Cross-Linking of Double-Helical Nucleic Acids with a Photoreactive Analogue of Ethidium†

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ABSTRACT: Intercalation of the ethidium analogue 3,5-diazido-5-ethyl-6-phenylphenanthridinium into double helices followed by irradiation with blue or ultraviolet light results in cross-linking between the two strands with an efficiency around 30% for DNA, RNA, and DNA–RNA hybrids. Details of this reaction and a convenient synthesis of the ethidium analogue are described. Stable tertiary structure in RNA impedes intercalation and thus reduces the efficiency of cross-linking. In contrast to the ethidium derivative, various acridine diazides show little or no cross-linking ability.

Recent years have seen a growth of interest in cross-linking methods, e.g., to immobilize a molecule, to "freeze" an equilibrium, or to demonstrate or localize contact between two macromolecules. A "cross-linker" is usually a bifunctional molecule (Friedman, 1977) tailored to produce a covalent linkage between the macromolecules of interest, according to criteria of (i) group specificity, (ii) reversibility (Ulmer et al., 1978), and (iii) the conditions under which it is required to react. Particularly convenient are cross-linkers that are activated by a pulse of light, since the moment at which they react can be more exactly controlled. A present, only one series of such reagents has been applied to nucleic acids, the psoralens [reviewed by Song & Tapley (1979)].

The replacement of the amine groups of polyaromatic diamines by azide groups, in order to induce a light-activated reaction with DNA, was first described by Bastos (1975). The synthesis and chemical characterization of ethidium derivatives with, respectively, one or both amino groups replaced by azide was later described by Graves et al. (1977), and the same group has since undertaken a fuller investigation of the physical (Garland et al., 1980; Graves et al., 1981) and biological (Hixon et al., 1975; Fukunaga & Yielding, 1979) aspects of the reaction of these compounds with DNA. These derivatives are of particular interest in that they not only attach themselves covalently to macromolecules but also provide fluorescent analogues of ethidium, the properties of which are well documented (Le Pecq, 1973; Morgan et al., 1979a,b). In this paper, the cross-linking property of ethidium diazide is demonstrated, its uses and limitations are described, and a comparison is made with other, similar compounds (Figure 1).

It will be shown that (i) intercalation is a necessary condition for cross-linking by ethidium diazide ($EthAz_2$),[1] (ii) cross-linking by $EthAz_2$ promotes, as expected, the renaturation of DNA, (iii) analogous acridine diazide derivatives do not yield satisfactory cross-linkers, (iv) most $EthAz_2$ molecules binding DNA do so by intercalation, and (v) cross-linking of nucleic acids with stable tertiary structure does not take place. Implications of the last point concerning tRNA and rRNA structure are discussed. The favorable balance between cross-linking and strand breakage indicates a potential use for ethidium diazide as a cross-linking reagent.

Experimental Procedures

(*a*) *Synthesis of $EthAz_2$.* Ethidium bromide (1 g, 2.5 mmol) is dissolved in 50 mL of water and 3 mL of 37% hydrochloric acid (30 mmol). Sodium nitrite (1 g, 14 mmol), in a small volume of water, is added dropwise at 0 °C. The resulting solution is added in portions at 0 °C to a solution of 1.8 g of sodium azide (27 mmol) in 40 mL of water and 40 mL of methanol. After reaction (10–20 min), 100 mL of chloroform is added to extract the product, which on neutralization with solid sodium bicarbonate passes into the organic phase as a neutral adduct between $EthAz_2$ and an excess azide ion [R_f 0.8 in alumina TLC with chloroform/methanol/concentrated ammonia (20:1:0.1 v/v) as eluent and ~0.5 in silica TLC with diisopropyl ether/methanol (9:1) as eluent].

To the filtered organic phase is added 50 mL of ethanol, and the chloroform and methanol are removed by rotary evaporation; 50 mL of water is then added, at which point the main impurities form an oily red precipitate. The supernatant is decanted and cleared by addition of 2–3 mL of 1 M sulfuric acid, which frees the excess azide; hydrazoic acid and ethanol are removed by rotary evaporation, and the resulting aqueous solution of ethidium diazide is neutralized (conveniently by shaking with barium carbonate). The purity is ≥95% (TLC) and can be improved by taking only 0.1 g of ethidium bromide and sodium nitrite in the above preparation. The UV spectrum corresponded to that of $EthAz_2$ (Graves et al., 1977).

The resulting aqueous solution is used directly for cross-linking. The success of the preparation depends on the observance of the correct molar excesses and the avoidance of buffers—particularly ammonia and Tris—that react with $EthAz_2$. Exact pH control is unnecessary. Ambient light should be kept low, but extreme precautions are unnecessary. Skin contact with $EthAz_2$, a potential carcinogen, should be avoided.

(*b*) *Cross-Linking of DNA. Hin*dIII restriction fragments of λ phage DNA [DNS-Längenstandards II, Boehringer, Mannheim) were used. A total of 1 μg of DNA in NaCl, 0.1 M, NaH_2PO_4, 10 mM, and EDTA, 1 mM, pH 7.0, was treated as indicated with $EthAz_2$ or other cross-linker and exposed to light (unless otherwise stated, 3 min at a distance ~5 cm from a 25-W UVSL hand lamp set to 366 nm). Samples were run on 0.8% agarose gels in denaturing (NaOH, 30 mM, and EDTA, 2 mM) or nondenaturing (Tris, 36 mM, NaH_2PO_4, 30 mM, and Na_2EDTA, 10 mM) buffer. Bands were stained with ethidium bromide (1 mg/L).

(*c*) *Cross-Linking of RNA and DNA–RNA Hybrids.* $(Ap)_9A$ (P-L Biochemicals) was tagged with ^{32}P at its 5′ end by standard methods (Gupta & Randerath, 1979). Aliquots

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[1] Abbreviations: $EthAz_2$, ethidium diazide, 3,8-diazido-5-ethyl-6-phenylphenanthridium cation; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; kb, kilobase.

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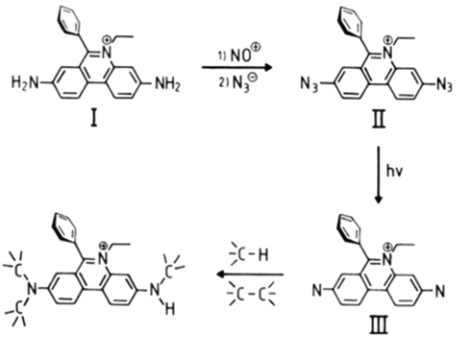


FIGURE 1: (I) Ethidium, (II) ethidium diazide, and (III) nitrene intermediate capable of insertion.

were mixed with excess poly(U) or poly(T) in the same buffer as in (b) above, treated with $EthAz_2$ and illuminated, applied to a 20 × 20 cm thin-layer plate (Polygram CEL 300, Macherey-Nagel, Düren, West Germany; prewashed in ammoniacal butanol), eluted by ascending chromatography at 50 °C (urea, 4 M, formic acid, 1 M, and pyridine to pH 4.3; Volckaert & Fiers, 1977), and autoradiographed.

(*d*) *Cross-Linking of tRNA and rRNA.* 16S rRNA from *Escherichia coli* was nicked by digestion with ribonuclease T_1 (Morgan & Brimacombe, 1973): 12 A_{260} units of 16S RNA was incubated with 60 units of the enzyme (Sigma Chemical Co.) in 80 μL of buffer [NaCl, 50 mM, $MgCl_2$, 5 mM, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), 20 mM, pH 7.8] at 20 °C for 30 min. $tRNA^{Phe}$ from yeast (Boehringer) was incubated with nuclease S_1 [10 units of enzyme (Sigma) with 1 A_{260} unit of tRNA in 30 μL of sodium acetate (20 mM, pH 5.5) for 1 h at 20 °C] so as to produce a strand break at the anticodon (Harada & Dahlberg, 1975); the two "halves" produced were resolved on a 12% polyacrylamide/urea gel. A higher cross-linker concentration was required (e.g., 0.2 mM); illumination was as in (b) above. Since $EthAz_2$ and its photolysis product inhibit nuclease S_1, the cross-linking was carried out after cleavage. For T_1 digestion, cross-linked tRNA was separated from excess dye by ethanol precipitation and incubated with RNase T_1 from Sigma (3 μg of enzyme/A_{260} unit of tRNA, pH 8, 37 °C, 2 h). Thin-layer chromatography was carried out on poly(ethylenimine)–cellulose (Macherey-Nagel, Düren, West Germany) with 35% v/v formic acid in 5 M urea; this separates T_1 oligonucleotides principally according to their number of U bases (Maly et al., 1980). Normally, 22% formic acid is used, but this does not move oligonucleotides with four U bases from the origin (Zwieb & Brimacombe, 1980). To increase their mobility, a higher proportion of formic acid is used here (cf. Volckaert & Fiers, 1977). Controls were carried out by omitting $EthAz_2$ and omitting tRNA.

Results

(*a*) *DNA Cross-Linking by $EthAz_2$.* Figure 2 shows the effect of increasing concentrations of cross-linker upon λ DNA fragments. The molecules denature on the gel with strand separation (lanes a and f); cross-linked strands cannot separate completely, and they migrate more slowly (lanes b–e). In a recent paper (Cech, 1981), cross-linking of DNA by psoralen has been demonstrated in a similar way.

The more dye is added, the greater is the yield of cross-linked DNA; however, radiation damage also takes place, so that the DNA begins to fragment and to disappear when too much dye is present. Lane d therefore represents an optimal dye:DNA ratio.

The following controls were also carried out. (i) Treatment of DNA with $EthAz_2$ without illumination showed neither

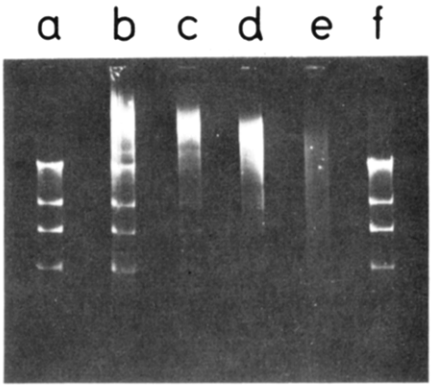


FIGURE 2: Cross-linking of DNA by ethidium diazide. Each sample contained 0.8 μg of DNA in 20 μL of buffer, and ca. 0.05, 0.15, 0.5, and 1.5 nmol of $EthAz_2$ was added to samples b–e, respectively, before photolysis. Photograph kindly provided by J. Köhler and S. Falkenberg.

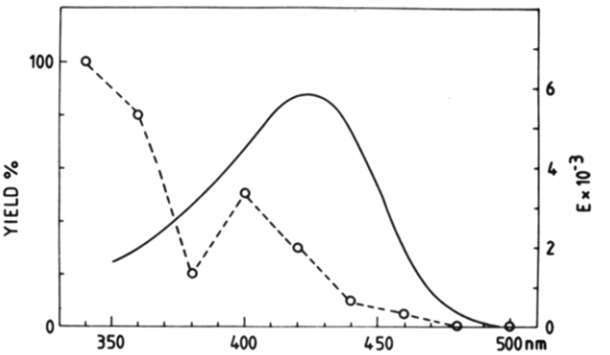


FIGURE 3: Wavelength dependence of cross-linking yield. The experiment of Figure 2 was carried out with the light source of an SLM 8000-DS fluorometer equipped with a Rhodamine quantum counter. Illumination was carried out at each wavelength with a constant number of photons. The yield of cross-linked DNA, estimated by eye from the band intensities on a photographic negative (cf. Figure 2), is shown (O---O) next to the absorption spectrum (—) of ethidium diazide.

cross-linking nor strand breakage. (ii) Likewise, treatment of DNA with ethidium and light showed neither cross-linking nor strand breakage, even at high ethidium concentrations. (iii) Prior illumination of $EthAz_2$ rendered it inactive in cross-linking. (iv) The inclusion of ethidium (ca. 0.2 mM) in the assay of Figure 2, lane d, virtually abolished the cross-linking property of $EthAz_2$; lower ethidium concentrations were less effective.

The first three controls confirm that both cross-linking by $EthAz_2$ and strand breakage proceed by way of the reactive nitrene intermediate, as expected from the known properties of aromatic azides (Knowles, 1972) and as shown in Figure 1. It remains uncertain where the nitrenes insert into the two DNA strands, since there is little agreement on precisely how polyaromatics are positioned in DNA. The fourth control suggests strongly that a condition for cross-linking is intercalation, and that occupation of the intercalating sites by ethidium prevents access, and thus cross-linking, by $EthAz_2$. The binding studies (below) confirm this.

Azide groups are as a rule excited to give nitrenes by energy absorbed by the aromatic part of the molecule. Figure 3 supports this assertion in the case of $EthAz_2$, since the yield of cross-linked product, after irradiation of DNA and $EthAz_2$ with a fixed number of quanta at various wavelengths, follows roughly the lowest electronic transition. The displacement of the peak to shorter wavelengths accords with the supposition that the azides are decomposed by the excess vibrational energy

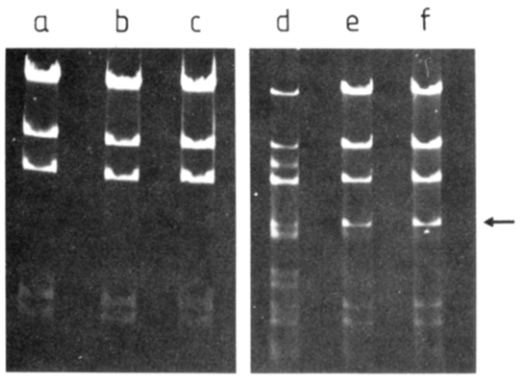


FIGURE 4: Analysis of DNA, cross-linked by $EthAz_2$, on a nondenaturing gel. Samples a–c were applied directly to the gel and samples d–f applied immediately after denaturation (2 min at 70 °C in 2 M urea): 1 μg of DNA and (lanes a and d) no $EthAz_2$, (lanes b and e) 0.05 nmol of $EthAz_2$, or (lanes c and f) 0.5 of nmol $EthAz_2$. The extra band appearing after denaturation (arrow) and the foreshortening of the largest fragment are due to dissociation of the "sticky end" of λ DNA. The lengths of the duplex bands (lanes a–c and f) are, in kilobases, from top to bottom 23.4, 9.5, 6.7, 4.3, 2.3, and 2.0.

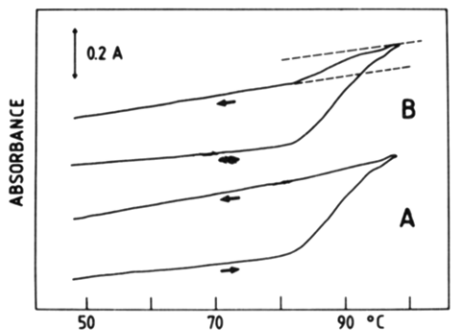


FIGURE 5: Melting of DNA (A) without and (B) with cross-links (see text).

of the excited molecule; however, the details of this photodecomposition are still unknown. It is in any case clear that, qualitatively, cross-linking takes place when and only when the aromatic chromophore absorbs radiant energy.

To confirm that cross-linking with $EthAz_2$ does not result in any fundamental damage to the DNA, λ DNA fragments were cross-linked and run on nondenaturing gels, in which they migrate as double helices. The pattern of double helices is unaffected (Figure 4, lanes a–c). If the DNA is denatured directly before application to the gel (Figure 4, lane d), then renaturation to give double helices requires ca. 10 min (Green & Tibbetts, 1981), and the strands, not having time to anneal, run separately. However, cross-linked strands can renature at once, with the result that the pattern of nondenatured double helices is restored (Figure 4, lanes e and f).

A further check on the efficiency of cross-linking is shown in Figure 5. Calf thymus DNA does not renature in a heating/cooling cycle at 1 °C/min, because of strand separation (curve A); however, cross-linking of some of the strands results in rapid reversal of the hyperchromicity upon cooling (curve B). (Complete reversal is not seen in Figure 5 because the DNA was heavily nicked.)

(*b*) *DNA Cross-Linking by Other Diazides.* Diazide derivatives of proflavin (3,6-diazidoacridine), of acridine yellow (3,6-diazido-2,7-dimethylacridine), and of acriflavin (3,6-diazido-10-methylacridinium) were prepared in a manner analogous to the synthesis of ethidium diazide. Their cross-linking properties for DNA were tested by the method of Figure 2 (denaturing gel). The proflavin and acridine yellow derivatives gave only a small yield of cross-linked product; all

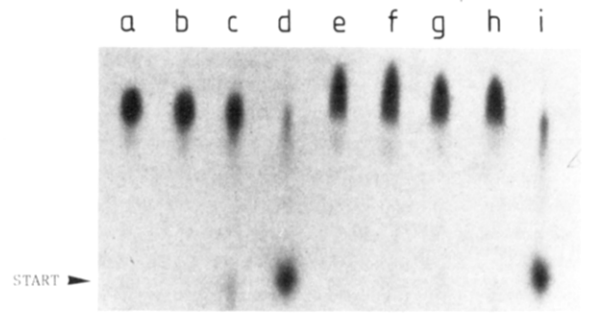


FIGURE 6: RNA–RNA and RNA–DNA cross-linking. Ascending PEI–cellulose TLC of $[^{32}P](pA)_{10}$ incubated with excess poly(U) (samples a–d), without complementary polymer (sample e), and with excess poly(T) (samples f–i). Increasing concentrations of $EthAz_2$ were used as follows: (a and f) none; (b and g) 10 μM; (c and h) 25 μM; (d, e, and i) 130 μM.

three led to extensive strand breakage. Furthermore, none of the products was as pure as $EthAz_2$ prepared by the same method.

(*c*) *RNA–RNA and RNA–DNA Cross-Linking.* Radioactively labeled $(pA)_{10}$ was allowed to form RNA–RNA [with poly(U)] or hybrid [with poly(T)] duplexes. After treatment with cross-linker and thin-layer chromatography under denaturing conditions as described under Experimental Procedures, the position of the radioactive marker showed the success or failure of cross-linking (Figure 6): poly(U) and poly(T) do not migrate, so cross-linked $(pA)_{10}$ remains at the origin. More reagent is needed for the short duplexes in this experiment than for the long DNA molecules in Figure 2; this is to be expected, since the cross-linking of all double helices requires one successful cross-link every few thousand base pairs for the DNA used and every 10 base pairs for the RNA.

(*d*) *tRNA Cross-Linking.* Transfer RNA has a single strong intercalating site for ethidium (Tao et al., 1970). This site lies in the acceptor stem between base pairs AU_6 and AU_7 both for yeast $tRNA^{Phe}$ (Jones & Kearns, 1975) and for *E. coli* $tRNA_1^{Val}$ (Kearns & Bolton, 1978). Binding of ethidium to the strong site does not otherwise affect the structure of tRNA, but the binding of further ethidium ions disrupts the tertiary structure of the molecule (Jones et al., 1978). A site in which the ethidium stacks onto two bases, U_8 and A_{13}, has been seen in the crystal (Liebman et al., 1977) but not in solution (Jones & Kearns, 1975).

Figure 7 shows the result of nicking tRNA at the anticodon loop and subsequent cross-linking with $EthAz_2$. Although some cross-linking takes place, the yield of cross-linked tRNA molecules never reaches 100%, even at high concentrations of the reagent (in this case 0.2 mM). Cross-linking of the 3′ and 5′ fragments, as seen on the gel, is the behavior expected if the intercalating site on tRNA behaves in the same way as the intercalating sites in the simple double helices described above.

Since there is only one intercalating site in tRNA, the yield of cross-linking in the acceptor stem must be equal to the product of the degree of saturation of the site and the effectiveness of cross-linking once the site is occupied. The saturation approached 100% under the conditions of the experiment (see below), so the efficiency of cross-linking in an occupied site is equal to the percentage reduction in area of the fragment peaks in lanes c and d of Figure 7 after normalization of the total area in each lane. Integration of the peaks led to a value for the cross-linking efficiency of 30 ● 5%.

Our assignment of the site of tRNA cross-linking is supported by three further observations. First, Bertrand & Kearns

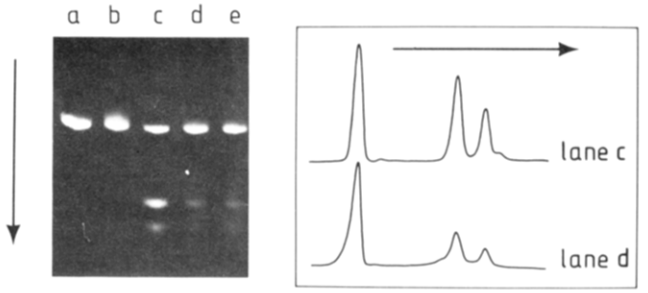


FIGURE 7: Cross-linking of tRNA. $tRNA^{Phe}$ (lane a) yields two fragments (lane c) when cleaved by S_1, as shown by polyacrylamide/urea gel electrophoresis and staining with ethidium. Cross-linking of the cleaved tRNA with $EthAz_2$ caused some of the fragments to stay together (lanes d and e), while uncleaved tRNA was unaffected by cross-linking (lane b). The two fluorescence scan traces (right) from a separate experiment give quantitatively the amount cross-linked, subject to the assumption that the different RNA polymers bind equal numbers of ethidium ions per base and that these have on average the same extinction coefficients and quantum yields. If the gel was completely destained (in 40% v/v ethanol/water), only the spots presumed to contain cross-linked tRNA retained their red fluorescence, attributed to the $EthAz_2$ photoproduct. (Scans taken with a prototype instrument by Sigma Instrumente, Berlin; courtesy of Dr. G. Salje.)

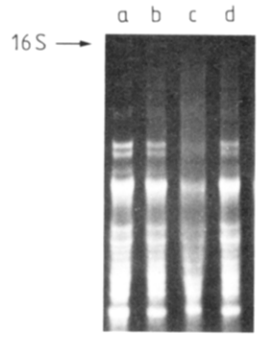


FIGURE 8: Attempted intramolecular cross-linking of 16S RNA: (lane a) rRNA pattern on a 3–8% polyacrylamide/urea gel; (lane b) after cross-linking with 0.2 mM $EthAz_2$; (lane c) as in lane b but with EDTA (excess over magnesium); (lane d) as in lane b but warmed to 40 °C before cross-linking.

(1982) have found by NMR spectroscopy that ethidium and ethidium 8-monoazide bind to one and the same site in nondenatured $tRNA^{Phe}$ from yeast. It is therefore to be expected that ethidium diazide should also bind to this site before photolysis. Second, we found that the addition of $EthAz_2$ to the tRNA–ethidium complex abolished the fluorescence of bound ethidium, confirming that $EthAz_2$ is capable of displacing ethidium from a common binding site. Third, when cross-linked yeast $tRNA^{Phe}$ was totally digested with RNase T_1 and the fragments were subjected to thin-layer chromatography, the red fluorescent fragment appeared with $R_f \simeq 0$, implying that it contained ≥5 U bases; this result is consistent with cross-linking across the acceptor stem and is not compatible with cross-linking across any other double-helical or known long-range base pair of yeast $tRNA^{Phe}$.

(*e*) *rRNA Cross-Linking.* To maximize the chance of cross-linking, 16S rRNA alone was used rather than complete 30S particles; it was nicked so as to reduce as far as possible constraints due to tertiary structure. These were further weakened in some assays by addition of EDTA and/or by warming to 40 °C.

Figure 8 shows the characteristic "ladder" of the nicked 16S RNA and the effect of treatment with $EthAz_2$ and photolysis: the background becomes somewhat brighter, but no new bands are formed. This effect, enhanced by EDTA and by warming, implies that cross-linking indeed takes place but that it is slight

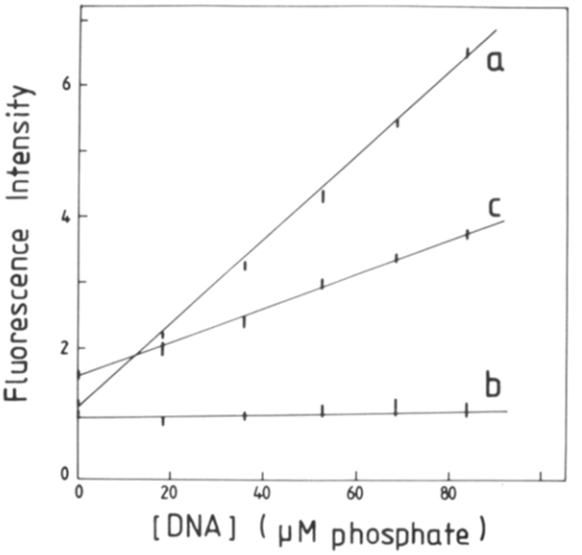


FIGURE 9: Competition between ethidium and $EthAz_2$ for binding sites on calf thymus DNA. DNA is added stepwise to (a) ethidium bromide, 0.1 mM, (b) $EthAz_2$, 0.44 mM, and (c) ethidium bromide, 0.1 mM, and $EthAz_2$, 0.44 mM (buffer similar to that used for cross-linking DNA). Ethidium fluorescence was monitored in an SLM 8000-DS (excitation 540 nm with Schott WG 340 filter; emission with Schott OG 570 filter).

in extent and not at all specific. The background enhancement is due to cross-linking rather than (as in Figure 2) to degradation, since the enhanced brightness is observed at a higher, rather than a lower, molecular weight.

(*f*) *Mode of Binding of Ethidium Diazide.* The equilibrium constants of binding of ethidium and ethidium diazide to calf thymus DNA have been reported to be 2.0×10^6 M^{-1} and 0.93×10^6 M^{-1}, respectively (Garland et al., 1980). It is interesting that the azide groups, with their comparative inability to form hydrogen bonds or to delocalize positive charge, do not reduce the affinity of the phenanthroline for intercalation sites in DNA. An explanation might lie in the mode of binding. By adding $EthAz_2$ to DNA nearly saturated with ethidium, competition between these two for intercalation sites resulted in the displacement of ethidium, observed by its decreased fluorescence; the ratio of the numbers of molecules of $EthAz_2$ and ethidium bound is proportional to the ratio of their concentration times that of their association constants for intercalation sites.

This is shown in a different way in Figure 9: addition of DNA to ethidium in the fluorometer caused the expected increase in fluorescence (a), and addition of DNA to $EthAz_2$ caused none (b). Addition of DNA to a mixture of both caused an intermediate rise in fluorescence (c). Since the dyes are in excess over DNA and their concentrations considerably exceed their reciprocal binding constants, we may assume near saturation of the DNA. There is also a substantial excess of free over bound dye. Application of the law of mass action to the competition between ethidium (E) and the diazide (EA) for a common site leads to the relation

$$\frac{\text{slope of c}}{\text{slope of a}} = \frac{K_E[E]}{K_{EA}[EA] + K_E[E]}$$

where K_E and K_{EA} are the respective binding constants. Inserting values from Figure 9 gives $K_{EA}/K_E = 0.35 \pm 10\%$. (This figure may err on the low side, since incomplete saturation of the DNA will make the mutual exclusion by the two dyes less rigorous and increase the slope in Figure 9c.) Comparison with the literature value of $0.93/2.00 = 0.47$ shows good agreement, verifying the starting assumption of

a common site for ethidium and $EthAz_2$ and thus implying that the binding of $EthAz_2$ is intercalative. [This is not at variance with the conclusion of Garland et al. (1980) that the exact mode of the interaction between DNA and these two dyes may differ.]

Discussion

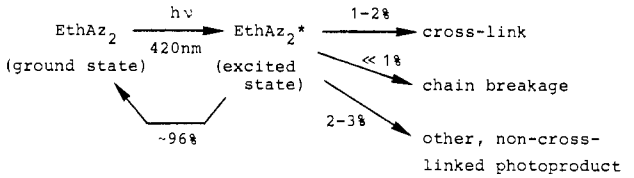
The results presented above have demonstrated that ethidium diazide is a good cross-linker for DNA, RNA, and hybrid double helices, while other diazide derivatives investigated do not make satisfactory cross-linking reagents. The advantages of ethidium diazide as a cross-linker lie in (i) its easy preparation and purification, as described above, (ii) the high ratio of cross-linking to strand breakage obtainable under correct conditions, (iii) its comparability to the well-documented ethidium ion, and (iv) the fact that it can be activated by long-wavelength ultraviolet or blue light, thus avoiding the absorption bands of most biological molecules and protecting the system against photodegradation. A particular advantage over psoralen is that the photolysis of psoralen appears to produce singlet oxygen as a biologically damaging side product (Singh & Vadasz, 1978; Lochman & Micheler, 1979). Disadvantages of ethidium diazide vis-à-vis the psoralens are that the photoreaction is less controllable (both ends react at once) and is irreversible.

We have shown above that the mode of binding of ethidium diazide to DNA is predominantly intercalative and that the condition for cross-linking is that $EthAz_2$ be allowed to intercalate and then be excited in its electronic absorption band centered at 420 nm or below. The requirement for intercalation means, in turn, that a sufficiently high concentration of $EthAz_2$ must be present (the concentration depending upon the length of the chain to be cross-linked) and that the double helix be unconstrained. This is the reason why tRNA, with ca. 20 base pairs, can only accommodate one intercalating ethidium in the A stem (Jones & Kearns, 1975): the lengthening and unwinding associated with intercalation are prevented by the stable tertiary structure of the molecule (Urbanke et al., 1973).

It is in this connection of interest that rRNA, even under the most favorable conditions, showed no sign of specific cross-linking. An attempt to cross-link 16S to 23S rRNA in whole ribosomes was also unsuccessful (data not shown): although 30S and 50S subunits could be cross-linked to each other in very low yield (<5%), as shown by sucrose gradient centrifugation, gel electrophoresis of the RNA showed only a trace of a 16S–23S cross-linked complex (yield ≪1%) (C. Glotz and R. Brimacombe, personal communication). This implies that the tertiary structure of 16S RNA in the ribosome is tight enough to be retained even when the proteins are removed and the RNA is extensively nicked, a postulate consistent with the known stable core of 16S RNA (Garrett et al., 1977). This raises the question of whether ethidium ions bound to ribosomal particles can be regarded as intercalated in the same sense as in double helices that are not constrained by tertiary structure; however, to attempt an answer to this would exceed the scope of the present investigation.

The yield of successful cross-linking in double helices was shown to be of the order of 30% in the case of tRNA. This figure is confirmed in the result shown in Figure 6 for RNA and hybrid double helices; the nearest-neighbor exclusion principle (Cairns, 1962; Bresloff & Crothers, 1981) implies that a duplex containing A_{10} should have five intercalation sites. If these are nearly all occupied (i.e., $[EthAz_2] \gg K_{EA}^{-1}$), then simple probability theory requires a 37% success rate in each site in order to obtain the approximately 90% overall success rate implied by the intensity ratios in Figure 6.

The yield in the wavelength-dependence experiment (Figure 3) is much lower because care was taken not to saturate the system with light. The geometry and optics of the fluorometer allow the conclusion that each of the 23-kb duplexes, with an occupation density of $EthAz_2$ approaching saturation, absorbed about 30 quanta at 420 nm, the absorption maximum. The loss by cross-linking of this band is around 30%, which implies that each quantum absorbed by intercalated $EthAz_2$ has a chance of about 1–2% of causing cross-linking. Combining this figure with the ultimate yield under saturating illumination of 37%, assumed to apply at all wavelengths, leads to the following approximate picture for the fate of $EthAz_2$ in a double helix, excited at 420 nm:



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Registry No. II, 67620-23-9; ethidium bromide, 1239-45-8; poly(A)-poly(U), 24936-38-7; poly(A)-poly(T), 27156-07-6.

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Dynamic Structure of DNA Complexes. Fluorometric Measurement of Hydrogen–Deuterium Exchange Kinetics of DNA-Bound Ethidium Dimer and Acridine–Ethidium Dimer†

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ABSTRACT: The hydrogen–deuterium (H–D) exchange kinetics of free and DNA-bound ethidium dimer and acridine–ethidium heterodimer were measured by stopped flow using fluorescence detection. This technique allowed a very accurate measurement of the exchange process. The H–D exchange kinetics were measured in various environments. In some cases, it was observed that the H–D exchange was much faster than the dissociation rate of dimer–DNA complexes. This showed that the exchange was taking place directly from the bound state. Furthermore, the action of a catalyst (imidazolium ion) on the rate of H–D exchange showed that a dynamic structural fluctuation of the ligand in its DNA complex was a necessary step on the exchange process.

Measurements of hydrogen exchange kinetics have furnished important information on the dynamic structure of proteins and nucleic acids [review by Woodward & Hilton (1979)]. Most of the data have been obtained by measuring tritium exchange. Recently, hydrogen exchange in nucleic acids has been studied by a new approach that takes advantage of the nucleic acid UV absorption change upon deuteration (Mandal et al., 1979).

Schreier & Baldwin (1976) demonstrated the usefulness of hydrogen exchange measurements in the study of the interaction kinetics of ligands with macromolecules. They showed that under some conditions, very small dissociation rate constants can be measured. Along this line, Mandal et al. (1980) measured the hydrogen–deuterium exchange of ethidium bromide in its complex with DNA using absorption spectroscopy. They have shown in their study the potentiality of this method to determine the binding parameters of a dye–DNA complex.

Furthermore, it is well-known that the fluorescence quantum yields of several dyes, including ethidium bromide, are from 2 to 4 times higher in D_2O than in H_2O (Stryer, 1966; Olmsted & Kearns, 1977). The kinetics of hydrogen–deuterium exchange of fluorescent compounds could therefore, in some cases, be very easily measured by fluorescence spectroscopy, as examplified by the work of Nakanishi et al. (1980) on tyrosine and tryptophan.

Recently, we have described the DNA binding properties of several bifunctional intercalators (Le Pecq et al., 1975, Roques et al., 1976; Delbarre et al., 1977; Gaugain et al., 1978a,b; Capelle et al., 1979; Markovits et al., 1979; Roques et al., 1979; Pelaprat et al., 1980; Markovits et al., 1981a,b; Delbarre et al., 1981; Reinhardt et al., 1982). We have shown that these molecules bind to DNA with a very high binding constant, which could be as high as 10^{10} M^{-1} in a physiological salt environment. Some of these molecules elicit strong antitumor properties (Roques et al., 1979; Pelaprat et al., 1980). Ethidium dimer (EthDi) and acridine–ethidium dimer (AcEthDi) are molecules that could represent interesting models for H–D exchange studies. Their fluorescence is

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