A STUDY OF THE BINDING OF METHYLENE BLUE, THIONIN, AZURE A, AZURE B, AND AZURE C TO DNA

Scott A. Burton and Raymond N. Castle*

Department of Chemistry, Brigham Young University Provo, Utah 84602 USA

Abstract — Methylene Blue, Thionin, Azure A, Azure B, and Azure C were found to bind strongly to DNA probably *via* intercalation. Binding affinity is the result of several factors including π moment along the long axis and dipole-dipole coupling both constrained by steric effects

INTRODUCTION

It has long been known that planar, heterocyclic molecules interact with,and pigment nucleic acids. Inhibition of synthesis and transcription of DNA[1] precipitating inhibition of protein synthesis[2] is observed when basic dyes are administered to biological systems. Among the many basic dyes, Methylene Blue, Thionin, Azure A, Azure B, and Azure C are excellent nuclear stains[3-5] and have been shown to inhibit nuclear division.[6] The pharmacological properties of these dyes very probably result from the binding of the drugs to DNA.

Deoxyribonucleic acid has been implicated as the *in vivo* receptor for antineoplastic drugs such as the anthracyclines,[7,8] actinomycin,[9] coralyne,[10,11] and ellipticine,[12] antitrypanosomal drugs such as the phenanthridines,[13] antibacterial drugs such as berberine[14] and echinomycin,[15] and antimalarial drugs such as the acridines[13] and isoquinolines.[16] These heterocyclic molecules have been shown to bind to DNA *via* intercalation where the DNA helix unwinds and the molecule situates itself between the base pairs[17-21] and *via* external stacking where the molecule is bound to the surface of the helix.[20,21]

Since many phenothiazine analogs bind to DNA *via* intercalation, it is probable that Methylene Blue, Thionin, Azure A, Azure B, and Azure C bind to DNA in like fashion The partly extended DNA molecule containing intercalated molecule layers differs from native DNA in several properties measurable in solution including. (a) The DNA helix is locally longer and stiffer enhancing its viscosity; (b) The micro-environment of the intercalated molecule is changed resulting in spectral alterations, and (c) The average mass per unit length of the DNA molecule is decreased reducing its sedimentation coefficient. The present work investigates the binding of Methylene Blue, Thionin, Azure A, Azure B, and Azure C to DNA using viscosity measurement, spectrofluorometric titration, ultracentrifugation methods, and MO calculations.

An increase in specific viscosity and a decrease in the sedimentation coefficient is observed when DNA interacts with these homologs. Each respective increase and decrease is generally proportional with the magnitude of each association constant.

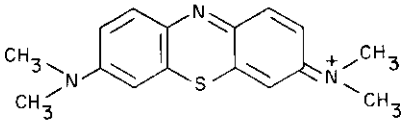
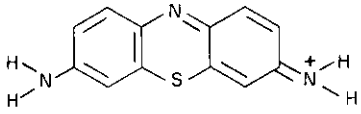
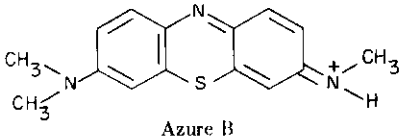
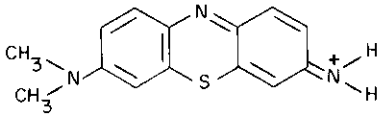
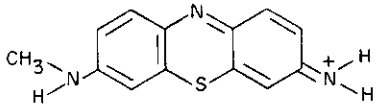
Spectroscopic studies with Methylene Blue,[23,24] Thionin, Azure B,[25] and Azure A[26] indicate that they bind to DNA with a maximum of 0.22 molecules per DNA phosphorus atom. This would be the expected maximum occupancy if the molecules enter at random, subject to the restriction that adjacent spaces may not be occupied.[27] All dyes in this study were found to bind to DNA with a maximum of 0.22 ± 0.02 molecules per DNA phosphorus atom.

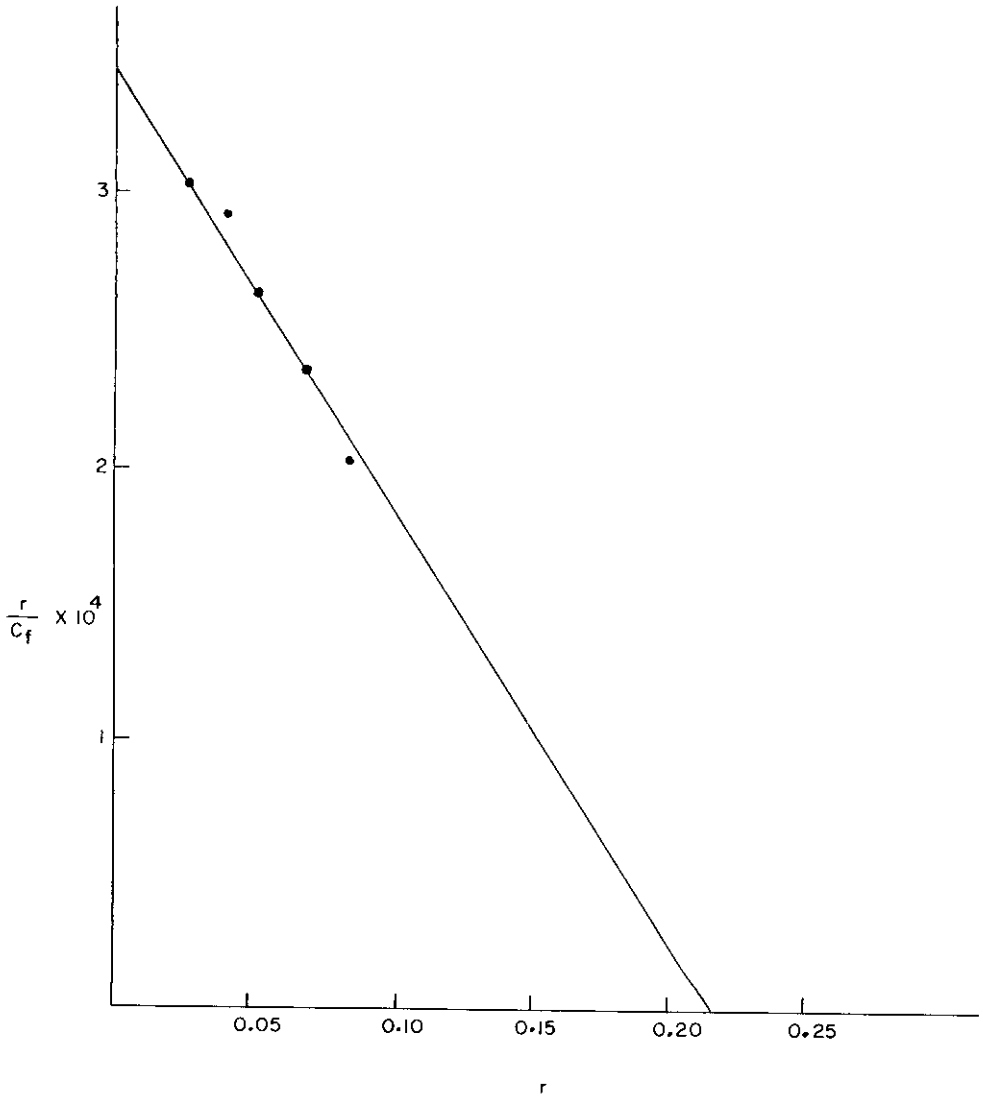
Upon binding with DNA all dyes display a bathochromic shift of 2 to 3 nanometers in the excitation spectra accompanied by a significant decrease in fluorescence. Both phenomena can be attributed to the interaction of the heterocyclic ring system of the bound molecule with the purine and pyrimidine bases of the DNA.[22]

Affinity of the phenothiazine dyes for DNA appears to involve a combination of factors. Hückel MO calculations reveal an apparent correlation between the π moments along the long axis of the molecules and binding affinity. Methylene Blue and Thionin which have no π moment along the long axis show similar association constants compared to the other homologs even though they are at opposite ends of the methylation spectrum Steric conformation of the molecule may also be a factor involved with binding affinity. Other than Thionin, addition of methyl groups to the parent ring structure generally decreases binding affinity. It is possible that methylation restricts binding so that it can occur only with the methylated amino groups protruding from the helix limiting the area that the molecule can be in contact with the base pairs of DNA. However methylation of the amino groups may only involve steric-electronic effects.

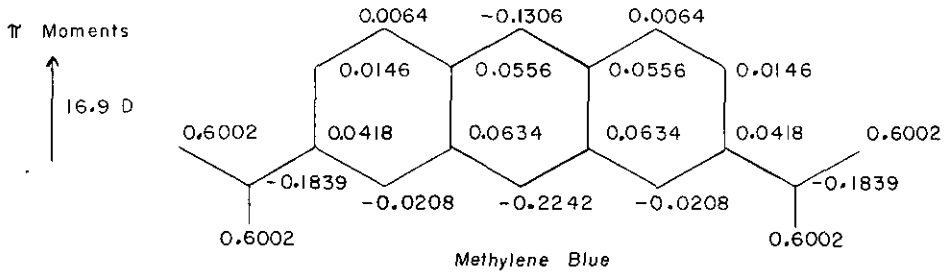
A correlation between fluoresence quenching and the association constant for each dye is apparent. The values for each molecule satisfy the following empirical equation: $K\,V^{0.637} = \text{constant}$ where K is the association constant, V is the ratio between the fluorescence intensity emitted by the bound and the free dye when excitation is produced under the same conditions of wavelength, concentration, temperature, and solvent, and the constant is 3.1×10^4. The quantity V is a measure of the charge transfer interaction. A small V value results from a large charge transfer indicating intimate dipole-dipole coupling between the heterocyclic molecule and the DNA base pairs. Affinity for DNA by these heterocyclic ligands is

the result of several factors including π moment along the long axis and dipole-dipole coupling both constrained by steric effects.[28]

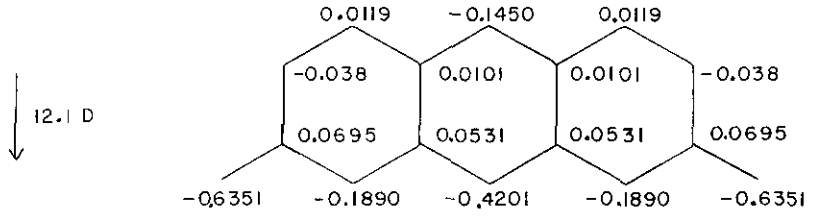
		K	V	η sp	S_{20}
DNA		–	–	0.0693	26.4
 Methylene Blue	Cl^-	9.0 X 10^4	0.190	0.0743	26 0
 Thionin	Cl^-	9.3 X 10^4	0.180	0 0740	25.9
 Azure B	Cl^-	1.0 X 10^5	0.157	0.0754	23.1
 Azure A	Cl^-	1.2 X 10^5	0.120	0.0769	24.1
 Azure C	Cl^-	1.6 X 10^5	0.077	0.0780	22.4



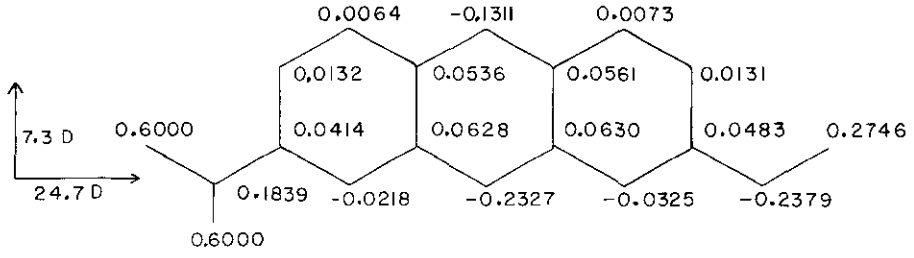
Plot of binding data for Azure C. C_f is the free dye concentration and r is the ratio of bound dye per nucleic acid phosphate.



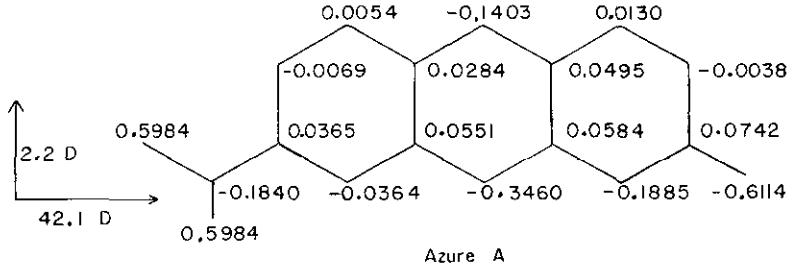
Methylene Blue



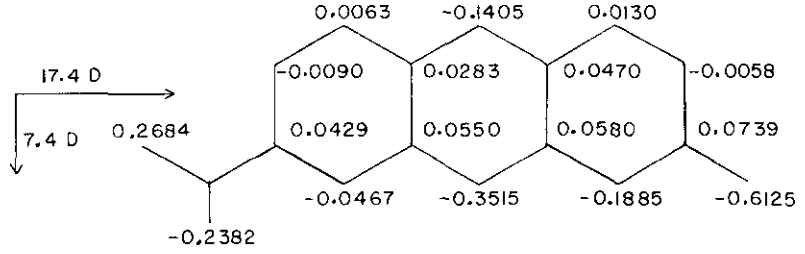
Thionin



Azure B



Azure A



Azure C

While none of these individual analytical procedures unequivocally proves whether or not a given molecule binds to DNA *via* intercalation, each result leads toward such a conclusion.

MATERIALS

Thionin, Azure A, Azure B, and Azure C were obtained from Eastman Kodak Co. Methylene Blue was obtained from MCB. All dyes were purified by column chromatography using neutral aluminum oxide as the adsorbent and an alcohol-benzene mixture as the eluent.[29] Calf thymus DNA was purchased from Calbiochem and used without further purification. Preliminary binding studies conducted using this DNA with proflavine or ethidium bromide gave results similar to the literature.[30-31] Phosphorus-DNA concentration was determined spectrophotometrically using the extinction coefficient 6412 $M^{-1}cm^{-1}$ at 260 nm. Bacterial phage λ DNA was purchased from Miles Laboratories and used without further purification. Phosphorus-DNA concentration was determined using the extinction coefficient 6700 $M^{-1}cm^{-1}$ at 260 nm.

FLUORESCENCE MEASUREMENTS

A Spex Fluorolog was used for measurement of excitation spectra. Excitation and emission monochrometers were positioned for maximum signal corresponding to the absorption and fluorescence maxima. Aliquots of 0.40 ml of a 1.00×10^{-4} *M* dye in 0.1 *M* Tris (tris(hydroxymethyl)aminomethane) and 0.001 *M* sodium chloride adjusted to *p*H 7.5 were added to three separate 2.00 ml solutions in a standard fluorometric cuvette: (a) solution 1: Buffer; 0.1 *M* Tris (*p*H 7.5) and 0.001 *M* sodium chloride; (b) solution 2: DNA; 1.4×10^{-4} *M* phosphorus-DNA, 0.1 *M* Tris (*p*H 7.5), and 0.001 *M* sodium chloride, and (c) solution 3. DNA Blank, 2×10^{-3} *M* phosphorus-DNA, 0.1 *M* Tris (*p*H 7.5), and 0.001 *M* sodium chloride. Titrations were run at room temperature. Spectrofluorimetric evaluation of the concentration of bound dye molecules was calculated as described by LePecq and Paoletti.[31]

$$c_b = \frac{I_2 - I_1}{(V - 1)k}$$

where c_b is the concentration of the bound dye in moles per liter, I_1 is the fluorescence intensity of a given concentration of dye in solution one, I_2 is the fluorescence intensity of a given concentration of dye in solution two, k is the number which relates fluorescence intensity with dye concentration in solution one ($I_1 = kc_1$), and V the ratio of I_3 to I_1 for a given concentration of dye. Due to the dimerization of the phenothiazine homologs at high concentration, the maximum $\bar{n}$ value of 0.22 molecules per DNA phosphorus atom was obtained by extropolation of the several points obtained at low dye concentration.

The equilibrium constants are computed using least-squares curve fitting of the data.[32]

ANALYTICAL ULTRACENTRAFUGATION.

Sedimentation coefficients were determined by boundary sedimentation in a Beckman model E analytical ultracentrifuge using uv optics. A 12 mm charcoal-filled Epon type II centerpiece and plain quartz windows were used. The sedimentation solvent consisted of 0.58 ml of 1.000 *M* sodium chloride and 0.100 *M* Tris (*p*H 7.5). Dye concentrations were 1.00×10^{-4} *M*. The solution in the sample well was 20 μl. of 1.5×10^{-4} *M* viral DNA in 0.100 *M* Tris (*p*H 7.5). Run conditions were 26,000 rpm in an An-D rotor at 20.0° with a photograph taken every 8 minutes. A Schoefel film densitometer was used to trace the absorption density. Sedimentation coefficients were determined at 20.0° and are uncorrected for viscosity, buoyancy or DNA concentration.

VISCOMETRY.

Viscosity was measured with an Ostwald viscometer at 25.0°. Solutions were composed of 2.0 ml. dye (1.00×10^{-4} *M*) in buffer and 5.0 ml. calf thymus DNA (1.43×10^{-4} *M*) in buffer. The buffer solution was 0.100 *M* Tris (*p*H 7.5) and 0.001 *M* sodium chloride. Densities were determined using a Sodev flow digital densimeter at 25.0°. Specific viscosity was determined using the buffer as the pure solvent.

MO CALCULATIONS

Hückel MO calculations were made using a computer program reported in the literature.[33]

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