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Ethidium Bromide and Its Photoreactive Analogues: Spectroscopic Analysis of Deoxyribonucleic Acid Binding Properties†

David E. Graves, Charles L. Watkins,‡ and Lerena W. Yielding*

ABSTRACT: In an effort to determine the in vivo targets for ethidium bromide, the promising new technique of photoaffinity labeling has been applied in the development of two photosensitive ethidium azido analogues. One of these, ethidium monoazide, 3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride, has been shown previously to have biological properties similar to those of the parent ethidium prior to photolytic activation. After photolysis, which results in the covalent attachment of the ethidium moiety, the monoazide demonstrates enhanced biological activity. The diazide, 3,8-diazido-5-ethyl-6-phenylphenanthridinium chloride, is much less active. Since nucleic acids are presumed to be one of the targets of ethidium, both the noncovalent and the covalent interactions of ethidium and these azides with calf thymus DNA were analyzed at several salt concentrations by using spectrophotometric and dialysis techniques. The results presented in this paper show that the noncovalent interaction of the monoazide with deoxyribonucleic acid (DNA) is essentially identical with that of the parent ethidium and is primarily intercalative in nature. The DNA interaction with the diazide, apparently a stacking interaction, is quite different as seen by the greater decrease in the apparent association constant at elevated salt concentrations. Furthermore, the covalent interaction of the monoazide with DNA formed with ~40% photolytic efficiency resembled that of the noncovalent complex which suggests that no reorientation of the noncovalently bound ligand is required for covalent attachment. These results demonstrate that the monoazido analogue of ethidium bromide may be useful in determining directly the targets responsible for biological activity.

The mechanisms responsible for ethidium bromide's diverse biological actions have not been elucidated despite extensive efforts devoted to the characterization of the interactions with nucleic acids. A major obstacle is the reversible nature of the drug interaction which precludes isolation of the in vivo and in vitro complexes. In an effort to solve the problem of reversible binding, ethidium was modified to the photosensitive monoazide (3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride) (Hixon et al., 1975; Graves et al., 1977) and the diazide (3,8-diazido-5-ethyl-6-phenylphenanthridinium chloride) (Bastos, 1975) shown in Figure 1 in order to provide a means of covalently attaching the parent ethidium to its target sites.

Azide photoaffinity labeling is a powerful technique which will hopefully provide the means of identifying and characterizing the biological targets responsible for biological activity. In the case of drugs interacting with macromolecules, the azido analogue should simulate the parent compound. Once the photoaffinity probe is bound to the target, the azido substituent is activated by light to a nitrene (DeTraglia et al., 1978; Bercovici & Gitler, 1978) which reacts instantaneously to

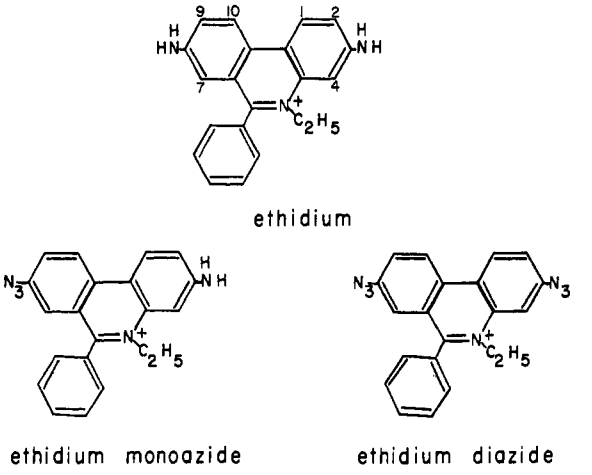

FIGURE 1: Chemical structures and numbering scheme of ethidium bromide and the photoreactive mono- and diazido analogues.

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covalently attach the bound ligand (Lwowski, 1967) without reorientating the ligand and without destroying the biological activity of the complex (Yielding & Yielding, 1980). The free ligand in solution is photolyzed simultaneously and is converted to hydroxylamine. It should be noted that the dissociation of the noncovalent ethidium–DNA complex is very slow (Bresloff & Crothers, 1975) compared with the short lifetime of the photogenerated nitrene (Lwowski, 1967); thus, nonspecific, extraneous interactions are kept at a minimum, and labeling can be accomplished at low drug concentrations.

The ethidium photoaffinity probes have proved interesting in studies of mitochondrial mutagenesis (Hixon et al., 1975; Morita & Yielding, 1977, 1978; Fukunaga & Yielding, 1979; Fukunaga et al., 1980), frame-shift mutagenesis (Yielding et al., 1976, 1979a; Yielding & Firth, 1980), and DNA repair (Cantrell & Yielding, 1977, 1981). Previously, the similarity of the in vivo binding sites of ethidium bromide, ethidium monoazide, and ethidium diazide was demonstrated by competition studies (Yielding et al., 1979b). By the use of *Salmonella* DNA[1] incorporated with [^{3}H]thymidine and [^{14}C]-ethidium monoazide, [^{14}C]ethidium diazide, and nonradiolabeled ethidium bromide, the decrease in the amount of covalently bound ^{14}C-labeled drug was found to be directly proportional to the increase in the ratio of ethidium bromide to [^{14}C]azide. Because of the potential importance of the photoaffinity probes, it was imperative to determine the detailed binding properties of such probes in comparison with the parent compound to interpret and extend these experiments.

The results of an extensive examination and comparison of the apparent association constants (K_a) and binding site sizes (n) for the noncovalent DNA complexes of ethidium and its azido analogues at varying ionic strength revealed that parent ethidium–DNA and monoazide–DNA complexes are qualitatively very similar. Furthermore, the covalent complex of the monoazide with DNA, formed with ~40% photolytic efficiency, shows spectral characteristics similar to those of the noncovalent complex. The noncovalent diazide–DNA interaction appears to be weaker than those of ethidium and the monoazide, since it is reduced to a greater extent with increasing salt concentrations. Furthermore, it is converted to the covalent adduct with only 10% photolytic efficiency. These results suggest that the greater biological activity of the monoazide may be explained by the "intercalative" interaction of the monoazide which more closely resembles that of the parent ethidium and allows for effective covalent attachment through photolysis.

Materials and Methods

Ethidium bromide (Calbiochem) was recrystallized once from methanol prior to use. Synthesis of the mono- and diazido analogues followed the procedure developed in this laboratory (Graves et al., 1977). Drug concentrations were determined spectrophotometrically by using the following extinction coefficients: ethidium bromide, ϵ_{476} = 5680 M^{-1} cm^{-1}; ethidium monoazide, ϵ_{458} = 5220 M^{-1} cm^{-1}; ethidium diazide, ϵ_{432} = 5850 M^{-1} cm^{-1} (Graves et al., 1977). Calf thymus DNA (Type 1) was purchased from Sigma Chemical Co. and purified according to the procedure of Muller & Crothers (1975); DNA solutions were prepared in 50 mM Tris-HCl (pH 7.5) by varying the ionic strength with 0.015, 0.036, and 0.2 M NaCl. DNA concentrations are stated in terms of nucleotide phosphorous by using the extinction coefficient ϵ_{260} = 6600 M^{-1} cm^{-1}.

Noncovalent Titration Measurements. The nonphotolyzed titration of the ethidium compounds with nucleic acids were performed with a 5-cm quartz cuvette by the addition of successive aliquots of concentrated DNA to a dilute stock drug concentration. The stock drug concentration was kept constant by simultaneous additions of the same volume of drug at twice the stock drug concentration. Low drug concentrations (1×10^{-5} M) were needed to negate cooperative effects of both the drug–drug and drug–DNA interactions (Turner et al., 1974; Pauluhn & Zimmerman, 1978). Spectra were obtained with a Cary 219 UV–vis recording spectrophotometer equipped with a Laudē thermoregulator maintaining the temperature at 25 °C. All measurements were taken from the digital display of the spectrophotometer at the wavelengths where the maximum change in absorbance was observed upon the binding of nucleic acid.

All procedures were performed under photographic safelights due to the reactivity of the azido analogues. Open shutter time on the spectrophotometer was minimized, although successive spectral scans in the 350–700-nm range showed no photolytic decomposition of the azido analogues.

Photolysis Conditions. Photolysis was performed with two light boxes (Buchler Instruments) each equipped with two General Electric daylight No. F15-T8D lightbulbs. Energy was delivered at a rate of ~80 J/(m^2 s). All samples were irradiated simultaneously and maintained at 25 °C throughout the 1-h photolysis.

Covalent Complex. [^{3}H]Ethidium bromide, [^{3}H]monoazide, and [^{3}H]diazide (sp act. = 106 mCi/mM) were utilized to quantitate the extent of covalent attachment resulting from photolytic activation. The starting concentrations of [^{3}H]-ethidium (and analogues) were determined from the visible absorbance. Approximately 5×10^{-6} M drug was used for each point. Samples with nucleotide/drug (N/D) ratios ranging from 30:1 to 0.5:1 were individually prepared and photolyzed in the manner described above. Samples were then dialyzed exhaustively against 50 mM Tris-HCl and 1.5 M NaCl (pH 7.5) to remove all noncovalently attached drug. Subsequently, samples were redialyzed in the original buffer for volume correction. Photolytic efficiencies of the drugs were determined by the comparison of the measurements of the radioactive counts of the samples, both prior to dialysis and after dialysis (Isocap 300 LSC, Searle), allowing direct conversion to molar concentrations by utilizing the known specific activity of 106 Ci/M.

Results

The interactions of the three ethidium compounds with calf thymus DNA resulted in both hypochromic and bathochromic shifts of the drug absorption spectra as shown in Figure 2. The λ_{max} of ethidium bromide shifted from 476 to 516 nm as the free drug became fully bound (Figure 2A), and an isosbestic point at 510 nm was observed, in agreement with Waring (1965), LePecq & Paoletti (1967), and Bittman (1969). The titration spectra for ethidium monoazide (Figure 2B) were very similar to those of ethidium bromide; there was a shift in the λ_{max} from 458 to 495 nm, with a well-defined isosbestic point appearing at 490 nm. The ethidium diazide, however (Figure 2C), demonstrated only a very slight bathochromic shift, but substantial hypochromicity upon binding DNA, characteristic of ethidium stacking interactions with mononucleotides or noncomplementary dinucleotides (Kastrup et al., 1978).

Scatchard analyses (Scatchard, 1949) of the UV–vis titrations of ethidium and the two photoreactive azido analogues

[1] Abbreviations used: DNA, deoxyribonucleic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; RNA, ribonucleic acid.

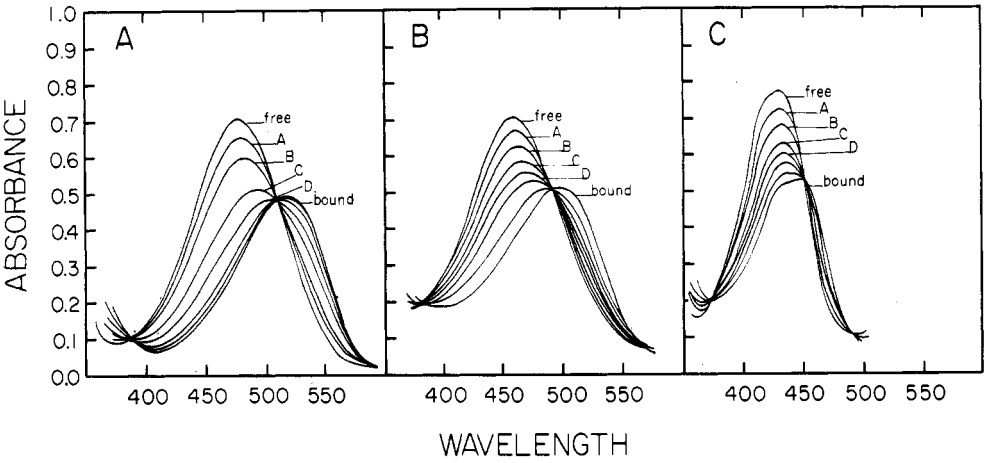

FIGURE 2: Representative absorption spectra resulting from the interaction of ethidium bromide (A), ethidium monoazide (B), and ethidium diazide (C) with calf thymus DNA. In (A), free ethidium bromide (2.46×10^{-5} M) was titrated with varying concentrations of DNA as shown in curves A, B, C, and D representing 1.25×10^{-5}, 2.5×10^{-5}, 5.0×10^{-5}, and 7.5×10^{-5} M, respectively. Fully bound ethidium bromide is also shown at saturating concentrations of DNA. (B) shows the titration curve of ethidium monoazide (free at 2.68×10^{-5} M) with DNA added in curves A, B, C, and D to give 1.25×10^{-5}, 2.5×10^{-5}, 5.0×10^{-5}, and 7.5×10^{-5} M DNA concentrations, respectively. Fully bound ethidium monoazide is shown at saturating DNA levels. (C) shows the titration curve of ethidium diazide (free at 2.65×10^{-5} M) with DNA added in curves A, B, C, and D, to give concentrations of 1.25×10^{-5}, 2.5×10^{-5}, 5.0×10^{-5}, and 7.5×10^{-5} M, respectively. Fully bound ethidium diazide is shown at saturating concentrations of DNA. All compounds were dissolved in 5 mM potassium phosphate buffer at pH 7.0 (no salt) at 25 °C, and the spectra were recorded with a Cary 219 UV–vis spectrophotometer with a 5-cm cell. These spectra were taken under low ionic strength condtions to illustrate maximum possible changes in absorbance of the compounds with titrations of DNA. The spectra used for calculation of binding parameters were taken under highe ionic strength conditions as described in thhe test. All experimental procedures were performed under safelight conditions.

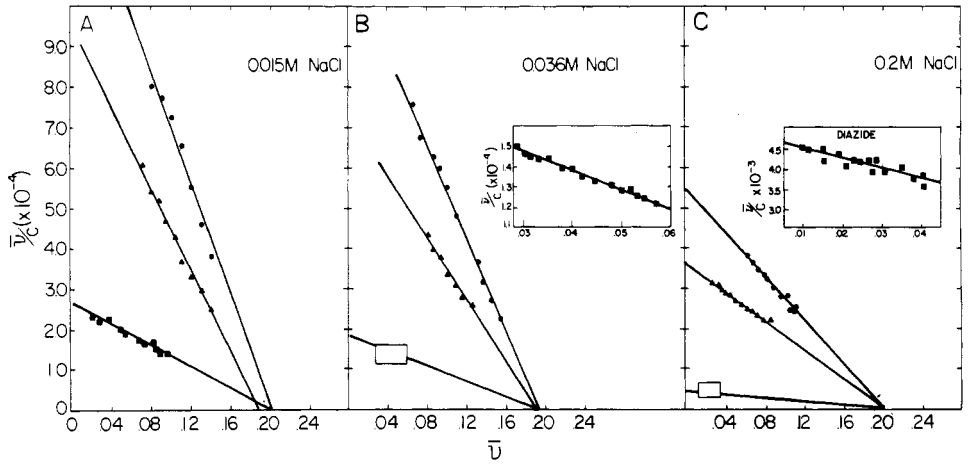

FIGURE 3: Scatchard analyses of the DNA binding with ethidium bromide (●), ethidium monoazide (▲), and ethidium diazide (■) at three salt concentrations. The correlation coefficients (R^2) for all plots were >0.95.

with calf thymus DNA at three salt concentrations are shown in Figure 3. These linear plots were obtained at 50 mM Tris-HCl (pH 7.5) and 0.015 (A), 0.036 (B), and 0.2 (C) M NaCl by using the range of α, the fraction of drug bound, from 0.2 to 0.8. Values of α outside this range yield unreliable results (Deranleau, 1969). The apparent association constant, K_a, and the number of drugs bound per nucleotide phosphate where all the binding sites are filled, $1/n$, were calculated and are given in Table I. Spectrophotometric analysis of ethidium–DNA interactions has been described previously (Waring, 1965; LePecq & Paoletti, 1967). A comparison of the monoazide and the parent ethidium binding to DNA reveals that the monoazide has essentially the same apparent association constant as the parent ethidium at the three salt concentrations examined. However, the diazide shows an apparent association constant of about half that for the parent ethidium at low ionic strength (0.015 M NaCl). As the ionic strength of the buffer is increased to 0.20 M NaCl, the diazide shows a 10-fold decrease in the apparent association constant, suggesting that most of its binding sites have been eliminated due to the competitive binding of the sodium ions. The "$1/n$" values were independent of ionic strength and were extrapolated to ~0.2 binding sites/nucleotide phosphate.

Table I: Apparent Binding Properties for the Interaction of Ethidium and the Mono- and Diazido Analogues with Calf Thymus DNA as a Function of Ionic Strength at 25 °C

drug	[Na^+] (M)	Scatchard, $K_a/(1/n)$	McGhee and von Hippel,[a] $K_a/[1/n$ (=0.5)]
ethidium bromide	0.015	$2.4 \times 10^6/0.20$	1.2×10^6
	0.036	$1.2 \times 10^6/0.19$	5.8×10^5
	0.200	$3.1 \times 10^5/0.20$	1.3×10^5
ethidium monoazide	0.015	$1.7 \times 10^6/0.19$	6.9×10^5
	0.036	$8.3 \times 10^5/0.19$	4.1×10^5
	0.200	$2.1 \times 10^5/0.20$	1.0×10^5
ethidium diazide	0.015	$9.8 \times 10^5/0.20$	4.2×10^5
	0.036	$3.2 \times 10^5/0.19$	1.1×10^5
	0.200	$3.1 \times 10^4/0.20$	1.3×10^4

[a] McGhee & von Hippel (1974).

An alternate approach to explain the dependence of K_a on ionic strength is to employ the condensation model for polyelectrolyte–counterion interactions as developed by Manning

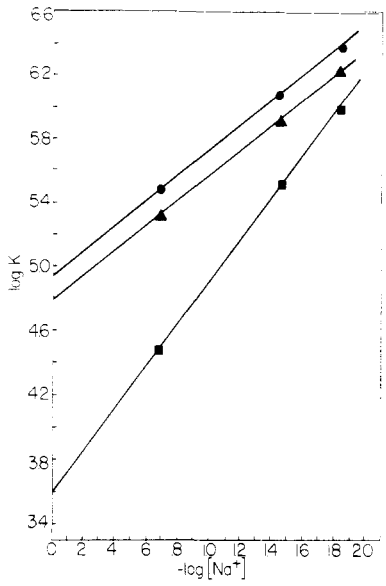

FIGURE 4: Dependence of the apparent association constant of ethidium bromide (●), ethidium monoazide (▲), and ethidium diazide (■) with calf thymus DNA on added salt concentration, expressed as $[Na^+]$.

(1978) and Record et al. (1978) and adapted for intercalating drug–DNA systems by Wilson & Lopp (1979). The data of Table I are plotted as log K vs. $-\log [Na^+]$ (Figure 4). Equilibrium constants were first determined as a function of total ionic strength (Tris buffer plus added sodium chloride). The association constants given in Table I were determined from the extrapolation to added sodium ion concentration (Reinhardt & Krugh, 1978; LePecq & Paoletti, 1967). As predicted by Manning (1978) and Record et al. (1978), each drug gave a linear plot. When the data were treated according to the method of Wilson & Lopp (1979) using

$$\frac{\delta \log K}{\delta \log [Na^+]} = -M'\Psi$$

where M' is the number of ion pairs formed between the ligand and DNA and the term Ψ is defined as the fraction of counter ions associated per phosphate, both ethidium bromide and the monoazido analogue showed equivalent slopes. However, the slope of the diazide curve was 2 times higher, suggesting that twice as many diazide molecules were needed as ethidium or monoazide to displace one sodium ion from the DNA.

Binding parameters for these drugs to DNA may be alternately determined by using the method of McGhee & von Hippel (1974). The data shown in Figure 3 (Scatchard plots) may be satisfactorily represented by eq 10 of McGhee and von Hippel, resulting in the intrinsic association constants (K) and the drugs bound per nucleotide ($1/n$) given in Table I. Neither the Scatchard nor the McGhee–von Hippel analysis provides exact quantitation of the binding parameters for the ethidium–DNA system (Krugh et al., 1975). It has been emphasized that ethidium will bind to sequences other than pyrimidine–purine sequences on DNA, RNA, and synthetic polynucleotides (Reinhardt & Krugh, 1978; Baguley & Falkenhaug, 1978). A complete analysis would require the determination of K_a and n for each of the unique intercalation sites on DNA. Thus, the term apparent and/or intrinsic association constant is often employed in Scatchard and von Hippel analyses. However, the qualitative conclusion of these studies, as demonstrated by both methods of analysis, is that the mode of binding of the monoazido analogue is quite similar to that of the parent ethidium bromide, while the diazide shows a much lower binding affinity.

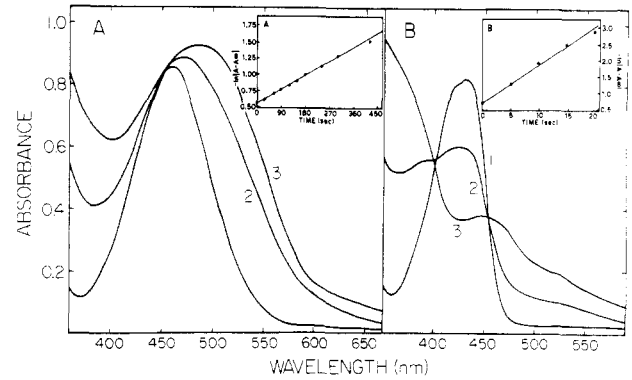

FIGURE 5: Visible absorption spectra of the photolytic decomposition products of ethidium monoazide (A) and ethidium diazide (B). In (A) curves 1, 2, and 3 represent photoperiods of 0, 5, and 30 min, respectively. (B) shows in curves 1, 2, and 3 photoperiods of 0, 5, and 30 s, respectively. The monoazide and diazide concentrations were 3.26×10^{-5} M and 2.78×10^{-5} M, respectively, in H_2O at pH 3.0 and photolysis was achieved by using General Electric daylight No. 715-TD8 fluorescent light boxes delivering an energy output of $\sim$80 J/(m^2 s). Spectra were run on a Cary 219 recording UV–vis spectrophotometer with a 5-cm cell. The inserts represent the determination of the apparent photolysis rate constants ($k_{photolysis}$) for ethidium monoazide (A) and ethidium diazide (B). The absorbance (A) was read at 485 nm for fully photolyzed monoazide and 432 nm for fully photolyzed diazide. The extinction coefficient for fully photolyzed drug (A_∞) was determined for each drug from a double-reciprocal plot of A vs. time in seconds. The slopes of the plots in the inserts are equal to the apparent photolysis rates.

Spectral Characterization of Ethidium Azide Photoproducts. In order to analyze the covalent complexes of ethidium monoazide and ethidium diazide with DNA and subsequently compare them with the noncovalent complexes, the consequences of photolytic activation of the unbound azido analogues had to be examined. This characterization is presented with the assumption that the photolysis process probably does not differ significantly for the bound and unbound ligand.

Photolysis of the azido analogues in an aqueous environment results in the formation of hydroxylamine photoproducts (Hixon et al., 1975). The visible spectra of the photolyzation of monoazide and diazide are shown in parts A and B, respectively, of Figure 5. Prior to photolysis, ethidium monoazide showed a single sharp band with an absorption maximum at 458 nm. Upon photolysis, however, this single band broadened, increased in absorbance, and shifted bathochromically to 485 nm. This value differs slightly from the results of Bolton & Kearns (1978), who found an absorption maximum of 475 nm. An isosbestic point was observed at 445 nm. The apparent rate constant of photolysis ($k_{photolysis}$) was determined for each azido analogue by measuring the absorbance at the wavelength showing the greatest absorbance difference between unphotolyzed and photolyzed drug. The wavelength chosen for the monoazide was 520 nm and for the diazide 432 nm. Samples were photolyzed for specified lengths of time and read spectrophotometrically, and the data were plotted at $1/A$ vs. $1/t$ where A is the observed absorbance for each sample and t is the time of photolysis in seconds. The y intercept is $1/A_\infty$, the reciprocal of the absorbance of the sample if it is 100% photolyzed. A plot of $-\ln |A - A_\infty|$ vs. t then gives a line whose slope is $k_{photolysis}$ (DeTraglia et al., 1978).

The photolysis for the monoazide was first order with respect to time (Figure 5A, insert, $R^2 = 0.99$) and characterized by a rate constant of $k_{photolysis} = 2.56 \times 10^{-3}$ s^{-1} with the photolyzing light delivered at a rate of $\sim$80 J/(m^2 s). The half-life was calculated as $\tau = 2.71 \times 10^2$ s.

The ethidium diazide photolysis profile shown in Figure 5B

differed significantly from that of the ethidium monoazide. Nonphotolyzed ethidium diazide exhibited one sharp band with an absorption maximum of 432 nm. As photolysis proceeded, the peak absorbance broadened bathochromically and extended from 420 to 460 nm with the λ_{max} at ~455 nm. Isosbestic points appeared at 455 and 400 nm. Upon photolysis, the drug absorbance was decreased between 400 and 455 nm and increased between 455 and 600 nm. The $k_{photolysis}$ was 1.14×10^{-1} s^{-1} and $\tau = 6.08$ s with a light source of 80 J/(m^2 s) (Figure 5B, insert, $R^2 = 0.99$).

The molar absorptivities were determined for fully photolyzed monoazide and diazide at the wavelength of maximum absorbance for each photolyzed analogue from a double-reciprocal plot of A vs. t, where the y intercept gave the reciprocal of the molar absorptivity for the concentration of the drug. The molar absorptivity for the fully photolyzed monoazide was found to be 5.66×10^3 M^{-1} cm^{-1} at 485 nm. This value is slightly higher than that observed by Bolton & Kearns (1978), who assessed the molar absorptivity of fully photolyzed monoazide to be 5.2×10^3. The slight variation in results could reflect incomplete photolysis which would be detected as a lower absorbance and a decreased bathochromic shift. The molar absorptivity for the fully photolyzed diazide was determined at 432 nm to be 2.28×10^3 M^{-1} cm^{-1}. The absorbance spectra and the photolytic process did not vary over the pH range 3–8 for both the monoazido and the diazido analogues.

Covalent Drug–Nucleic Acid Complex. Photolysis of the drug–nucleic acid complex results in covalent attachment of the photoreactive azido analogues in situ. In an effort to examine directly this covalent complex without contaminating noncovalently bound drug, the samples were dialyzed after photolysis in 50 mM Tris-HCl (pH 7.5) and 1.5 M NaCl buffer.

Examination of the extent of covalent attachment was done by direct measurement of the ^{3}H-labeled ethidium monoazido and diazido analogues (sp act. = 106 mCi/mM). [^{3}H]-Ethidium bromide was used to determine the efficiency of this dialysis system in order to remove all noncovalently attached drugs. Figure 6 shows the results of photolytic activation of the monoazido and the diazido analogues in situ as a function of both the nucleotide/drug ratio and ionic strength. The efficiency for photolytic attachment of the monoazido analogue was around 34–45%, depending on the ionic strength at nucleotide/drug ratios of 20:1. In contrast, removal of the noncovalently bound [^{3}H]ethidium bromide and nonphotolyzed ethidium monoazide was 90–95% effective until nucleotide/drug ratios of 20:1 were reached. At these ratios, both are difficult to remove from the DNA, and levels of noncovalently associated drug appeared to be ~20–25%. Thus, at N/D ratios of less than 20:1, direct examination of the properties of the covalent complex with only minor contributive effects by noncovalent drug molecules was possible. By use of ^{3}H-labeled drug, concentrations both prior to and after dialysis revealed the actual extinction coefficient at λ_{max} 500 nm to be $\sim 4.1 \times 10^3$ M^{-1} cm^{-1}. This molar absortivity for the covalently attached drug is similar to that presented by Bolton & Kearns (1978). Calculation of ϵ_{500} thus did not necessitate the assumption that the covalently attached drug maintained the same spectra properties as the noncovalent drug molecule. The diazide showed minimal binding, <10%, even at nucleotide/drug ratios of 20:1.

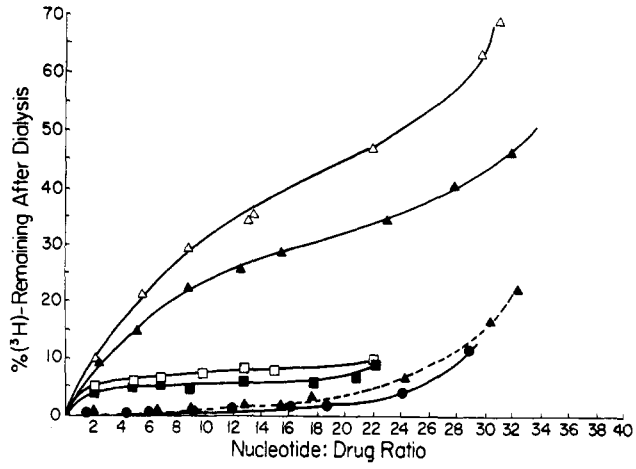

FIGURE 6: Percentage of [^{3}H]ethidium bromide, [^{3}H]ethidium monoazide, and [^{3}H]ethidium diazide (sp act. = 106 mCi/mM) remaining after dialysis as a function of the nucleotide/drug ratio prior to dialysis. Samples with N/D ratios ranging from 0.5:1 to 30:1 were prepared in 50 mM Tris-HCl buffer (pH 7.5) with added salt and photolyzed for 1 h at 25 °C with two General Electric daylight No. F15-T8D light bulbs delivering energy at 80 J/(m^2 s). Aliquots (0.1 mL) taken from each sample were counted prior to dialysis against 50 mM Tris-HCl buffer (pH 7.5) and 1.5 M NaCl. Subsequently, samples were redialyzed in the original buffer. Aliquots were taken from the dialyzed samples and were counted with a liquid scintillation counter. Photolyzed ethidium monoazide (~5×10^{-6} M)–DNA solutions with 0.015 M NaCl (△); photolyzed ethidium monoazide (~5×10^{-6} M)–DNA solutions with 0.20 M NaCl (▲); nonphotolyzed ethidium monoazide (~5×10^{-6} M)–DNA solutions with 0.20 M NaCl (▲---▲); photolyzed ethidium diazide (~5×10^{-6} M)–DNA solutions with 0.015 M NaCl (□); photolyzed ethidium diazide (~5×10^{-6} M)–DNA solutions with 0.20 M NaCl (■); ethidium bromide (~5×10^{-6} M)–DNA solutions with 0.20 M NaCl (●).

Discussion

The present studies have demonstrated, by comparing the apparent association constants for the DNA interactions with the parent ethidium and its azido analogues, that the monoazide–DNA complex is identical with that of ethidium. The apparent association constant for the diazide–DNA interaction is lower, indicative of a lower affinity type interaction. The binding site size of the diazide is the same, however, as that of ethidium and the monoazide.

A nonintercalative stacking interaction is further indicated by an extension of the analysis of the binding parameters as a function of salt concentration which shows that twice as many diazide molecules were needed to generate the displacement of one sodium ion from the DNA as were required of the parent ethidium or the monoazide. The decrease in K_a with increasing ionic strength (Na^+Cl^-) for ethidium binding to DNA was interpreted by LePecq & Paoletti (1967) in terms of a dominant intercalative mechanism at high salt concentration and both intercalative and stacking mechanisms at low salt concentrations. A qualitative model was proposed to explain the role of the sodium ion whereby competitive binding between sodium ions and ethidium ions exists for negatively charged sites on the DNA. An equilibrium constant for binding of sodium ion to the DNA was calculated. Recently, Pauluhn & Zimmermann (1978) reinvestigated the influence of ionic strength on the binding properties of the ethidium–DNA system and proposed a three-step mechanism involving noncovalent intercalative binding of the drug and competitive binding of the metal cation and ethidium to DNA, the mechanism being similar to that of LePecq & Paoletti (1967). Such a rationale has been employed in this discussion to demonstrate the similarity in binding between ethidium and the monoazide derivative and the differences in binding between ethidium and the diazido derivative to DNA. Thus, the diazide interaction appears more ionic, characteristic of a stacking interaction probably dependent on the ionic binding

of the quaternary nitrogen of stacked diazide moieties with the phosphate anions of the DNA backbone.

The difficulty in dissociating the ethidium–DNA complex and the noncovalent monoazide–DNA complex with high salt concentration at nucleotide/drug ratios of 30:1 is a demonstration of a very strong interaction occurring when there is a large excess of binding sites. In contrast, the noncovalent diazide–DNA interaction at high nucleotide/drug ratios is easily reversed with increasing salt concentrations which means that the strong interaction seen in the cases of ethidium and monoazide is not detectable between the diazide and DNA. These results may explain the high photolytic efficiency, 35–45%, of converting the noncovalent monoazide–DNA complex to a covalent adduct as opposed to the low efficiency, <10%, for the conversion of the noncovalent diazide–DNA complex to a covalent one. Since the monoazide does demonstrate the strong intercalative binding to DNA observed for ethidium, this binding of the monoazide may provide an orientation of the ligand which is coveniently cross-linked upon photolysis. However, at most, only a small fraction of the diazide apparently intercalates so that the diazide can efficiently covalently attach. Covalent attachment could occur without intercalation if, in fact, the diazide stacked on the outside of the DNA and, with some probability, was activated when in close proximity to the phosphate–ribose backbone. Subsequent rearrangement leading to intercalation may account for minimal biological activity.

These results explain, to some extent, the large difference in the enhancement of biological activity with photolytic activation between the monoazide and the diazide. The monoazide intercalates like the parent ethidium and is properly oriented for covalent attachment upon photolytic activation. Thus, once the monoazide–DNA interaction is rendered covalent, the ethidium binding and consequently the biological activity dependent upon that binding are enhanced many-fold. However, at most, only a small fraction of the diazide apparently intercalates with the proper orientation at the time of activation to permit covalent attachment.

In conclusion, these results suggest that an intercalative interaction may be the binding mechanism responsible for the biological activity of ethidium bromide seen in several systems. Furthermore, the monoazide promises to be an exciting new probe which can be used to identify and characterize the mechanisms and consequences of the ethidium binding in vivo and in vitro.

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