EQUILIBRIUM CONSTANTS FOR THE BINDING OF AN HOMOLOGOUS SERIES OF MONOFUNCTIONAL AND BIFUNCTIONAL INTERCALATING DIACRIDINES TO CALF THYMUS DNA

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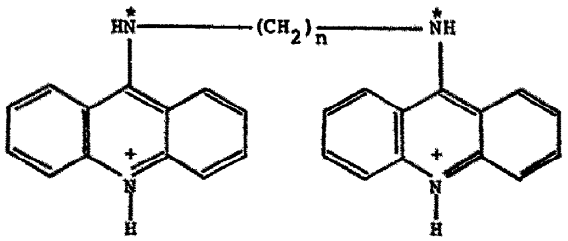
1. Introduction

The search for drugs having enhanced affinity and specificity for binding to DNA centres largely on bifunctional intercalating agents [1–9]. The expectation is that the free energy of binding of chromophores may be additive in polyfunctional compounds. However, many factors influence association constants, e.g., entropy and enthalpy effects due to hydration and stereochemical constraints imposed by the polymer, and it has not yet been established to what extent the transition from mono- to bifunctional reaction is accompanied by an enhanced binding constant. The present paper reports equilibrium binding measurements performed with an homologous series of diacridines in which two 9-aminoacridine rings are joined via their amino groups with a simple methylene chain [3]. For simplicity these compounds are referred to by the pseudonym C*n* where *n* is the number of carbon atoms in the connecting link (see table 1). The experiments were conducted in a buffer of high ionic strength specially selected to weaken the interaction sufficiently to bring the binding constants into the measurable range while avoiding precipitation of drugs and/or complexes so far as possible. We find that C2 and C4, previously shown to behave as monofunctional intercalators [5], have binding constants very similar to that of the 'parent' intercalating drug 9-aminoacridine whereas those of the bifunctional homologues C6 and C8 are 10- and 15-fold greater, respectively. C5, whose mode of interaction with DNA is peculiar [5], displays a cooperative Scatchard plot with an intrinsic association constant indistinguishable from that of 9-aminoacridine and a cooperativity parameter of approx. 10.

Table 1
Molar extinction coefficients



Compound	Buffer	50% (v/v) buffer–dimethyl sulphoxide
9-aminoacridine	$E_{400} = 1.08 \times 10^4$	$E_{403} = 1.04 \times 10^4$
C2	$E_{406} = 1.75 \times 10^4$	$E_{408} = 1.38 \times 10^4$
C4	$E_{408} = 1.80 \times 10^4$	$E_{411} = 1.94 \times 10^4$
C5	$E_{406} = 1.64 \times 10^4$	$E_{410} = 1.87 \times 10^4$
C6	$E_{411} = 1.78 \times 10^4$	$E_{414} = 1.61 \times 10^4$
C8	$E_{410} = 1.78 \times 10^4$	$E_{411} = 2.20 \times 10^4$

+ M.R.C. Training Fellow.

2. Materials and methods

The buffer contained 2 mM MES, 1 mM EDTA and 0.5 M NH_4F adjusted to pH 6.3 with ammonia solution. Reagent grade water from a Millipore Milli-Q2 system was used throughout. The bis-acridines

were synthesized and purified as the crystalline hydrated hydrochlorides by Dr. R. G. McR. Wright and stored in a desiccator in the dark at 0–4°C. Drug solutions were freshly prepared, avoiding the use of any organic solvents, and were maintained in the dark at room temperature. Siliconised glassware was used to minimise losses by adsorption to glass surfaces. Calf thymus DNA (highly polymerised sodium salt, type 1) was purchased from Sigma Chemical Company. Solutions of 1–2 mg/ml were obtained by homogenizing the DNA together with buffer till dissolved and sonicating lightly at 0° to reduce the viscosity. DNA concentrations were determined assuming a molar extinction coefficient of 6600 per nucleotide at 260 nm.

Binding curves were measured by equilibrium dialysis using an M. S. E. Dianorm apparatus. Dialysis cells having two 5-ml compartments separated by a Spectrapor 2 regenerated cellulose membrane (nominal mol. wt. cut-off 12–14 000) were loaded with approximately 4 ml of 152 μM calf thymus DNA in one chamber and the same volume of the appropriate drug solution in the other. The cells were rotated to establish equilibrium in a water bath at 25°C for 20 h, after which the drug concentration in each chamber was determined spectrophotometrically in 40 mm light-path quartz semi-micro cuvettes with a Unicam SP500 series II spectrophotometer. For the free drug side the molar extinction coefficients determined by direct weighing were used (see table 1); for the other side the complex was dissociated by addition of an equal volume of dimethyl sulphoxide (DMSO), and the total drug concentration was estimated using molar extinction coefficients determined for 50% (v/v) buffer–DMSO mixtures, also listed in table 1. Controls were performed to verify complete dissociation of the DNA complexes by DMSO over the entire range of binding levels studied. The concentration of bound ligand, equal to the difference between total and free drug concentration (c), was divided by the DNA concentration to yield the binding ratio r (mol drug bound/mol of nucleotide) and equilibrium isotherms were constructed in the form of Scatchard plots (r/c vs. r). The intrinsic association constant for an isolated potential binding site, $K(0)$, and the number of nucleotides occluded by a single bound drug molecule, n, were estimated by an iterative procedure designed to satisfy eq. (10) of McGhee and Von Hippel [10]:

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

given the experimentally determined values of r and c and an initial guess of n. Details of the computation were as previously described [4].

3. Results

Figure 1 shows a Scatchard plot for 9-aminoacridine in which the data are best fitted with an association constant of 4.64×10^3 M^{-1} and an occluded site size of 4.30 nucleotides per bound drug molecule. Binding parameters for all the compounds studied are collected in table 2. At the ionic strength used here (0.5) typical aminoacridines tend to self-associate with dimerization constants of approximately 2×10^3 M^{-1} [11]; thus the measured free drug concentration may over-estimate the true free monomer concentration at equilibrium. Neglect of dimerization has no effect on the value of $K(0)$ but increases the apparent occluded site size: for 9-aminoacridine

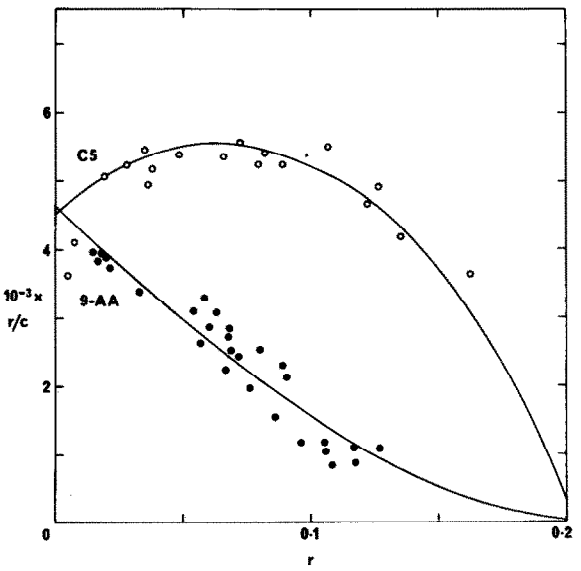


Fig.1. Scatchard plots for the interaction between 9-aminoacridine (•) or the diacridine C5 (○) and calf thymus DNA. The curves are theoretical, computed to fit eq. (10) (9-aminoacridine) or eq. (15) (C5) of McGhee and Von Hippel [10] using the parameters listed in table 2.

Table 2
Binding parameters

Compound	$10^{-3} \times K(0)$ (M^{-1})	n (nucleotides per bound drug molecule)
9-aminoacridine	4.64	4.30
C2	3.09	1.00
C4	4.15	2.22
C5	4.5	4.8
C6	44.5	3.34
C8	70.6	3.84

With the exception of C5 estimates of $K(0)$ and n were determined by a non-linear least squares fit to eq. (10) of McGhee and Von Hippel [10]. Data for C5 were fitted to their eq. (15) with $\omega = 9.5$

analysis of the data assuming a dimerization constant of 2×10^3 M^{-1} lowers n by 10% from 4.30 to 3.84 nucleotides.

Scatchard plots for all the diacridines examined extend over a limited range of binding ratios due to slow precipitation of the complexes above $r = 0.12$–0.16. It was not possible to determine dimerization constants for these compounds by optical methods since they failed to deviate from

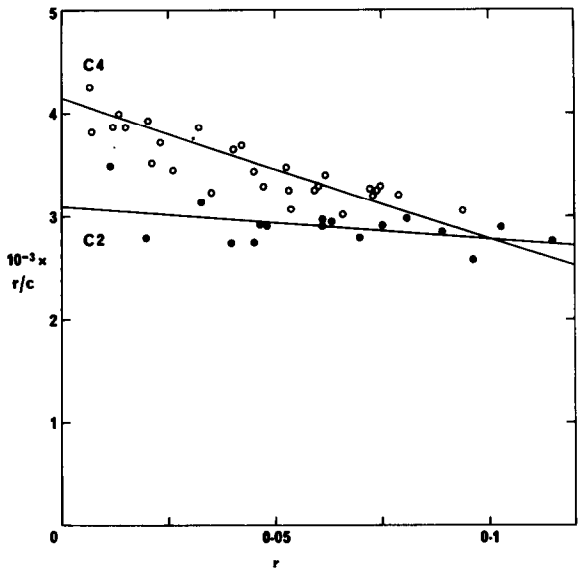


Fig.2. Scatchard plots for the binding of diacridines C2 (•) and C4 (○) to DNA. The curves are theoretical, computed to fit eq. (10) of McGhee and Von Hippel [10] using the values of $K(0)$ and n listed in table 2.

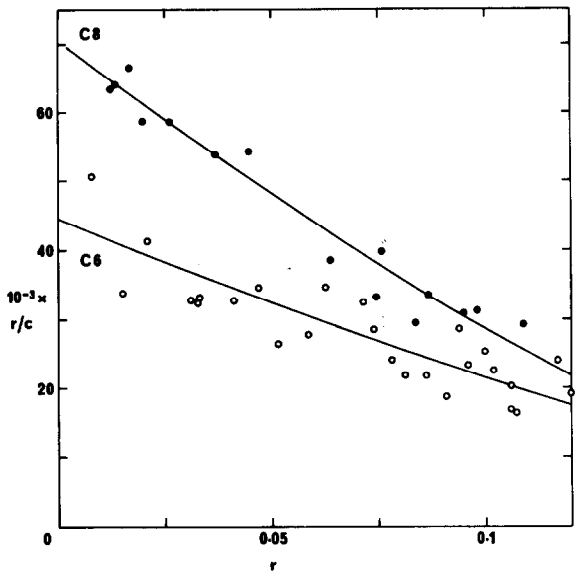


Fig.3. Scatchard plots for the binding of bifunctional diacridines C6 (○) and C8 (•) to DNA. The curves represent eq. (10) of McGhee and Von Hippel [10] with $K(0)$ and n as listed in table 2.

Beer's law at the highest accessible concentrations, despite clear evidence of aggregation in n.m.r. experiments [12]. For these reasons the values of n given in table 2 are best regarded as upper limits. The binding isotherms for C2 and C4 (fig.2) yield values for $K(0)$ and n of 3.09×10^3 M^{-1} and 1.0 nucleotides and 4.15×10^3 M^{-1} and 2.22 nucleotides, respectively. These association constants are similar to that of 9-aminoacridine though the shapes of the curves are noticeably more shallow, reflecting the very low apparent values of n. In contrast, the association constants for the longer chain bifunctional C6 and C8 derivatives (4.45×10^4 M^{-1} and 7.06×10^4 M^{-1}, respectively; fig.3 and table 2) are at least an order of magnitude greater than those for 9-aminoacridine and the monofunctional homologues C2 and C4. Thus, there is a marked increase in binding constant associated with the transition from monofunctional to bifunctional reaction [5] in this series of diacridines. The enhanced affinities of C6 and C8 for DNA diminish the possible effects of drug dimerization on their Scatchard plots because the total free ligand concentrations did not exceed 6 μM over the measured range. Allowing for a dimerization

constant of 10^4 M^{-1}, a reasonable estimate for diacridines [12], decreases n for C8 by only 6% from 3.86 to 3.60 nucleotides.

As in previous studies [5] the data for C5 are conspicuously different from those for either the monofunctional or bifunctional diacridines. The Scatchard plot is humped, concave downwards, indicative of a cooperative binding process which is not satisfactorily described by eq. (10) of McGhee and Von Hippel [10]. A cooperativity parameter, ω, must be introduced as in eq. (15) of ref. [10] which yields values of $K(0) = 4.5 \times 10^3$ M^{-1}, $n = 4.8$ nucleotides and $\omega = 9.5$. The binding constant for C5 is thus indistinguishable from that of 9-aminoacridine and is similar to the values found for the monofunctional diacridines. Allowing for possible dimerization serves only to enhance the cooperativity parameter from 9.5 to 11.2 and to reduce n from 4.5 to 4.2 nucleotides.

4. Discussion

The values of $K(0)$ determined for C2, C4, C6 and C8 provide clear evidence that progression from mono- to bifunctional intercalation in an homologous series of diacridines is accompanied by substantially enhanced affinity constants. If the free energy of binding of the two chromophores were simply additive, one would expect the association constant of a bifunctional compound to approximate to the square of that for the corresponding monofunctional molecule. The binding constants for C6 and C8, being 10- and 15-fold greater than those for C2 and C4, fall well below the value of approximately 10^7 M^{-1} estimated in this way. This suggests that there may exist considerable entropy effects related to solvent rearrangement around the DNA, and/or unfavourable enthalpies and entropies associated with distortion of both nucleic acid and drug in the formation of the intercalated complex. In other studies with dimeric acridines and phenanthridines [2,8] it appeared that binding constants for bis-intercalators compared to simple monomers might reach those predicted by the simplistic model. However, the linker chains in some of these compounds contained functional groups able to form additional ionic and hydrogen bonds to the DNA which were lacking in the monomeric units. The existence of energetically unfavourable components in dimer binding is also implicit in the affinity constants for C2 and C4 which are similar to that for 9-aminoacridine: one might naively have expected them to be greater because of additional electrostatic attraction between the non-intercalated chromophore and the sugar phosphate backbone. Perhaps the expected increase in enthalpy is counteracted by unfavourable entropy terms of the sort suggested above.

It is unfortunate that the apparent values of n, the parameter of site-size, determined in this work are too unreliable to provide effective comparison with theoretical expectations for bis-intercalation with or without neighbour exclusion [5]. However, apart from the intrinsic uncertainty in n occasioned by possible dimerization of drugs leading to inaccuracy in the estimation of true free monomer concentration, it is dubious whether eq. (10) of McGhee and Von Hippel [10] would ever provide reliable results for n in this situation. Indeed, there may not be a unique value of n that describes the entire binding isotherm. For compounds capable of both mono- and bifunctional intercalation, such as C6 and C8, the apparent value of the excluded site size might depend upon the binding level since bifunctional reaction is expected to dominate at low values of r with monofunctional reaction becoming significant at high free-ligand concentrations [13].

The enigmatic character of C5, first revealed by hydrodynamic measurements [5], extends to its binding behaviour as shown by the cooperative Scatchard plot (fig.1). Its intrinsic binding constant to an isolated site is typical of those found for the monofunctionally intercalating ligands so that the interaction could be viewed as simple intercalation with moderate cooperativity arising from ligand–ligand or ligand–site interactions. On the other hand, a humped binding curve may also result from mixed modes of intercalation where monofunctional behaviour dominates at low r and gives way to (conformationally induced) higher affinity bifunctional reaction with increasing levels of binding. Whilst this interpretation runs contrary to arguments based on straightforward thermodynamic principles [13], it does accord with the apparent increase in extension of the helix associated with binding of C5 as r rises [5].

Acknowledgements

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