Increased accessibility of bases in DNA upon binding of acridine orange*

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ABSTRACT

Acridine orange (AO) forms 1:1 complexes with dsDNA which are insoluble in aqueous media, exhibit red luminescence, have minimal green luminescence and resemble complexes of AO with ss nucleic acids. During formation and/or dissociation of these complexes, accessibility of DNA bases to two conformational probes, formaldehyde and diethyl pyrocarbonate is increased, suggesting that the base pairing is destroyed and DNA at least partially denatured. Adriamycin and Ellipticine, but not Ethidium Bromide exert similar destabilizing effects. The results confirm our earlier predictions based on thermodynamic calculations that the double helix undergoes destabilization upon binding an intercalator characterized by high cooperativity in interaction with ss nucleic acids. Thus, the highly cooperative ligand binding to ss sections during the "breathing" of the polymer may progressively destabilize the adjacent ds structure.

INTRODUCTION

Mechanisms of interaction of nucleic acids with acridine dyes in general and acridine orange (AO) in particular were extensively investigated during the past four decades (Reviews 1-3). There is a general consensus that at low binding density AO binds to double stranded (ds) nucleic acids by intercalation (4-6). This type of interaction ("strong binding") enhances the stability of the double helix against thermal denaturation (7,8) and the intercalated dye exhibits green luminescence (Review 9).

Interactions of AO with single stranded (ss) nucleic acids or with ds nucleic acids at high binding density are less understood. These interactions ("weak" or "external" binding) were originally visualized as formation of dye stacks attached electrostatically to the phosphates of the nucleic acid backbone; the dye-dye interactions in the stacks have been held responsible for the long wavelength (red) luminescence of the AO-ss nucleic acid complexes (10-12, Review 9).

Our earlier data could not support the dye-dye stacking model, and to explain the data a new model was proposed in which AO partially intercalates between the bases forming stacks of alternating dye-base composition (13,14). In this model

the dye molecules are inserted between adjacent bases of the ss polymer not only transiently during complex formation (as visualized in earlier models, see refs. 15,16) but throughout the entire range of the binding density. In accordance with this model, the dye-base interactions are observed even at a 1:1 (dye/phosphate) binding density. The dye-base stacks are strongly hydrophobic which manifests in precipitation of the complexes during titration; the solute-solid transition of the complexes is believed to be responsible for the appearance of the red luminescence (13).

The structure of AO-ss nucleic acid complexes, as mentioned above, as well as high cooperativity in AO binding (14) both bear important implications with regard to the influence of AO on the stability of ds nucleic acids. Namely, taking into account the thermodynamics and stoichiometry of binding of AO to ss vs ds-nucleic acids, it is expected that under certain conditions the dye will denature the double helical structure (14).

The differential staining of ss vs ds nucleic acids disappears at higher AO concentration; above that concentration only red luminescence, which is characteristic for ss nucleic acids, can be seen (17). It was also observed that AO prevented DNA renaturation (18,19). The possibility that AO may denature dsRNA was raised based on cytochemical observations (13,20), but the direct evidence for the destabilizing effect of the dye on the double helix was never obtained.

In the present studies using two conformational probes of nucleic acids, which preferentially modify bases not involved in base-pairing, formaldehyde (CH_2O) and diethyl pyrocarbonate (DEP), we provide experimental data which support the contention that AO destabilizes the double helical structure of DNA. Namely, we observe that in the AO-DNA complexes, which at high AO concentration precipitate from solution, accessibility of the bases to these probes is increased.

MATERIALS AND METHODS

Highly polymerized calf thymus DNA (Type I, Sigma Chemical Co., St. Louis, Missouri) was dissolved in 0.1 M NaCl, 5mM HEPES, 1mM EDTA-Na at pH 7.0, and sheared by sonication. This treatment lowered the MW to about 3×10^5 (21). The final preparation of DNA had extensive ds structure as evidenced by its hyperchromicity ($H = 1.48$) and typical thermal denaturation curves ($T_m = 82^\circ\text{C}$ at 0.1M NaCl, pH 7.0). Concentrations of DNA were determined by UV light absorption measured at 260nm; the molar extinction coefficient was assumed to be $6.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (22).

Chromatographically purified acridine orange, 3,6-bis(dimethylamino)

acridine hydrochloride (AO), was obtained from Polysciences (Warrington, Pa.). The concentration of the dye was estimated colorimetrically at the isosbestic point (470nm) using the molar absorption coefficient $4.33 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (15).

Diethyl pyrocarbonate (diethyl oxydiformate, diethyl dicarbonate, DEP) was obtained from Eastman Kodak Co. (Rochester, N.Y.). The mixture of DEP with ethanol was prepared just before using (23,24).

Titration of calf thymus DNA with AO

Aliquots containing 2.5 ml of DNA (P-concentration 53 μ M) dissolved in the buffer containing 0.15M NaCl, 5mM Hepes, 1mM EDTA, pH 7.0 in a quartz cuvette with teflon-coated magnetic stirrer, were placed in the thermostatic holder ($25 \pm 0.1^\circ\text{C}$) of an SLM 4800 spectrofluorimeter (SLM Instruments Inc., Urbana, IL.) interfaced to a Hewlett-Packard (HP) 9826 computer. The luminescence and light scatter were measured at 90° geometry with polarizers oriented at 55° (25). The width of excitation and emission bands was 8nm. The sample and the blank (buffer only) were titrated with small volumes (5-10 μ l) of AO stock solution ($C_{\text{AO}} = 0.18$ or 1.9mM) with extensive stirring. Following addition of each portion of AO the stirring was stopped and the light scatter (emission monochromator at 350nm) green- (emission at 530nm) and red-luminescence (emission at 650nm) were measured. The excitation monochromator was set at 350nm. At this wavelength both AO and the AO-DNA complex have relatively small light absorption which allows light scatter measurements; yet the absorption was adequate for excitation. The results of green luminescence and light scatter measurements were expressed as I_s/I_b where I_b and I_s are intensities of luminescence or light scatter of the blank and sample, respectively. Because red luminescence of the blank at low AO concentration was too low to be measured accurately the results were expressed as $I_s - I_b$ (arbitrary units). The data were then processed by computer using the "cubic spline" interpolation program, smoothed by a multiple pass (up to 6 times) regression analysis program and drawn by digital plotter (HP 7225A).

Interactions of DNA-AO complex with DEP or CH_2O

Procedure 1 consisted of four consecutive steps:

A) Precipitation of the AO-DNA complex by admixing 2 ml of AO solution with 10 μ l of DNA stock solution and incubation for 24 hours at 25°C . The final concentrations of AO and DNA-P were about 0.5mM and 0.05mM, respectively, in buffer 10mM NaCl, 5mM Hepes, 1mM EDTA, pH = 7.0.

B) Separation of the AO-DNA complex (precipitate) by low speed centrifugation (500g, 10 min).

C) Solubilization of the precipitate in 2 ml of the buffer containing 25% (v/v) ethanol and incubation at 25°C for 2 hours.

D) Separation of DNA from A0 by passing the solution through a AG-50W-X8 Na⁺ cation-exchange column (13) followed by dialysis against the buffer containing 10mM NaCl (3 times, 300 ml, 3 hours each). The probes (CH₂O, 1% final concentration or 30μl DEP) were included either before addition of A0 (Step A) or before separation of the precipitate (Step B), or together with ethanol (Step C). When CH₂O was used, the probe was also present at 1% concentration throughout all consecutive steps including the melting.

In the control experiments DNA was treated with DEP and CH₂O but in the absence of A0 in the same way as described above (Step D).

Procedure 2

The experiments with A0-DNA complexes in solution were made in the buffer containing 150mM NaCl in the way similar to that described in Procedure 1 except that the A0 concentration was varied (up to 0.1mM) and separation of the precipitate by centrifugation was omitted. DEP (30μl per sample) was added to the samples together with ethanol. Before melting, samples were dialyzed against buffer containing 10mM NaCl.

Interactions of DNA-Co³⁺ complex with DEP

Aliquot of 2 ml of DNA (50μM) in a buffer containing 10mM NaCl, 5m Hepes, pH 7.0 has been precipitated by admixing with a small volume of stock solution of Cobaltic Hexamine Chloride = [Co(NH₃)₆] Cl₃ (Eastman Kodak Co, Rochester, N.Y.) to final concentration 0.2mM. After 24 hours at room temperature, the complex was separated by centrifugation (700g, 15 min). The precipitate was then treated with a freshly prepared (23,24) mixture of 2 ml buffer with 30μl of DEP and then with 4N NaCl to a final concentration of 0.15M, followed by Step D, Procedure 1.

Melting profiles of DNA were measured with a Zeiss PM-6 spectrophotometer equipped with a thermoelectric control unit (1°C/min) in 1 cm light path quartz cuvettes against the blank (buffer only). The results, expressed as the absorbance changes at 260nm, were recorded, processed (smoothing, derivation) and plotted by the HP computer (as described above). The melting temperature, T_m, was determined by one-half the numerically integrated area under the differential-absorbance melting curve. The renaturation profiles were usually recorded (1°C/min) and the renaturation temperature T_r were calculated in the same way as the T_m. A second melting were also occasionally recorded and the results confirmed with the expected properties of denatured DNA (not shown).

In addition to A0, DNA complexes with Ellipticine, Adriamycin and Ethidium Bromide were studied in the same manner as described above (Procedure 2).

Fig. 1. The effect of increasing AO concentration on spectral and thermal properties of calf thymus DNA. A - Titration of DNA (53 μ M in 0.15M NaCl, 5mM Hepes, 1mM EDTA, pH 7.0) with AO; green luminescence (at 530nm) $\times 10$ - Δ - and light scatter - $\circ$ - changes; (left ordinate), red luminescence (at 650nm) changes - + - (right ordinate); I_s , I_b intensity of luminescence or light scatter of the sample or blank respectively. B - Decrease of the melting temperature (ΔT_m) of DNA recovered from the complexes treated with DEP in the presence of various concentrations of AO (Procedure 2). The results have not been corrected for the effect produced by AO alone ($0.8 \pm 0.3^{\circ}$ C).

RESULTS

Fig. 1 illustrates changes in green (530nm) and red (650nm) luminescence, as well as in light scatter, which occur during treatment of DNA with increasing concentrations of AO. At the onset of the titration ($<5\mu$ M AO), the green luminescence of the sample is higher than that of the free dye (relative luminescence >1), which is consistent with the higher quantum yield of luminescence of the intercalated vs free dye monomer (26), then it decreases, reaching a minimum of $\sim 80\mu$ M of AO. The red luminescence of the sample increases during the titration (up to

Fig. 2. The effects of the probes CH₂O (A) and DEP (B) on the differential-absorbance melting curves of the DNA recovered from the complexes with AO. The probes were added at different steps (A,B,C) of the experiment (Procedure 1) as indicated.

A. Curve 1 - control DNA, Curve 2 - DNA + CH₂O, Curve 3 - DNA + AO + CH₂O (Step C).

B. Curve 1 - Control DNA, Curve 2 - DNA + DEP, Curve 3 - DNA + AO + DEP (Step A), Curve 4 - DNA + AO + DEP (Step C).

75μM AO) and this curve exhibits several phases. The initial portion of the curve (4-40μM AO), represented by a relatively flat slope, is followed by a steep increase (40-75μM AO) and then by a drop (~75μM AO) of red luminescence. The light scatter of the sample starts to increase after 50μM AO and the maximal rate of increase is observed at 70-90μM AO. The final light scatter of the sample at

Table 1: Variations in the thermal transitions of calf thymus DNA recovered from complexes with acridine orange

	CONFORMATIONAL PROBE ^{a)}															
	None		CH ₂ O				DEP									
	-	+	-	+	(A)	+	(B)	+	(C)	-	+	(A)	+	(B)	+	(C)
AO	-	+	-	+	(A)	+	(B)	+	(C)	-	+	(A)	+	(B)	+	(C)
Melting temperature (T _m)	70.0	69.2	67.9	62.8	63.1	63.2	69.6	60.5	60.5	60.0						
Renaturation temperature (Tr)	41.0	40.0	37.0	35.0	27.5	32.0	37.5	33.5	33.5	30.0						
% renaturation	33	32	6	4	2	2	35	20	19	16						
ΔT _m ^{b)}	--	0.8	2.1	7.2	6.9	6.8	0.4	9.5	9.5	10						

a) The probes were added at different steps (A,B,C) of the experiment (Procedure 1) as indicated

b) The difference between T_m of DNA not exposed to AO or the probe and T_m of the sample

the end of the titration exceeds by a factor of more than 40 that of the sample at the the onset of the titration.

The drop in red luminescence observed between 75 and 100μM AO correlates with maximum light scattering. The decrease in red luminescence, therefore, is most likely a consequence of the formation of colloidal particles (13) which have a tendency to sediment and adhere to the cuvette walls.

Thus, in summary, during the titration of DNA with AO, a product is formed which exhibits red luminescence, has minimal green luminescence and scatters light. Light scattering is the consequence of the collapse of polymer structure. A few hours following the titration the precipitate becomes visible and the product undergoes precipitation to such an extent that it can be separated by low speed (500g) centrifugation. From the precipitate formed at an AO concentration of 0.4mM (D/P >1), over 95% of the original DNA can be recovered using the cationic exchange resin separation as described earlier (13).

The structure of the red luminescing product was investigated using two conformational probes of nucleic acids which react preferentially with bases of ss nucleic acids. Thus, formaldehyde (CH₂O) (27,28) or diethyl pyrocarbonate (DEP) (23,24) were included at various stages of complex formation and/or dissociation and after removal of the dye the recovered DNA was analysed by thermal denaturation and renaturation. The results are presented in Fig. 2 and Table 1.

In the absence of AO, CH₂O produced a modest decrease in the transition temperature (ΔT_m = 2.1°C) consistent with the known reactivity of this probe with DNA during "breathing". In combination with AO, however, the shift of the transition to a lower temperature was much more extensive (~7°C). The most dramatic effect was observed during combined treatment of DNA with AO and DEP; such treatment lowered the T_m of the recovered DNA by as much as 10°C. Neither AO nor DEP alone produced significant changes in DNA stability (Table 1).

Fig. 3. The absorbance (at 260nm) renaturation profile of the DNA recovered from its complexes with AO. The probes (CH_2O or DEP) were added at different steps (A, B, C) of the experiment (Procedure 1) as indicated. Curve 1 - Control DNA; Curve 2 - DNA + DEP; Curve 3 - DNA + AO + DEP (Step A); Curve 4 - DNA + CH_2O ; Curve 5 - DNA + AO + CH_2O (Step C). A_T - absorbance at temperature T ($^{\circ}\text{C}$), A_{25} - absorbance of the sample at 25°C before melting.

Not only the denaturation, but also the ability of the recovered and then denatured DNA to renature is affected when the AO-DNA complexes are treated with CH_2O or DEP (Fig. 3). Both the extent of renaturation as well as the renaturation temperature (T_r) of DNA are lowered and the degree of changes in general parallels a decrease in T_m (Table 1). The ability to renature, however, calculated from hyperchromicity at 95° vs 25°C after cooling, in the presence of CH_2O is very small and is also observed in the absence of AO. Because CH_2O was present in the sample it interacted with the bases during the heating and cooling (28) and this obscures the original modification of DNA by the probe in complexes with AO. Thus, these data reflect both modifications of DNA bases accessible in complexes with AO and subsequent modification when DNA undergoes heat-denaturation. As a consequence, the recovered DNA shows very low renaturability.

The experiments reported in Table 1 were done on AO-DNA complexes obtained at low (10mM NaCl) salt concentration (Procedure 1). In addition, several experiments were done at higher ionic strength (0.15M NaCl) and the product forming at various AO concentrations was analysed (Procedure 2). Results of these experiments are shown in Fig. 1B. In comparison with 10mM NaCl, the changes observed at 0.15M NaCl were of lesser magnitude. These changes correlate with AO concentration. A near constant decrease in T_m of about 1.7°C is seen at

A0 concentration between 2 and 50 μ M. At 75 and 100 μ M, however, the decrease is 5 and 6 $^{\circ}$ C, respectively. At these concentrations the product exhibits maximal red luminescence (75 μ M), maximal light dispersion (100 μ M) and has little green luminescence (29), (Fig. 1A).

To investigate whether or not the observed variations in T_m are specific to DNA interaction with A0, or are a consequence of the nonspecific collapse of DNA structure (DNA condensation, precipitation), an experiment was made to analyse DNA structure in complexes with molecules other than A0. It is known that cations with triple (or higher) valency produce a collapse of the DNA structure (30 and references cited therein). We have investigated the complex of DNA with Co^{3+} using cobaltic hexamine chloride in the same manner as described above for A0. No differences in T_m of DNA recovered from the DNA- Co^{3+} complex in the presence vs absence of DEP were observed (results are not shown). Thus, the phase transition of DNA from solute to solid state per se does not seem to be responsible for the observed increase in accessibility of DNA bases in complex with A0.

Other intercalators, such as Adriamycin ($c = 0.2\text{mM}$ and Ellipticine ($c = 0.1\text{mM}$) also precipitated DNA and in the presence of DEP (Procedure 2) had a similar effect on DNA, lowering T_m by 4.8 and 5.1 $^{\circ}$ C, respectively. In contrast, Ethidium Bromide tested under the same conditions at concentrations up to 0.5mM neither precipitated DNA nor had any effect on its T_m .

DISCUSSION

The present data are consistent with the notion postulated before (13,14) that A0 may destabilize the double helix. The results of the titration indicate formation of a product which luminesces in red, undergoes precipitation and shows minimal green luminescence. A decrease in green luminescence during the titration is apparently the result of two processes. Because the dye intercalated into DNA has a higher quantum yield of luminescence than free dye in solution (26), at low A0 concentration the sample containing DNA exhibits higher green luminescence than the blank containing free dye. During the titration, after the intercalative binding sites become saturated, the relative luminescence of the sample is expected to decrease asymptotically to 1.0 reflecting the increasing ratio of free to intercalated dye. The observed decrease in green luminescence (Fig. 1A), however, is steeper than it would be due to the increased content of the free dye, and at A0 concentration above 50 μ M the relative luminescence of the product is actually below 1.0. Thus, during the titration, the product shows a progressive loss of green luminescence (29) which may indicate (although per se

cannot be considered a proof) that DNA within the product has less double strandedness, i.e. has fewer intercalation sites. The decrease of green luminescence below 1.0 occurs at AO concentrations which induce the formation of colloid particles which scatter the light.

The structure of DNA in the product was investigated using CH_2O and DEP. Both probes react with bases of nucleic acids forming N-derivatives; following such a modification the bases lose the ability for pairing (23,24,27,28). Because the amino- or imino-groups are inaccessible in dsDNA, reactivity of DNA provides information about conformational motility of DNA ("breathing" of the ds structure) and is often used to discriminate ss vs ds conformation. One of the differences between these two probes relates to the reversibility of the binding; the reaction with CH_2O is reversible while with DEP it is not (23,24,27,28). Also, in contrast to CH_2O , DEP is unstable having an approximately 30 min half-life time under the present conditions.

The probes were added during the different stages of the AO-DNA complex formation and dissociation. Following decomposition, the dye was removed by cation-exchange resin (13) and thermal denaturation of the recovered DNA was then analysed (Fig. 2, Table 1). The results suggest that during the formation and/or dissociation of the DNA-AO complexes there are stages when the DNA strands separate, making the bases accessible to the probes. The separation of strands, however, does not appear to be complete, because otherwise the DNA recovered from complexes with AO alone would be extensively denatured, which was not found. Thus, most likely, during precipitation some base-pairing is preserved. Perhaps the polymer trapped in the precipitate is protected against the further denaturing effect of AO. When the complex undergoes dissociation in the presence of ethanol, the preserved double stranded sections maintain the base pairing sequence in register and help in the renaturation in a manner similar to that when DNA is crosslinked (31).

When DEP was added after formation of the precipitate, the effect was still as high as that when the probe was added early during formation of the complex. This observation is consistent with the proposed intercalative structure of the AO-ss nucleic acid complexes (13,14). Namely, interactions with the probes are expected to be most effective during the transition phase, when the complex undergoes dissociation prior to renaturation of DNA. Within the complex itself, a protection against base reactivity with the probes may be ensured by the close interactions with AO.

As can be expected, the increase in ionic strength lowered the reactivity of the bases with DEP (Fig. 1B). While high ionic strength enhances the stability

of the double helix (Review 32) it also decreases interactions of AO with ss nucleic acids (Kapuscinski *et al*, manuscript in preparation). More surprising was the observation that DEP in the presence of low AO concentration (1-30 μ M) lowered the T_m by 1.3-1.8 $^{\circ}$ C (Fig. 1B). Because the changes in T_m in this region parallel changes in red luminescence, yet the complex does not show an increase in turbidity, it is possible that the preparation of DNA used (M.W. $\sim 3 \times 10^5$) contained small sections highly sensitive for the denaturing action of AO (end-portions of the polymer?).

The mathematical model for helix-coil transition of nucleic acid in the presence of ligands which bind to both ss and ds nucleic acids was developed by McGhee (33) and according to this model, small ligands such as AO are expected to be especially effective in altering the temperature of the transition. The present data support this model. In addition, our earlier studies (14) provided thermodynamic data and calculations indicating that AO may destabilize the double helix. These calculations have shown that the energy required for dissociation of the intercalative complex of AO with RNA (and likely DNA) and the strand separation may be compensated for by highly favorable free energy change of the cooperative AO binding to single strands. It means that AO, especially at high dye to DNA-P ratio, may bind to ss sections of nucleic acid during the "breathing" of the double helix and, because of high cooperativity, the reaction will expand. The 1:1 complexes of AO with ss RNA and ss-DNA are insoluble in aqueous media and exhibit red luminescence (13,14) similar to that observed presently in the precipitates formed during titration of dsDNA with AO.

Precipitation of DNA with different intercalators and other organic aromatic monocations was observed frequently, but was either neglected or explained as due to charge neutralization. The precipitate of AO-DNA complexes was reported to have uninterpretable x-ray diffraction patterns (34). It was also observed that solutions of AO-DNA complexes containing more than 0.3 mol of AO/DNA-P cannot be prepared without formation of precipitates (35). The precipitation of dsDNA after neutralization of one-third of the polymer charges by monocations cannot be explained, especially in view of the Manning theory of polyelectrolyte solutions (36). According to this theory, spontaneous DNA structure collapse (condensation) occurs when more than 90% of DNA-P charges are neutralized by counterions (32). It has been shown that such a degree of saturation in aqueous solutions can be achieved only by polyvalent cations with a charge not less than 3 (e.g. 30). Furthermore, the cation-induced toroidal condensation of DNA in the presence of Co^{3+} is very sensitive to the presence of mono- or divalent cations and can be reversed at 20mM NaCl (30). In contrast

condensation induced by AO is observed in 0.15M NaCl (Fig. 1).

According to the anticooperative model of self-association (37), under conditions (ionic strength, pH, temperature) similar to those used during the titration (Fig. 1) at 100 μ M AO, fewer than 7% AO molecules can form aggregates containing more than 3 dye molecules. Therefore, at DNA concentration 53 μ M during the whole range of the titration up to 100 μ M AO, no conditions existed whereby aggregates of AO larger than 3 molecules could be responsible for the precipitation of any significant portion of DNA, due to charge neutralization according to the Manning theory (36). On the other hand, AO-induced condensation of dsDNA can be explained assuming denaturation of DNA and formation of the hydrophobic complex of the structure proposed before.

The question may be asked as to whether or not the increase in reactivity of ds nucleic acids with CH₂O or DEP may be a result of an acceleration of the reaction rate by the dye itself, e.g. as observed with morpholine in the reaction of CH₂O with nucleosides (28). In the case of ds nucleic acids, however, the rate-limiting step is an opening of the double helix and not the base-probe interactions (24,27,28). Furthermore, acceleration of the reaction with both probes, differing in structure, is of rather low probability. Another possibility, namely, that the probe is transported in the form of a complex with the intercalator into the double helix is also very remote if one considers the sterical requirements for the intercalation and the fact that the effect was observed with two different probes and several intercalators.

It should be cautioned that the increased accessibility of the bases to CH₂O and DEP, although generally recognized as indicating an "opening" of the double helix (23,24,27,28) is an indirect test of the conformational changes of DNA. Unfortunately, the optical properties of AO (as well as those of other intercalators), which exhibit strong absorption in the UV spectrum, make orthodox spectral techniques for direct evidence of strand separation (e.g. UV hyperchromicity changes, C.D.) useless. The problem is additionally hampered by the complexity of interactions involving the presence of AO in monomer and aggregate forms, its intercalation (all involving changes in absorption spectra) and especially by the precipitation of the product. The insolubility of the product makes it difficult, if not impossible, to apply such techniques as NMR or to study hydrodynamic properties of DNA by gel electrophoresis, sedimentation, or direct observation by electron microscopy.

Ethidium Bromide is a strong intercalator, but in contrast to AO, Ellipticine (38) or Adriamycin (39) has low affinity towards ss nucleic acids (40). This dye did not precipitate DNA and did not affect T_m of DNA recovered

from the complex. Thus, the intercalation per se does not involve any transient change in DNA structure that in some way may facilitate reactivity of the bases with the probes.

DNA denaturation in situ may be induced by higher AO concentration in living cells and the precipitates, having high electron density, can be observed by electron microscopy (41). The pattern of AO-induced DNA denaturation in living cells resembles denaturation of DNA in situ by heat or acid and is useful for characterization of condensed vs diffuse chromatin (41).

The observed, novel mechanism of DNA denaturation induced by the intercalating ligand, characterized by high cooperativity of binding to ssDNA may be representative of more general processes such as these operating during replication or transcription (e.g. unwinding proteins 33,42,43) and provide a model in studies of other mutagens and antitumor agents (44,45) and dyes which form precipitates with nucleic acids such as pyronin-Y(G), methyl green, malachite green and crystal violet (46,47).

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