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The interaction of ethidium with synthetic double-stranded polynucleotides at low ionic strength

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ABSTRACT

The interaction of ethidium with synthetic DNA and RNA double-stranded polymers at 0.01 M ionic strength, pH 7.0, has been studied by fluorimetry at low drug to nucleotide ratios. Binding constants have been calculated assuming an excluded-neighbouring site model for the interaction of ethidium with double-stranded polymers. The values obtained are poly d(AT).poly d(AT), 9.5×10^6 M^{-1}; poly dA.poly dT, 6.5×10^5 M^{-1}; poly d(GC).poly d(GC), 9.9×10^6 M^{-1}; poly dG.poly dC, 4.5×10^6 M^{-1}; poly d(AC).poly d(GT), 9.8×10^6 M^{-1}; poly d(AG).poly d(CT), 1.3×10^6 M^{-1}; poly rA.poly rU, 4.1×10^7 M^{-1}. The displacement of ethidium from poly d(AT).poly d(AT) by 9-aminoacridine and an acridine-containing antitumour agent (NSC 156303; 4'-(9-acridinylamino)methanesulphon-m-anisidide) has also been examined.

INTRODUCTION

Ethidium bromide is a trypanocidal drug which interacts with double-stranded DNA and RNA by intercalating between adjacent base pairs[1,2]. It has found many uses, including the isolation of covalently closed circular DNA[3], the analysis of different topological forms of DNA[4] and the probing of chromatin structure[5]. The approximately 50-fold increase in fluorescence yield which occurs when it intercalates[6] makes it a useful probe to measure changes in DNA or RNA sites available during, for example, the binding of another drug species. We have been interested in using an ethidium displacement technique[6] to measure the binding constants of a number of DNA-binding anti-tumour drugs, in particular a series of compounds related to the acridine derivative m-AMSA[7] (NSC 156303), synthesised by Cain and Atwell[8]. Before comparing the interactions of acridine

derivatives with defined intercalation sites, the interaction of ethidium itself with double-stranded polynucleotides of known sequence had first to be determined.

A low ionic strength buffer has been chosen because of the low solubility of many intercalating drugs in the presence of salt. It was found in preliminary experiments that, at low ionic strength, ethidium fluorescence is not directly proportional to the concentration of bound ethidium, i.e. some self-quenching occurs (cf ref. 6). Therefore, the technique used by Le Pecq and Paoletti at high ionic strength[6] cannot be applied directly. The approach used here has been to measure both the free drug concentration and the drug:base pair binding ratios at low ethidium concentrations. By assuming an excluded-neighbouring site model for ethidium binding[2] and using a mathematical treatment for such a model[9] the binding constants for a number of synthetic double-stranded polynucleotides have been measured.

METHODS

Polynucleotides: Poly d(AG).poly d(CT) was kindly provided by Prof. A.R. Morgan, Dept. of Biochemistry, University of Alberta, Canada. Poly dA.poly dT, poly d(GC).poly d(GC), poly dG.poly dC, poly d(AC).poly d(GT) and poly rA.poly rU were obtained from Boehringer Mannheim, Germany. Poly d(AT).poly d(AT) and other samples of poly d(GC).poly d(GC) and poly dG.poly dC were obtained from Miles Research Products, U.S.A. Both samples of poly dG.poly dC had first to be brought to pH 12.5 with NaOH, then dialysed back to pH 7 as described by Wartell et al.[10], in order to give reproducible results. All polymers were dissolved in 0.01 SHE buffer[11] (9.4 mM NaCl, 20 µM EDTA, 2 mM HEPES adjusted to pH 7.0 with NaOH) before use.

Agents: m-AMSA was provided by Dr. B.F. Cain, Cancer Chemotherapy Laboratory, Auckland. Ethidium bromide and 9-aminoacridine were purchased from the Sigma Chemical Co., U.S.A.

Fluorimetry: A Zeiss PMQ-2 spectrophotometer with a ZFM-4 fluorescence attachment was used at the maximum sensitivity.

Excitation of the dust-free buffer solution (1 cm cuvettes containing initially 3.04 ml 0.01 SHE buffer) was achieved using a high pressure mercury lamp and 546 nm (M546) filter). Fluorescence emission was measured at 595 nm (slit width 0.75 mm). Polynucleotide and ethidium solutions in 0.01 SHE buffer were added to the cuvette with an Agla micrometer syringe through a Portex catheter.

RESULTS

Determination of ethidium : base pair ratios and free ethidium concentrations: A small amount (0.2-2 μM in base pairs) of the polynucleotide was introduced into the fluorescence cuvette, and a range of ethidium concentrations (0.01-0.16 μM) was delivered by microsyringe from a stock solution (26 μM in 0.01 SHE buffer). The fluorescence readings, after substraction of the reading for buffer plus polynucleotide alone, were divided by the corresponding base pair concentration (corrected for volume changes) to give the specific fluorescence. Corrections for absorption of incident light and for the fluorescence of free ethidium were negligible. The specific fluorescence was plotted against total ethidium concentration for a range of base pair (S_t) concentrations (Fig. 1). For selected values on the ordinate (0.1-0.15 $\log_{10}$ intervals) sets of values of total ethidium (E_t) and base pair (S_t) concentrations could be determined. These were plotted as in Fig. 2, together with least-squares regression lines.

If ν_E is the ratio of bound ethidium (E_b) to total sites (S_t), and E_f is the concentration of free ethidium, it follows that the total ethidium concentration (E_t) is given by the equation:

$$E_t = \nu_E S_t + E_f \quad \ldots\ldots\ldots \quad (1)$$

The slope and intercept of each line in Fig. 2 therefore correspond to ν_E and E_f respectively.

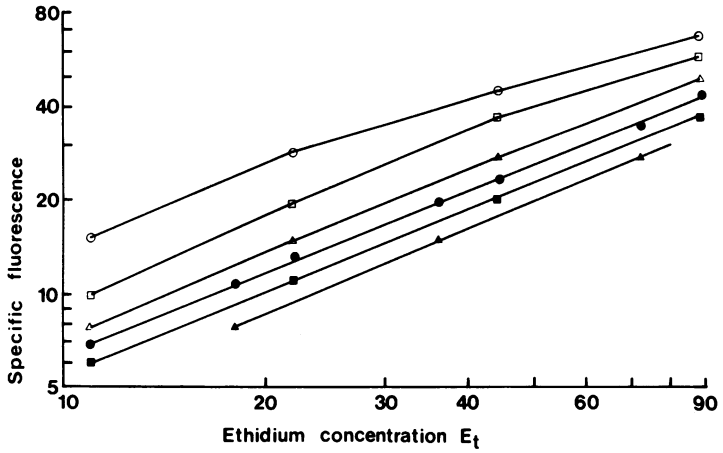

Fig. 1

Relationship between specific fluorescence (measured fluorescence divided by DNA concentration, arbitrary units) and total added ethidium concentration (E_t) for a series of concentrations (0.12, 0.24, 0.36, 0.48, 0.60, 0.72 μM in base pairs) of poly d(AT).poly d(AT).

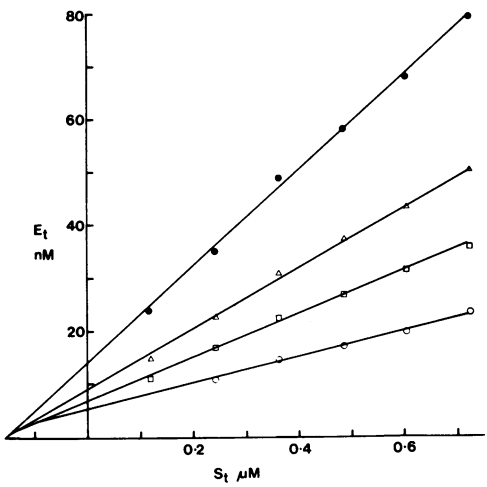

Fig. 2

Relationship between total added ethidium (E_t) and total added poly d(AT).poly d(AT) (S_t) at the following values for specific fluorescence: 10 (o—o); 15 (□—□); 20 (Δ—Δ) and 30 (●—●). Values are taken from Fig. 1.

Determination of ethidium binding constants: The rationale adopted here assumes that the double-stranded polynucleotide behaves as a lattice containing S_t binding sites[9]. The binding constant (K_E) for ethidium is given by the expression:

$$K_E = \frac{E_b}{E_f \cdot S_f}$$

where S_f is the concentration of free ethidium binding sites. If R is defined as the ratio of free binding sites to total lattice sites (i.e. $R = S_f/S_t$), the equation may be re-written as

$$K_E = \frac{\nu_E}{E_f R} \quad \ldots\ldots\ldots \quad (2)$$

In the mathematical treatment of McGhee and von Hippel[9], if a bound drug molecule occupies n lattice sites, R is given by:

$$R = (1-n\nu)\left(\frac{1-n\nu}{1-(n-1)\nu}\right)^{n-1}$$

Assuming that a bound intercalating molecule occupies two lattice sites[2], then

$$R = \frac{(1-2\nu)^2}{1-\nu} \quad \ldots\ldots\ldots \quad (3)$$

and

$$\frac{\nu}{R} = \frac{\nu(1-2\nu)^2}{1-\nu} \quad \ldots\ldots\ldots \quad (4)$$

The relationships between ν, R and $\frac{\nu}{R}$ can be calculated and expressed in graphical (Fig. 3) or tabular form. In the case of Fig. 2, for each value found for ν_E, the corresponding value of $\frac{\nu_E}{R}$ may be found using equation (4). The corresponding value of E_f, together with equation (2), gives the binding constant. The mean value and standard deviation of K_E for each of the polynucleotides tested is shown in Table I.

Determination of binding constants for acridine derivatives: Both 9-aminoacridine and m-AMSA are known to bind to double-stranded DNA by intercalation[11], and it was assumed that they have the same site size as ethidium[2]. The procedure outlined above for poly d(AT).poly d(AT) was repeated in the presence

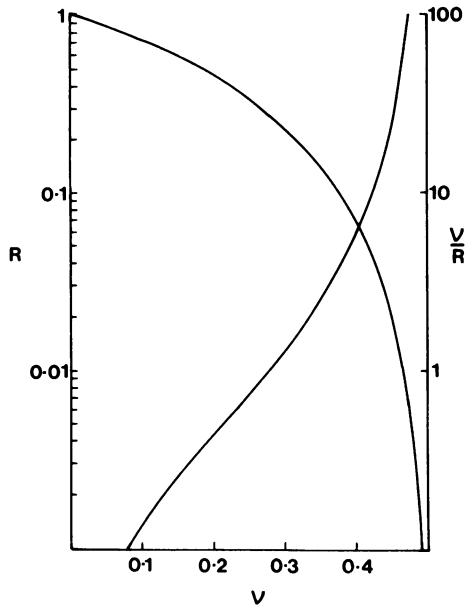

Fig. 3

Theoretical relationships between R (line with negative slope) or $\frac{\nu}{R}$ (line with positive slope) and ν.

Table I. Binding affinities of ethidium for double-stranded polynucleotides

Polynucleotide	λ_{max}	ε_m^* x10^{-3}	K_E x10^{-6} M^{-1}
Poly d(AT).poly d(AT)	260	6.8	9.5 ± 0.9
Poly dA.poly dT	260	6.0	0.65± 0.04
Poly d(GC).poly d(GC)	254	8.4	9.9 ± 0.5
Poly dG.poly dC	253	7.4	4.5 ± 0.5
Poly d(AC).poly d(GT)	260	6.5	9.8 ± 1.7
Poly d(AG).poly d(CT)	260	5.7	1.3 ± 0.1
Poly rA.poly rU	257	5.8	41 ± 6

* ε_m values (in terms of nucleotide concentration) are quoted from Wells et al.[12] for the DNA samples, and from the manufacturers' specifications for poly rA.poly rU.

of 1.6 μM 9-aminoacridine or 2.2 μM m-AMSA (Figs 4 and 5). At these concentrations, the specific fluorescence of ethidium was reduced by 50-60% compared to that in the absence of drug.

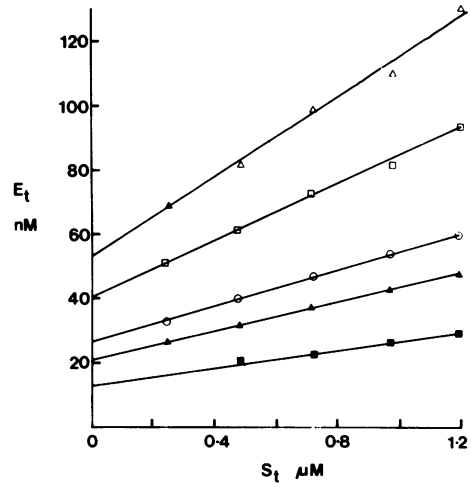

Fig. 4

Relationship between total added ethidium (E_t) and total added poly d(AT).poly d(AT) (S_t) in the presence of 9-aminoacridine (1.65 μM) and at the following values for specific fluorescence: 5 (■—■); 8 (▲—▲); 10 (o—o); 15 (□—□) and 20 (Δ—Δ). Methods as for Figs 1 and 2.

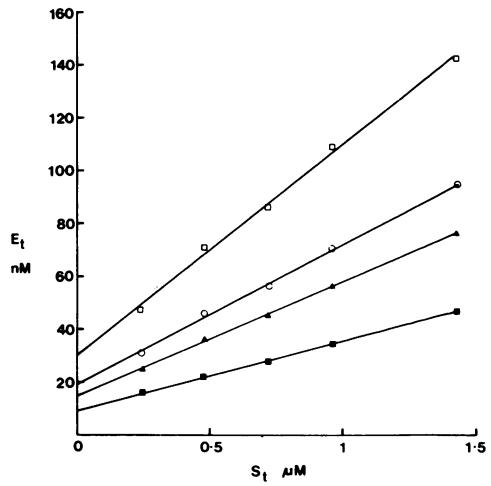

Fig. 5

Relationship between total added ethidium (E_t) and total added poly d(AT).poly d(AT) (S_t) in the presence of m-AMSA[7] (2.2 μM) and at the following values for specific fluorescence: 5 (■—■); 8 (▲—▲); 10 (o—o) and 15 (□—□). Methods as for Figs 1 and 2.

Control experiments indicated that neither acridine derivative fluoresced itself under these conditions, and neither affected the fluorescence of free ethidium. From the values of the

slopes and intercepts of the lines in Figs 4 and 5, ν_E and E_f were determined, and using equation (2), R values were calculated. The fraction of the total sites occupied (ν) is ($\nu_E + \nu_D$), where ν_D is the fraction of the total sites occupied by the acridine drug. Since ν can be calculated from R using equation (3) (Fig. 3), corresponding values for ν_D can be determined. The binding constant (K_D) is then calculated using the equation (cf equations 1 and 2):

$$K_D = \frac{\nu_D}{D_f R} = \frac{\nu_D}{(D_t - \nu_D S_t)R}$$

An average value was used for S_t. The values obtained for the binding constants of 9-aminoacridine and m-AMSA were $1.9 \pm 0.4 \times 10^6$ M^{-1} and $4.3 \pm 0.8 \times 10^5$ M^{-1} respectively.

Quenching of ethidium flucrescence by bound m-AMSA: In graphs such as Figs 2, 4 and 5, the relationship between the specific fluorescence and the corresponding value of ν_E measures the fluorescence quantum yield of bound ethidium. A decrease in specific fluorescence at constant ν_E would indicate quenching of fluorescence. In the case of m-AMSA, the values for specific fluorescence were 50% lower than that for ethidium alone at the same ν_E value, indicating considerable quenching. In the case of 9-aminoacridine, values for specific fluorescence were less than 5% lower, indicating little or no quenching.

DISCUSSION

In the case of ethidium there is now considerable evidence from binding isotherms[1] model building studies[2] X-ray diffraction data of ethidium-dinucleotide complexes[13,2] and X-ray diffraction data of DNA bound to a platinium-phenanthridine derivative[15], that intercalation excludes occupancy of the two neighbouring inter-base pair sites, thus justifying the use of this model. The treatment used to derive binding constants assumes that in this case neither positive nor negative interactions occur between bound drug molecules [9].

It is clear from Table I that DNA polymers containing alternating purine-pyrimidine sequences bind much more strongly to ethidium than those containing purine-purine and pyrimidine-pyrimidine sequences. This is consistent with the preference of ethidium to form complexes with purine-pyrimidine dinucleotides [13,14]. It should be noted that the alternating polymers have two types of intercalation site, and that the treatment used would give an average value if the binding constants for the two sites are different. If one takes the logarithmic mean of the different binding constants, in the proportions represented in calf thymus DNA, one obtains a binding constant of 3.5×10^6 M^{-1}. Using spectrophotometric measurements in 0.02 M buffer, Wakelin and Waring[16] obtained a value of 2.5×10^6 M^{-1}.

The strong binding of ethidium to the ribopolymer poly rA· poly rU was unexpected. However, this result is consistent with the presence of a strong binding site for ethidium between successive A-U pairs in phenylalanyl transfer RNA[17].

The derivation of binding constants for 9-aminoacridine and m-AMSA demands a number of assumptions, both in terms of the binding model (i.e. that the acridine derivatives and ethidium bind to equivalent sites) and in the mathematical calculation. Combination of equations (1) and (2) gives the expression:

$$E_t = \nu_E S_t + \frac{\nu_E}{K_E R}$$

The second part of the expression is a constant only if R is independent of S_t. However, as S_t increases, the concentration of the free acridine derivative will decrease, and R will increase slightly. The slopes of the lines in Figs 4 and 5 therefore correspond to slightly less than the true values of ν_E. Alternative methods for calculating K_D values by ethidium displacement will be discussed elsewhere (W.R. Wilson and B.C. Baguley, manuscript in preparation).

At the concentration used for the data in Fig. 5, m-AMSA causes approximately 50% quenching of the fluorescence of bound ethidium. The values for ν_D found in this experiment were in the range 0.23 ± 0.03. The simplest model to explain this

quenching is that m-AMSA fully quenches the fluorescence of any ethidium bound to an adjacent (non-excluded) site. The chance that a bound ethidium molecule is adjacent to m-AMSA (i.e. on either side) is equal to 2 ν_D, or 0.46 ± 0.06. Thus one would expect quenching of 46 ± 6%. Such quenching does not occur with 9-aminoacridine.

In conclusion, ethidium is an extremely useful fluorescent probe in investigating interactions between double-stranded polynucleotides and other molecules. It should be kept in mind, however, that ethidium does not bind with equal affinity to all inter-base pair sites, and that decreases in ethidium fluorescence may occur by fluorescence quenching as well as by displacement.

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REFERENCES

1 Waring, M.J. (1965) J. Mol. Biol. 13, 269-282
2 Sobell, H.M., Tsai, C.-C., Jain, S.C., and Gilbert, S.G. (1977) J. Mol. Biol. 114, 333-365
3 Bauer, W., and Vinograd, J. (1965) J. Mol. Biol. 33, 141-171
4 Morgan, A.R., and Pulleyblank, D.E. (1974) Biochem. Biophys. Res. Commun. 61, 396-403
5 Lawrence, J.-J., and Daune, M. (1976) Biochemistry 15, 3301-3314
6 Le Pecq, J.-B., and Paoletti, C. (1967) J. Mol. Biol. 27, 87-106
7 Abbreviation: m-AMSA: 4'-(9-acridinylamino)methanesulphon-m-anisidide.
8 Cain, B.F., and Atwell, G.J. (1974) Eur. J. Cancer 10, 539-549
9 McGhee, J.D., and von Hippel, P.H. (1974) J. Mol. Biol. 86, 469-489
10 Wartell, R.M., Larson, J.E., and Wells, R.D. (1974) J. Biol. Chem. 249, 6719-6731

11 Waring, M.J. (1976) Eur. J. Cancer 12, 995-1001
12 Wells, R.D., Larson, J.E., Grant, R.C., Shortle, B.E., and Cantor, C.R. (1970) J. Mol. Biol. 54, 465-497
13 Tsai, C.-C., Jain, S.C., and Sobell, H.M. (1977) J. Mol. Biol. 114, 301-315
14 Krugh, T.R., and Reinhardt, C.G. (1975) J. Mol. Biol. 97, 133-162
15 Lippard, S.J., Bond, P.J., Wu, K.C., and Bauer, W.R. (1976) Science 194, 726-728
16 Wakelin, L.P.G., and Waring, M.J. (1974) Mol. Pharmacol. 9, 544-561
17 Jones, C.R., and Kearns, D.R. (1975) Biochemistry 14, 2660-2665