the ligands in question the value of n is the same and that they do not bind significantly to the random-coil form. These assumptions appear to be reasonably well founded for any two quinoxaline compounds.

Solvent-partition analysis for 'TANDEM'

The partition coefficient of 'TANDEM' between '70/30 IPA/heptane' solvent and 0.01 SHE buffer was determined essentially as described by Waring *et al.* (1975). Eight tubes containing 0.5ml of '70/30 IPA/heptane' solvent and 2ml of buffer with various amounts of 'TANDEM' were shaken for 2h at 20°C. One of these was a blank containing no antibiotic, to be used as reference for subsequent absorption measurements. After separation of the phases the concentration of 'TANDEM' in the organic phase was determined from the A_{316} in 10mm-light-path semi-micro cuvettes after a 5-fold dilution had been made. The concentration in the aqueous phase was determined from the A_{325} in 40mm-light-path semi-micro cuvettes after addition of an equal volume of dimethyl sulphoxide. The partition coefficient was estimated to be 67.6 ± 1 (Fig. 2).

An identical procedure was also used to measure binding curves, except that the aqueous phase now contained nucleic acid at a concentration of $150\,\mu$M. Absorption measurements on the organic phase together with the partition coefficient yielded an estimate of c, the free 'TANDEM' concentration in the aqueous phase. Thus the bound 'TANDEM' concentration could be determined by difference and divided by the nucleotide concentration to give r (mol of 'TANDEM' bound/mol of nucleotides). As described previously (Lee & Waring, 1978) these values of r and c were analysed in terms of eqn. (10) of McGhee & Von Hippel (1974) to yield estimates of $K(0)$, the intrinsic association constant, and n, the number of nucleotides occluded by the binding of one molecule.

Results

'TANDEM'

The effect of a large excess of calf thymus DNA on the u.v.-absorption spectrum of 'TANDEM' is shown in Fig. 3. The hypochromic and bathochromic shifts are very similar to those found for echinomycin (Waring *et al.*, 1975) and an isosbestic point at 348nm can also be observed. Thus as regards its spectral properties, 'TANDEM' appears to behave like a typical quinoxaline compound and to interact well with DNA.

The effect of 'TANDEM' on the sedimentation coefficient of bacteriophage-PM2 DNA is shown in Fig. 4. Examination of the densitometer tracings in these experiments clearly revealed the presence of unbound 'TANDEM' at ratios within the equivalence region (filled symbols in Fig. 4), in contrast with the situation with naturally occurring quinoxaline

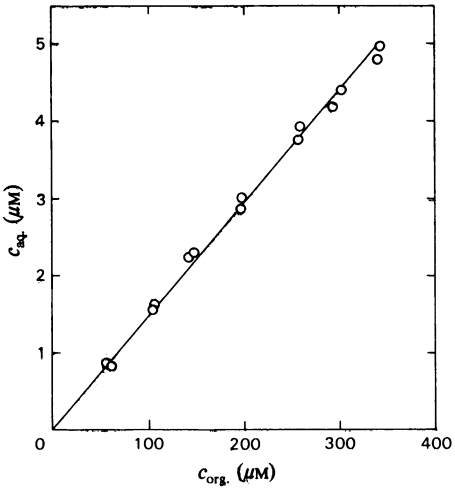


Fig. 2. *Partition of 'TANDEM' between '70/30 IPA/heptane' solvent and 0.01 SHE buffer*
The ordinate and abscissa represent the concentrations of 'TANDEM' in the aqueous and organic phases ($c_{aq.}$, $c_{org.}$ respectively) after equilibration by shaking for 2h at 20°C. The line drawn was fitted to the points by the method of least squares and constrained to pass through the origin; its slope ($c_{org.}/c_{aq.}$) is 67.6 ± 1.

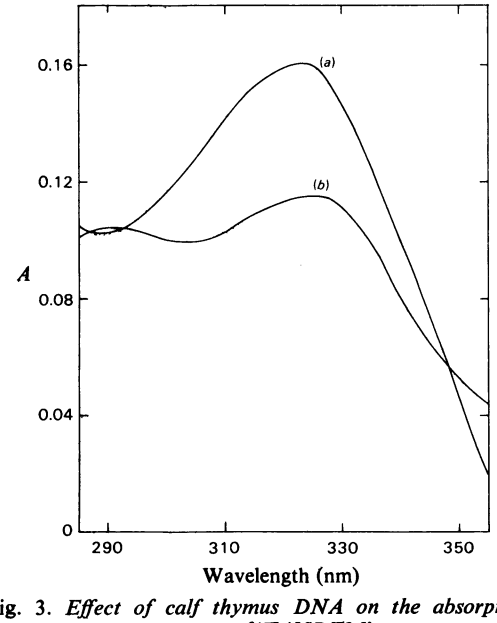


Fig. 3. *Effect of calf thymus DNA on the absorption spectrum of 'TANDEM'*
(*a*) Spectrum of 3.3μM-'TANDEM' in 0.01 SHE buffer measured in 40mm-light-path quartz cuvettes; (*b*) as (*a*), with calf thymus DNA present at a concentration of 61μM.

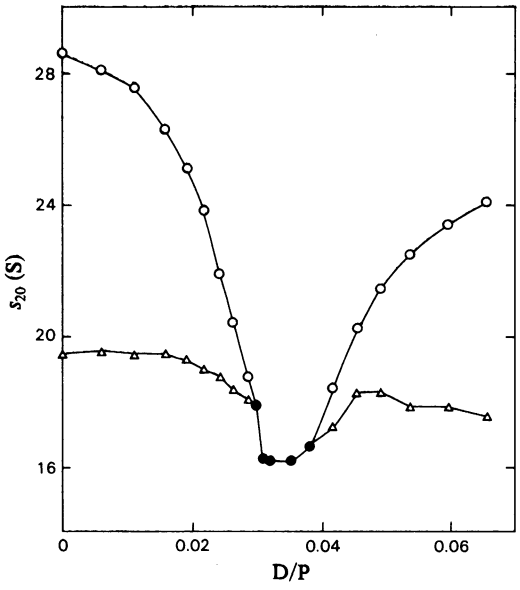


Fig. 4. *Effect of 'TANDEM' on the sedimentation coefficient of bacteriophage-PM2 DNA*
The DNA preparation contained 85–90% closed circular duplex molecules, whose s_{20} is represented by ○. The s_{20} of the nicked circles is represented by △, and when the two components co-sedimented as a single unresolved boundary the symbol ● is plotted. The abscissa shows the molar ratio of 'TANDEM' molecules to total DNA nucleotides (D/P).

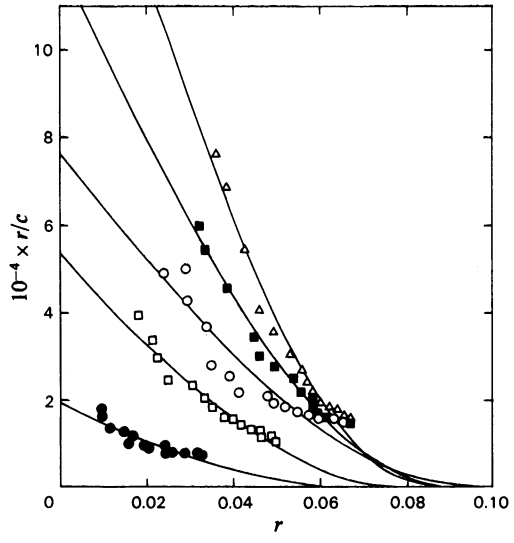


Fig. 5. *Effects of base-composition on the binding of 'TANDEM' to DNA*
The data are presented in the form of Scatchard plots where r is the binding ratio ('TANDEM' molecules bound per nucleotide) and c is the free ligand concentration. ●, *Micrococcus lysodeikticus* DNA (72% G+C); □, *Escherichia coli* DNA (50% G+C); ○, calf thymus DNA (42% G+C); ■, nicked bacteriophage PM2 DNA (42% G+C); △, *Clostridium perfringens* DNA (30% G+C). The curves are theoretical, computed to fit eqn. (10) of McGhee & Von Hippel (1974), the values of $K(0)$ and n listed in Table 3 being used.

antibiotics described previously (Lee & Waring, 1978), thus suggesting that the binding of 'TANDEM' is considerably weaker. The 'TANDEM'/nucleotide ratio (D/P) at the midpoint of the equivalence region is 0.035, which in itself suggests a mechanism of binding involving at least sesquifunctional (1½-fold) intercalation (Waring & Wakelin, 1974). However, the true equivalence binding ratio could be determined accurately by reference to a binding isotherm for nicked bacteriophage-PM2 DNA (see Fig. 5). This yielded an equivalence value of 0.0292 ± 0.0040 molecule bound per nucleotide, similar to that found for the naturally occurring quinoxaline compounds, and corresponding to a helix-unwinding angle 1.75 ± 0.23 times that of ethidium (Wakelin & Waring, 1976; Lee & Waring, 1978). Thus, as with echinomycin, quinomycin C and the triostins, the intercalation of 'TANDEM' into DNA at $I 0.01$ is convincingly bifunctional in character.

Scatchard plots for the binding of 'TANDEM' to five naturally occurring DNA species are presented in Fig. 5. The curves drawn are isotherms calculated from the McGhee & Von Hippel (1974) treatment of the data points. In general the experimental points lie on a significantly more concave curve than can be accommodated by the calculated best-fit isotherms. Indeed at low levels of binding the calculated value of r/c increases dramatically, reflecting the fact that the value of c, the free drug concentration, falls sharply to such an extent that it cannot be measured accurately. These points are not shown in Fig. 5, nor were they used in the analysis of binding parameters, but this evidence suggests that there exist binding sites in these DNA molecules with very much higher binding constants than the average. Thus in this case the treatment of McGhee & Von Hippel (1974), although revealing the correct trends, cannot be expected to give as good an estimate of the intrinsic association constant as for the other quinoxaline compounds. In general, the extrapolation to the r/c axis becomes worse as the binding constant increases, because of the lack of data at low values of r.

There is, however, little doubt that the interaction between 'TANDEM' and natural DNA species is characterized by considerable specificity. More important is the fact that the pattern of specificity observed is essentially reversed compared with that found for echinomycin, quinomycin C and triostin

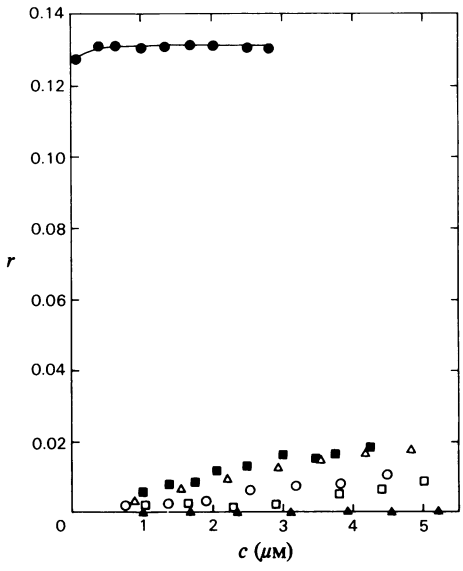


Fig. 6. *Interaction between 'TANDEM' and polynucleotides*
The binding ratio r ('TANDEM' molecules bound per nucleotide) is plotted as a function of the free ligand concentration c. ●, Poly(dA-dT); ■, poly(dI-dC); □, poly(dG-dC); ○, poly(dA)·poly(dT); △, poly(rA)·poly(rU); ▲, rRNA from *E. coli* B.

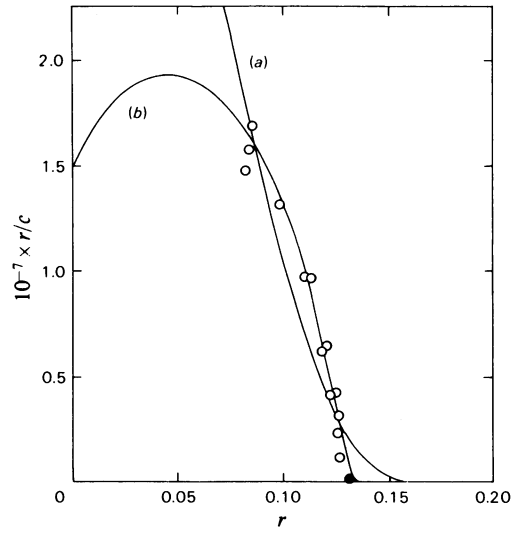


Fig. 7. *Scatchard plot for the interaction of 'TANDEM' with poly(dA-dT)*
Curve (*a*), describing eqn. (10) of McGhee & Von Hippel (1974), was fitted to the experimental points by computer. Curve (*b*) describes eqn. (15) of McGhee & Von Hippel (1974) for a co-operative binding process; it was fitted to the points by trial and error. ●, A superimposition of eight independent determinations at this value of r, with r/c values so low as to be virtually coincident with the abscissa.

A (Wakelin & Waring, 1976; Lee & Waring, 1978). In this case the preference is clearly for (A+T)-rich, not (G+C)-rich, DNA. Moreover, as for triostin A (Lee & Waring, 1978), the binding to nicked bacteriophage-PM2 and calf thymus DNA, both containing 42% (G+C), is significantly different, but again the order of preference is reversed.

Binding isotherms for the interaction of 'TANDEM' with RNA and synthetic polynucleotides are shown in Fig. 6. The complete lack of binding to rRNA provides a useful standard of reference against which to assess the data for poly(rA)·poly(rU), poly(dI-dC), poly(dA)·poly(dT) and poly(dG-dC), suggesting that the binding to these synthetic double-helical polymers is small but definitely significant. The binding to poly(dI-dC) in particular is readily detectable and significantly greater than that found for poly(dG-dC).

The isotherm for poly(dA-dT) is virtually flat over the whole range of measurable free ligand concentration and thus shows extremely tight binding. A Scatchard plot of these data is simply a vertical straight line, showing that the binding constant is too large to be accurately determined. In this case the chosen partition coefficient is too low to permit analysis of the interaction by the standard procedure. However, some further data points were obtained by increasing the volume of the organic phase to 4ml, so that absorption measurements could be made in 40mm-light-path cuvettes without dilution. This resulted in a 20-fold decrease in the smallest free ligand concentration that could be measured compared with the standard procedure. In this way the Scatchard plot shown in Fig. 7 was produced. The filled circle represents eight data points at this value of r, but with r/c values so low as to be virtually coincident with the abscissa. The curves drawn through the data points represent the simple binding isotherm calculated from eqn. (10) of McGhee & Von Hippel (1974) by using all 21 points, and also the best-fit 'humped' co-operative binding curve defined by their eqn. (15) fitted by trial and error as previously described (Lee & Waring, 1978). There is little doubt that the co-operative treatment is preferable, especially at low values of r/c, and in this instance the co-operative character of the interaction is much more convincingly evident than was the case for quinomycin C and triostin A binding to the same polymer (Lee & Waring, 1978). Because of lack of data at low values of r in the important region of the 'hump', the binding constant $K(0)$ and co-operativity parameter ω could not be estimated accurately. However, in general, co-operative isotherms that gave a reasonable fit had the product of $K(0)$ and ω

Table 3. *Parameters of binding of 'TANDEM' to nucleic acids*

$K(0)$, 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), except for poly(dA-dT), where the eqn. (15) for co-operative binding was used; for details see the text. For poly(dG-dC) and poly(dA)·poly(dT) the very low levels of binding observed did not permit any theoretical analysis; the values of $K(0)$ quoted are upper-limit estimates based on the maximum value attained by r/c.

Nucleic acid	$10^{-4} \times K(0)$ (M^{-1})	n
M. lysodeikticus DNA	1.94	12.13
E. coli B DNA	5.35	10.55
Calf thymus DNA	7.64	8.89
Nicked bacteriophage-PM2 DNA	12.10	9.50
Cl. perfringens DNA	18.46	10.02
Poly(dG-dC)	<0.2	—
Poly(dI-dC)	0.63	8.41
Poly(dA)·poly(dT)	<0.3	—
Poly(rA)·poly(rU)	0.52	8.01
Poly(dA-dT)	1500 for $\omega = 15$	7.40

approximately constant, with values of $K(0)$ between 1.0×10^7 and 2.0×10^7. The curve shown in Fig. 7 is characterized by parameters $K(0)$, n and ω of 1.5×10^7, 7.4 and 15 respectively.

The complete set of binding parameters for 'TANDEM' is given in Table 3. They are all deduced in terms of eqn. (10) of McGhee & Von Hippel (1974), except for poly(dA-dT), where the co-operative eqn. (15) was used. The values quoted for poly(dA)·poly(dT) and poly(dG-dC) are upper-limit estimates based on the very low degrees of binding observed, but no sensible estimates of n could be deduced. In general, the values of the parameter n are larger than those found with other quinoxalines (Wakelin & Waring, 1976; Lee & Waring, 1978), which is consistent with a greater degree of specificity and more concave curvature in the Scatchard plots. The overall specificity pattern is completely consistent with a very large preference for (A·T) base-pairs.

'ELSERTA'

The absorption spectrum of 'ELSERTA' is typical of a quinoxaline compound (cf. Fig. 3), but large excesses of calf thymus DNA or poly(dA-dT) had no detectable effect on either the position or magnitude of the principal absorption peaks. Likewise, 'ELSERTA' has no effect on the sedimentation coefficient of closed circular duplex bacteriophage-PM2 DNA up to D/P values of approx. 0.085 (Fig. 8). These two experiments by themselves do not prove conclusively that there is little interaction between 'ELSERTA' and DNA, but it certainly does not behave like a typical quinoxaline compound.

To probe further the possibility of interaction, if any, with DNA, thermal-denaturation experiments were performed with poly(dA-dT) and *Clostridium perfringens* DNA, the latter being chosen in preference to calf thymus DNA because the 'melting' transition is sharper and occurs at a lower temperature. The results are shown in Fig. 9, with the effect of 'TANDEM' for comparison, so that estimates of the binding constant can be made. For 'ELSERTA' and *Cl. perfringens* DNA the change in the 'melting' temperature (ΔT_m) is not greater than 1°C, the proposed limit of accuracy of the technique. For 'TANDEM' a ΔT_m value of 1°C would occur at a D/P value of approx. 0.003, which by reference to the binding isotherm corresponds to an r value of approx. 0.003 at the T_m. Thus if the value of n is the same for 'ELSERTA' and 'TANDEM', it can be stated that there was less than 0.003 molecule of 'ELSERTA' bound/nucleotide at the T_m. Consequently an estimate of the minimum free ligand concentration can be made for 'ELSERTA', from which the binding constant can be evaluated by using the relationship $K_T c_T = K_E c_E$ (the subscripts refer to 'TANDEM' and 'ELSERTA'). In this way the binding constant of 'ELSERTA' to *Cl. perfringens* DNA is estimated to be less than $1 \times 10^3 M^{-1}$.

'ELSERTA' does, however, produce a significant effect on the T_m of poly(dA-dT). In this case comparison with the data for 'TANDEM' shows that at a ΔT_m of 2°C the ligands have r values of approx. 0.005. The binding constant for 'ELSERTA' on poly(dA-dT) estimated in an analogous manner is approx. $2 \times 10^3 M^{-1}$. It is difficult to put limits on the errors in these binding-constant measurements, especially since they necessarily incorporate any errors in the binding constants of 'TANDEM'. However, there is no doubt that the interaction between 'ELSERTA' and DNA must be several orders of magnitude weaker than that of 'TANDEM'.

'BOCANTA' and 'ACIMBO'

Investigations of the effect of DNA on absorption spectra were not possible with 'BOCANTA' and 'ACIMBO' because the benzyloxycarbonyl substituent lacks an absorption peak distinct from that of DNA. Also there was no detectable effect of 'BOCANTA' on the s_{20} of supercoiled bacteriophage-PM2 DNA up to D/P values of 0.22 (Fig. 8). However, the possible interaction of these compounds with DNA was amenable to investigation by determination of thermal-denaturation profiles.

Their effects on the 'melting' temperature of poly(dA-dT), *Cl. perfringens* DNA and poly(rA)·(rU)

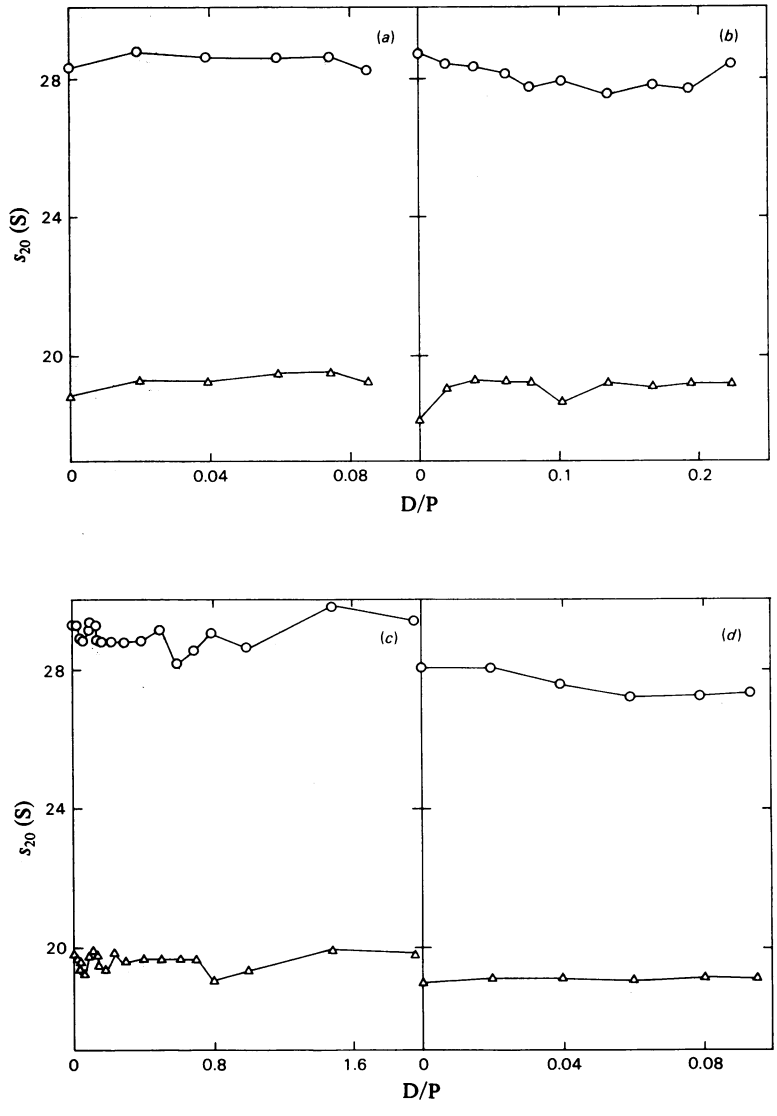


Fig. 8. *Effects of quinoxaline analogues and derivatives on the sedimentation coefficient of bacteriophage PM2 DNA* Symbols and abbreviations are as in the legend to Fig. 4. (*a*) 'ELSERTA'; (*b*) 'BOCANTA'; (*c*) echinomycinic acid dimethyl ester; (*d*) echinomycin split product 1. Note the different scales used for the abscissae.

are recorded in Table 4. Results for 'TANDEM' on these nucleic acids are included for comparison. The effect of 'BOCANTA', though small, is reproducibly detectable, whereas that of 'ACIMBO' is barely detectable at much higher D/P values. Poly(rA)·poly(rU) was included in this study, since 'TANDEM' binds to it only weakly (Fig. 6 and Table 3) and therefore it might provide a good reference for comparison with the weak interaction of 'BOCANTA'. Binding constants for 'BOCANTA', estimated by the same means as above, were $4\times10^2\,M^{-1}$, $8\times10^2\,M^{-1}$ and $2\times10^2\,M^{-1}$ for poly(dA-dT), *Cl. perfringens* DNA and poly(rA)·poly(rU) respectively. Because the measured T_m changes were only a few degrees larger than the accuracy with which they could be measured it is difficult to assess the errors in these estimates. They are probably of the order of 25%, and thus the binding constants of 'BOCANTA' to nucleic acids should be thought of as lying in the range 10^2–$10^3\,M^{-1}$. For 'ACIMBO' the T_m changes are barely significant at much higher values of D/P, so that the binding constants are estimated to be less than $50\,M^{-1}$.

Echinomycinic acid dimethyl ester and echinomycin split product 1

The u.v.-absorption spectra of these derivatives of echinomycin were not significantly perturbed in the presence of calf thymus DNA at a 20-fold molar excess of nucleotides. Nor did the compounds affect the sedimentation coefficient of bacteriophage-PM2 DNA at concentrations up to saturation of the solution, corresponding to D/P values of 2.0 and 0.096 respectively (Fig. 8). Furthermore, neither substance had any significant effect on the thermal-denaturation profile of *Cl. perfringens* DNA. By the method described in the section on 'ELSERTA' the binding constant of echinomycinic acid dimethyl ester to this DNA can be estimated to be less than $30\,M^{-1}$, and that for echinomycin split product 1 is less than $10^3\,M^{-1}$. Again it is the aqueous solubility that determines the upper limit that can be placed on the binding constants. Thus it appears that breaking either the lactone linkages or the cross-bridge of echinomycin practically abolishes the interaction with DNA.

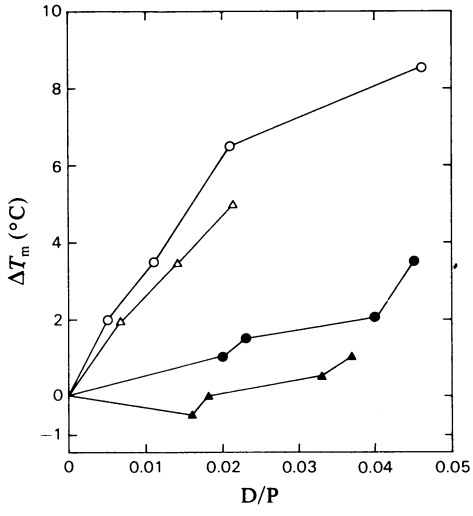


Fig. 9. *Effect of 'TANDEM' and 'ELSERTA' on the thermal-denaturation transition midpoint (T_m) of poly(dA-dT) and Clostridium perfringens DNA*

The change in 'melting' temperature (ΔT_m) is shown as a function of the molar ratio of ligand to total nucleotides (D/P). ○, △, Effects of 'TANDEM' on poly(dA-dT) and *Cl. perfringens* DNA respectively; ●, ▲, effects of 'ELSERTA' on poly(dA-dT) and *Cl. perfringens* DNA respectively.

Discussion

Of the six quinoxaline-type compounds studied in this work only 'TANDEM' interacted strongly with DNA. The mechanism of its binding is clearly via bifunctional intercalation, as seems to be typical of the naturally occurring quinoxaline antibiotics (Wakelin & Waring, 1976; Lee & Waring, 1978). This behaviour contrasts strongly with that of 'ELSERTA'. It seems reasonable to suppose that the conformation of the peptide ring in the two molecules is very similar, since the quinoxaline chromophores themselves do not appear to be the cause of any steric congestion, as revealed by inspection of space-filling molecular models. Indeed there is experimental evidence that this is so, from the similarity in coupling constants deduced from n.m.r. studies (J. Kalman, personal communication). Thus if in 'TANDEM' the chromophores are positioned roughly parallel to allow for bifunctional intercalation into the DNA helix, the chromophores of 'ELSERTA' would be expected to lie in approximately the same plane as the peptide ring. Hence bifunctional intercalation by 'ELSERTA' would require a radically different peptide-ring conformation. In the light of these considerations it is perhaps

Table 4. *Effect of 'TANDEM', 'BOCANTA' and 'ACIMBO' on the thermal denaturation of double-helical nucleic acids*

	'TANDEM'		'BOCANTA'		'ACIMBO'	
	D/P	ΔT_m (°C)	D/P	ΔT_m (°C)	D/P	ΔT_m (°C)
Poly(dA-dT)	0.005	+2.0	0.10	+1.0	0.44	+1.0
	0.011	+3.5	0.10	+1.0	0.81	+0.5
	0.021	+6.5	0.16	+2.0		
	0.046	+8.5	0.16	+2.0		
Poly(rA)·poly(rU)	0.014	+1.0	0.08	+0.5	0.43	+1.0
	0.016	+1.5	0.08	+1.5	0.81	+1.5
	0.021	+3.0	0.12	+2.5		
	0.025	+3.5	0.12	+3.0		
Cl. perfringens DNA	0.007	+2.0	0.10	+2.5	0.42	+0.5
	0.014	+3.5	0.12	+1.5	0.82	+1.0
	0.022	+5.0	0.14	+3.5		
			0.18	+2.5		

less surprising that the interaction of 'ELSERTA' with DNA is weak than that it can be detected at all [in fact only with poly(dA-dT)].

It also seems highly probable that the conformation of the peptide ring in 'BOCANTA' is essentially the same as in 'TANDEM'. The benzyloxycarbonyl moiety of 'BOCANTA' has two additional connecting atoms to the aromatic ring system compared with the quinoxaline-2-carboxyl moiety of 'TANDEM'. In addition, the 'chromophores' are only single rings similar to ones which, in general, have been reported to intercalate only partially, if at all (Gabbay *et al.*, 1972, 1973; Jacobsen & Wang, 1973; Kapicak & Gabbay, 1975). Consequently the behaviour of 'BOCANTA' provides something of a stringent test for allowable modifications of the chromophores. The fact that 'BOCANTA' retains the property of binding to nucleic acids, albeit weakly, therefore suggests that more 'conservative' chromophore replacements could be made to the basic quinoxaline antibiotic ring structure without disastrous loss of DNA-binding ability.

The importance of integrity of the peptide portion has been established by the results with echinomycinic acid dimethyl ester and echinomycin split product 1. It might be expected that by breaking the lactone linkages of echinomycin to produce echinomycinic acid dimethyl ester the structural rigidity of the quinoxaline antibiotics would be lost. Unless specific internal hydrogen bonds can be formed, the molecule would then be able to adopt many different conformations in solution having similar energies. In consequence the inability of echinomycinic acid dimethyl ester to bind to DNA is readily explained [it must be remembered that echinomycinic acid dimethyl ester is a mixture of several closely related compounds, and thus the upper limit placed on the binding constant ($30 M^{-1}$) is an average]. Perhaps more surprising, therefore, is the lack of detectable binding of echinomycin split product 1 to DNA, since this molecule retains an intact octapeptide ring even though the cross-bridge has been broken. It might be argued that the thiol proton of echinomycin split product 1 is ionized and thus the molecule is repelled by the negatively charged DNA helix. However, thiol groups in similar compounds have pK_a values greater than 8.33 (Perrin, 1965) and thus in 0.01 SHE buffer the thiol group would be expected to be less than 8% ionized. Perhaps more important is the fact that potential-energy calculations reveal that closure of the cross-bridge in echinomycin and triostin A severely restricts the number of allowed conformations of the peptide ring (G. Ughetto, personal communication). Consequently the conformation(s) adopted by echinomycin split product 1 may well be quite different from those that characterize 'TANDEM' and the naturally occurring quinoxaline antibiotics. The very much lower binding constant of 'ACIMBO' compared with that of 'BOCANTA' reinforces the conclusion that some form of cross-bridge is a necessary requirement for strong interaction with DNA.

In general, therefore, the structure–activity relations for quinoxaline antibiotics that emerge from this and earlier studies suggest that if further derivatives are to be produced the best approach might be to change the chromophores but retain the basic octapeptide backbone. In this way the molecule could retain a high affinity for DNA but be endowed with properties of particular use to medicine or molecular biology. For example, by changing the chromophores the hydrophilic/lipophilic balance could perhaps be altered to improve the chemotherapeutic index. Indeed, some success has already been claimed for this approach from the results of antibiotic-activity assays of quinoline analogues of echinomycin (Katagiri *et al.*, 1975; D. Gauvreau, personal communication). Further analogues could be produced containing chromophores that themselves possess considerable base preference (Müller *et al.*, 1975). In this way the base-sequence specificity of the molecule could probably be increased severalfold over that shown by the parent antibiotic.

The pattern of specificity displayed by 'TANDEM' in its interaction with different nucleic acids is quite remarkable; in complete contrast with the naturally occurring quinoxaline antibiotics (Wakelin & Waring, 1976; Lee & Waring, 1978), it shows a marked preference for (A+T)-rich DNA. The α parameter for 'TANDEM' as defined by Müller & Crothers (1975), calculated from the binding constants to *M. lysodeikticus* and *Cl. perfringens* DNA, is 0.105. Thus the compound apparently shows a distinct preference for sites containing three (A·T) base-pairs (cf. the values of α calculated by Lee & Waring, 1978).

Some degree of co-operativity has been suggested for the binding of quinomycin C and triostin A to poly(dA-dT) (Lee & Waring, 1978) and now a more striking example has been discovered with 'TANDEM'. The origins of this co-operativity remain obscure, but the phenomenon, though rare, is not unknown in ligand–nucleic acid interaction: co-operativity can apparently occur in the binding of ethidium to poly(dA-dT) (J. L. Bresloff & D. M. Crothers, unpublished work) and to poly(dG-dC) (Pohl *et al.*, 1972).

The extremely weak interaction of 'TANDEM' with poly(dG-dC) compared with poly(dA-dT) is intriguing. Because of the ability of these two polymers to bind the naturally occurring quinoxaline antibiotics reasonably tightly (Wakelin & Waring, 1976; Lee & Waring, 1978), it might be concluded that their conformations in solution share some similarities. Such a conclusion was reached by Arnott *et al.* (1974) from X-ray-diffraction studies on

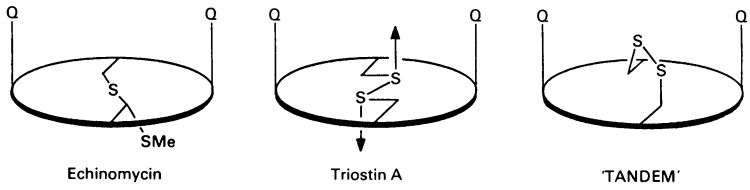


Fig. 10. *Schematic illustration of probable cross-bridge configurations in quinoxaline compounds*
The convention adopted is that proposed by Cheung *et al.* (1978); the peptide ring is represented as lying in a plane perpendicular to the page, with the quinoxaline chromophores (Q) projecting 'up' in the plane of the paper. In echinomycin the asymmetric $-SCH_3$ function of the thioacetal cross-bridge is represented in the favoured 'down' orientation (Cheung *et al.*, 1978); in 'TANDEM' the sulphur atoms of the disulphide cross-bridge are represented 'up', and in triostin A the two sulphur atoms are free to move above or below the average plane of the peptide ring as indicated by the arrows. See the text for discussion.

oriented fibres, although c.d. spectra did reveal differences in their solution configurations (Wells *et al.*, 1970; Pohl & Jovin, 1972). However, the natural DNA species studied also revealed the preference of 'TANDEM' for (A·T) base-pairs, and thus it must be considered that the specificity of 'TANDEM' may be determined by interaction with specific functional groups on the base-pairs. Now in the minor groove of the DNA helix, which would appear to be the most preferable binding site for quinoxaline compounds (Wakelin & Waring, 1976), the hydrogen-bond-forming groups on both types of base-pair are identical, except for the 2-amino group of guanine. Consequently, one plausible hypothesis for the strong (A·T)-base-pair specificity of 'TANDEM' is that the 2-amino group of guanine (or a hydration shell around it) causes steric hindrance with the peptide portion of 'TANDEM'.

Support for this view is provided by the binding to poly(dI-dC), which, compared with poly(dG-dC), simply lacks the 2-amino group of guanine. The binding of 'TANDEM' to this polymer is weak, but yields a binding constant at least three times that of poly(dG-dC). Neither echinomycin nor triostin A binds well to poly(dI-dC), and there is good evidence that it can adopt an unusual configuration in solution (Mitsui *et al.*, 1970). Thus the weak binding of 'TANDEM' is not unexpected, but still provides a strong contrast with the very feeble interaction with poly(dG-dC).

If this view of the origin of the (A·T)-base-pair specificity of 'TANDEM' is correct, then it may be possible to relate it to molecular structure by reference to the conformation adopted by 'TANDEM' compared with that adopted by the quinomycins and triostin A. The conformation adopted by echinomycin in solution has been extensively studied by n.m.r. and by empirical potential-energy calculations (Cheung *et al.*, 1978). The model proposed has a rigid symmetrical peptide ring with parallel chromophores extending roughly perpendicular to the mean plane of the peptide ring. The space between the chromophores is sufficient to accommodate two base-pairs. The thioacetal cross-bridge is oriented with the *S*-methyl group 'down' (the convention adopted is in relation to the chromophores, which are said to point 'up'). The polar conformation of triostin A and the conformation of 'TANDEM' as revealed by n.m.r. studies appear to be very similar to that of echinomycin (J. Kalman, personal communication). The conformation of the peptide in all cases shows only small differences.

This immediately suggests that to account for the observed differences in specificity special attention must be paid to the properties of the sulphur-containing cross-bridge. J. R. Kalman, T. J. Blake, D. H. Williams, J. Feeney & G. C. K. Roberts from unpublished work propose that the cross-bridge of triostin A in the polar conformation is in rapid equilibrium between three different conformers. Two of these can be described as having one sulphur atom up and one down, and the other has both sulphur atoms up. 'TANDEM', on the other hand, maintains a more rigid cross-bridge configuration with both sulphur atoms always up (J. Kalman, personal communication). This is to be compared with echinomycin, for which the preferred conformation has the *S*-methyl group of the cross-bridge down (Cheung *et al.*, 1978). These proposed conformations are presented schematically in Fig. 10.

The (A·T)-base-pair specificity of 'TANDEM' may thus be attributable to steric interference between the 2-amino group of guanine (or its hydration shell) and the sulphur atoms of 'TANDEM's' cross-bridge. For echinomycin, on the other hand, with the *S*-methyl group of the cross-bridge down there is little interference between the cross-bridge and the 2-amino groups of guanine nucleotides between the chromophores. Indeed, it appears possible to form hydrogen bonds between the 2-amino group and the carbonyl groups of the alanine residues of echinomycin or quinomycin C. For triostin A the cross-

bridge can adopt different conformations with similar energies (J. R. Kalman, T. J. Blake, D. H. Williams, J. Feeney & G. C. K. Roberts, unpublished work), one of which, with the two sulphur atoms up, would again interfere with the 2-amino group of guanine, whereas the other two conformations would not be expected to cause steric hindrance. Consequently by adopting different cross-bridge conformations, triostin A may be able to bind to many different types of site and thus show little base-sequence specificity.

Therefore, to some extent at least, the specificity patterns shown by quinoxaline compounds can be related to the structure and conformation of the sulphur-containing cross-bridge. It is possible that steric interference with the 2-amino group of guanine (or its hydration shell) is a general mechanism by which ligands can achieve (A·T)-base-pair specificity. This would, in part, explain why many (G·C)-specific ligands are intercalators, whereas many (A·T)-specific ligands bind to the outside of the helix (Müller & Gautier, 1975; Müller *et al.*, 1975).

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