DAMAGE BY VISIBLE LIGHT TO THE ACRIDINE ORANGE–DNA COMPLEX

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ABSTRACT Salmon DNA has been irradiated with visible light in the presence of acridine orange. If the dye is bound to the DNA, there results: (*a*) a decrease in sedimentation coefficient, (*b*) a lowering of viscosity, and (*c*) a decrease in the thermal denaturation temperature. CsCl banding experiments show that the first two effects reflect depolymerization of the DNA. Depolymerization apparently occurs by single-strand scission although some double-strand scission is not excluded. The destabilization of secondary structure results probably from chemical attack on the components of the individual strands.

INTRODUCTION

The fluorescent diaminoacridine dyes bind strongly to nucleic acids in solution (1). When applied to living materials as fluorochromes they become localized in nucleic acid–containing structures (2). One of these dyes, acridine orange (3,7-*bis*-dimethylaminoacridine) has recently been shown to sensitize living cells to the action of visible light that is absorbed by the dye. For example, irradiation with visible light after addition of AO[1] reduces survival (ability to form visible colonies on agar plates) in yeast and bacteria (3) and produces chromosome abnormalities in *Vicia faba* root tips (4). From an analysis of the yeast survival curves, and in view of the very strong binding in solution of DNA compared with RNA (5), it has been concluded that the principal damage is probably mediated through the DNA.[2]

[1] Abbreviations used in this paper are as follows—

AO = acridine orange

AET = S,2-aminoethyl isothiouronium bromide hydrobromide

$s_{20,w}$ = sedimentation coefficient in Svedbergs corrected to the buoyancy and viscosity of water at 20°C

Tris = tris-(hydroxymethyl)aminomethane

η_{sp} = specific viscosity extrapolated to zero shear gradient

$\eta_{sp,0}$ = specific viscosity, extrapolated to zero shear gradient, of unirradiated solution

[2] Photosensitization may also play a role in the mutagenic action of the related dye, proflavin (3,7-diaminoacridine) (6).

In the present experiments we have studied the effects of visible light irradiation of the native AO-DNA complex in solution. We have found that: (*a*) irradiation decreases the viscosity and sedimentation coefficient and hence, by inference, the molecular weight of the DNA; (*b*) depolymerization requires the binding of dye molecules and the presence of atmospheric oxygen; (*c*) degradation involves single breaks in each of the individual strands of the DNA helix; and (*d*) secondary structure is destabilized.

MATERIALS AND METHODS

1. *Materials.* Three samples of salmon testis DNA (samples C, ST-1, and D) were prepared according to the method of Simmons (7, 8) and stored as air-dried fibers at 4°C. Solutions were prepared by dispersing the fibers in 0.01 M NaCl at 1°C. After 2 days' shaking, the sedimentation distribution became reproducible, showing a maximum at $s_{20,w} = 24$ and little high *s* material. All DNA preparations gave large hyperchromic shifts on denaturation. Sample D had a 40 per cent increase in OD_{260} upon heat denaturation in 0.01 M NaCl (measured at the ambient temperature).

Fisher (histological grade) AO was extensively purified by a method described elsewhere (9) and was spectroscopically pure. Before purification the crude material contained 1 mole $ZnCl_2$ per mole dye. (Recrystallization from ethanol did not remove the Zn contaminant.) AET was the generous gift of Dr. N. Frigerio.

2. *Preparation of Solutions.* For irradiation experiments, AO-DNA solutions in 0.01 M NaCl were made by adding AO ($2 - 4 \times 10^{-5}$ M) dropwise, with stirring, to equal volumes of DNA ($1 - 1.2 \times 10^{-2}$ per cent). In such solutions, more than 95 per cent of the dye is bound to DNA. In order to reduce possible aggregation effects, AO-DNA solutions were equilibrated at least 12 hours at 0—6°C before irradiation. If smaller volumes of more concentrated AO were added to DNA or if the AO was added too rapidly, small fibers often precipitated.

Prior to irradiation solutions were handled under a red safelight.

For some experiments AO-DNA solutions were deoxygenated with a stream of nitrogen ("purified") which had been washed with alkaline pyrogallol and water. The solutions were then frozen in dry ice–ethanol under a continuous stream of nitrogen, after which the vials were evacuated and sealed.

3. *Physical Measurements.* Viscosity was measured in a Cannon-Ubbelohde multigradient viscometer immersed in a 25 ± 0.02°C thermostat. Average shear gradients for water at 25°C were in the range of 50 to 200 sec^{-1}. Experiments were performed in red or subdued light to avoid further photolytic effects.

Sedimentation velocity was determined in a Spinco model E ultracentrifuge using ultraviolet optics. AO-DNA solutions in 0.01 M NaCl were diluted with 2 volumes of 0.3 M NaCl to give an ionic strength of 0.2 and an OD_{260} less than 0.8. At this high ionic strength the solutions could be handled in white light since the dye binding is considerably weakened (10). Boundary sharpening is greatly reduced at 0.2 ionic strength.

Density gradient centrifugation was performed in 7.5 molal CsCl (Maywood optical grade) at 44,770 R.P.M., 25°C, for 24 hours in the Spinco model E ultracentrifuge. A sample of *Pseudomonas fluorescens* DNA served as an internal standard.

4. *Irradiation.* Two light sources were used : (1) a General Electric AH-6 high pressure Hg arc filtered with 5 cm of 12 per cent $CuSO_4$ in 5 per cent H_2SO_4, and

(2) a similarly filtered General Electric R-52 floodlamp.[3] At the distance of irradiation the incident energy was 10^6 erg/cm²/sec and 10^5 erg/cm²/sec, respectively. The actual values are not significant since the spectral distributions of the two sources are rather different.

5. *Sonic Degradation.*

(*a*) For sedimentation studies samples of 0.04 per cent DNA in 0.01 M NaCl were degraded by exposure for 1 to 5 minutes to 10 kc sound waves produced by a Raytheon sonic oscillator model F 101 set at 1.1 to 1.2 amperes.

(*b*) For denaturation studies 0.1 per cent DNA solutions (sample C) in 0.01 M NaCl were irradiated in a Raytheon 9 kc sonic oscillator. Prior to sonic degradation, AET (11) was added to a concentration of 3.7×10^{-4} M, solutions were deoxygenated with pyrogallate-washed N_2, and sealed in polyethylene-topped glass vials. After varying periods of irradiation, sodium acetate, pH 7, was added to a concentration of 0.3 M and DNA was precipitated with 2 volumes of ethanol. The DNA was redissolved in 0.01 M NaCl, reprecipitated from pH 7 buffered sodium acetate with ethanol, washed with ethanol and acetone, and finally air-dried. The air-dried fibers (containing about 15 to 20 per cent moisture) were stored at 4°C.

6. *DNAase Digestion.*

(*a*) For the experiments described in Table I stock DNAase solutions (Nutritional Biochemical Corporation, 1 × crystallized) were freshly prepared at a concentration of 0.02 mg/ml in 0.01 M NaCl. To 2 ml of 0.04 per cent DNA in 0.01 M NaCl containing 10^{-3} M $MgCl_2$ was added 0.0005 to 0.002 ml of the DNAase stock solution (all solutions at 38°C). After 20 seconds' digestion, the sample was placed in an ice-water mixture. However, even without cooling, degradation proceeded only slightly further. Degraded solutions were then stored overnight at 1°C, after which time all enzyme activity had been lost.

(*b*) For the methanol thermal denaturation experiments DNAase (0.02 per cent in 0.01 M NaCl) was prepared from a 1 mg/ml stock solution. Small (0.002 to 0.02 ml) volumes of enzyme were added to 4 ml aliquots of 0.025 per cent DNA in 0.01 M NaCl, 0.001 M $MgCl_2$, pH 7, at 37°C. After 20 to 120 seconds, the mixtures were plunged into ice and 1 ml ice cold 0.01 M versene, pH 7.0, was added.

7. *Thermal Denaturation.*

(*a*) In the presence of formaldehyde: DNA and AO-DNA solutions were fully denatured (40 per cent hyperchromicity) at 70°C in 0.005 M NaCl, 0.01 M phosphate buffer, pH 7.6-7.8, 2 per cent freshly neutralized formaldehyde. All solutions were checked for full hyperchromicity before being run in the ultracentrifuge.

(*b*) In the presence of methanol: The thermal stability of DNA which had been irradiated in the presence of AO was measured in 44 weight per cent methanol at low ionic strength. Under these conditions the AO-DNA complex is dissociated.

Solutions for thermal analysis were made as follows:—4 ml AO-DNA in 0.01 M NaCl and 1 ml 10^{-3} tris pH 7.1 were mixed and cooled in ice. 5 ml iced methanol (spectroscopic grade) was added dropwise, with stirring. Optical densities at 259 mμ were measured as a function of temperature in a Beckman model DU spectrophotometer.

8. *Control Experiments.* Control experiments yielded the following results: (*a*) Irradiation in the absence of AO has no effect on the viscosity or $s_{20,w}$ distributions of DNA solutions. (*b*) Bubbling N_2 or O_2 through solutions and subsequent freezing has

[3] The floodlamp set-up is that which was used in the yeast irradiation experiments (3).

no effect on the $s_{20,w}$ distribution of unirradiated solutions. (*c*) Addition of AO at 0.2 ionic strength to unirradiated DNA does not measurably alter its $s_{20,w}$ distribution. (*d*) Addition of AO increases viscosity at 0.01 ionic strength. At 0.2 the effect is much less pronounced, and in 0.5 M NaCl it is undetectable. (*e*) No after-effect could be detected. The viscosity of irradiated AO-DNA solutions was insensitive to the time elapsed between irradiation and viscosity measurements.

RESULTS AND DISCUSSION

1. *Degradation of DNA.* The principal conclusion from the present experiments is that irradiation by visible light of a AO-DNA solution results in degradation of the DNA; *i.e.,* a decrease in molecular weight. Evidence comes from two sources.

(*a*) With increasing dose of radiation the sedimentation coefficient and viscosity decrease simultaneously (Figs. 1 and 2) implying a decrease in molecular weight.

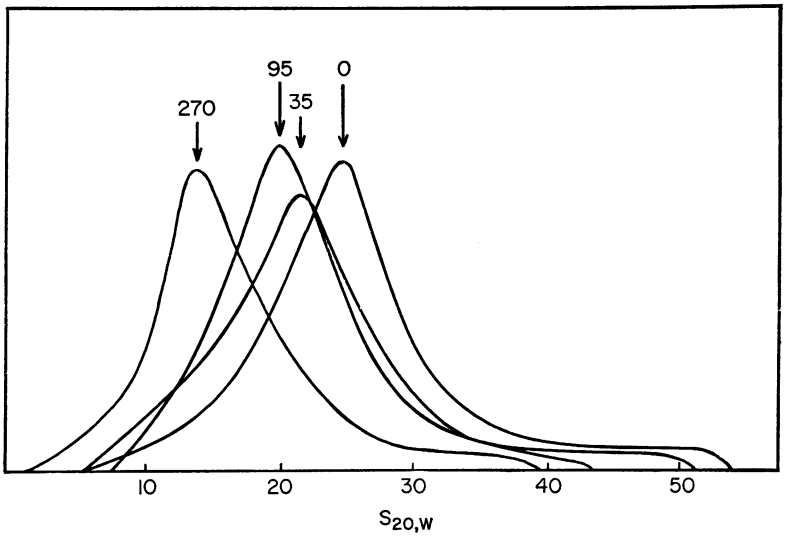

FIGURE 1 Distribution of sedimentation coefficients for AO-DNA after different periods of irradiation. Irradiation time in minutes as indicated.

With long irradiation the minimum median $s_{20,w}$ thus far produced has been 9.6; further degradation would probably be effected by addition of more AO to the degraded product (to replace that consumed by bleaching) and additional irradiation.

In a number of experiments, the influence of AO concentration on DNA photolysis was investigated. It was found that the specific ability to decrease DNA viscosity (η_{sp}/η_{sp0} *versus* C_{AO}) decreased with increasing AO concentration in the range of 0.5 to 2 $\times$ 10^{-5} M AO (5 $\times$ 10^{-3} per cent DNA). The optimal ratio of AO to DNA has not been determined. Consequently the conditions used in the present work were selected to give maximum binding.

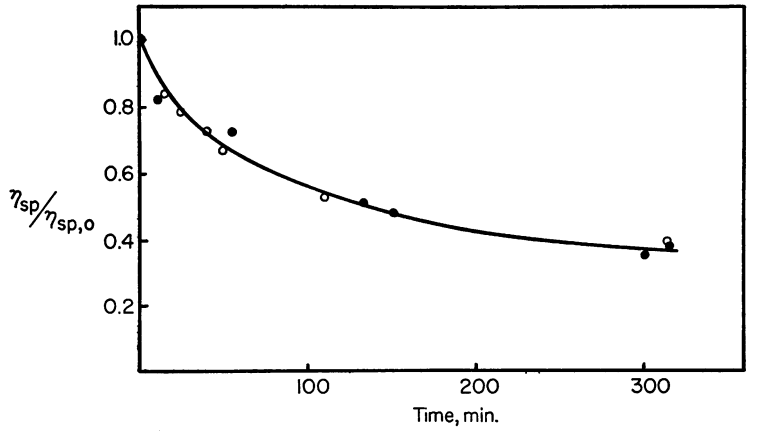

FIGURE 2 Effect of irradiation on the viscosity of AO-DNA. Irradiation at 10^5erg/cm^2/sec in 0.01 M NaCl.

o: Viscosity measured in 0.1 M NaCl.

●: Viscosity measured in 0.5 M NaCl.

η_{sp}: Specific viscosity (extrapolated to zero shear gradient).

$\eta_{sp,o}$: Specific viscosity of unirradiated control.

(*b*) Density gradient centrifugation in a CsCl gradient shows that these viscosity changes cannot be the result of denaturation alone. In Fig. 3 are shown two densitometric traces for irradiated and unirradiated salmon DNA. These curves have been arranged so that *Pseudomonas fluorescens* DNA bands, added as an internal standard, are superimposed. Mean apparent densities of control and irradiated salmon DNA differ by only 0.001. (This small difference, within our experimental precision, is in the opposite direction from that corresponding to denaturation.) The apparent density of denatured salmon DNA is also indicated. The increased width of band II is consistent with a decrease in molecular weight. The slight skewness of this band suggests density heterogeneity, and may result from selective base destruction or other chemical changes (see below).

2. *Bleaching.* In the course of irradiation, AO is noticeably bleached and its fluorescence in solution decreases markedly. The identification of the bleached product has not been made. It absorbs weakly in a broad band at 310 to 430 mμ and does not fluoresce. The existence of this bleaching complicates the interpretation of the kinetics of the degradation process, because, as bleaching continues, there are fewer AO molecules absorbing light. Consequently the effective dose rate decreases with time, so that time of irradiation is not proportional to dose received. Under conditions of AO-DNA binding this bleaching increases as the sedimentation coefficient and viscosity of the DNA are lowered. However, AO is bleached by light and O_2 even in the absence of binding to DNA.

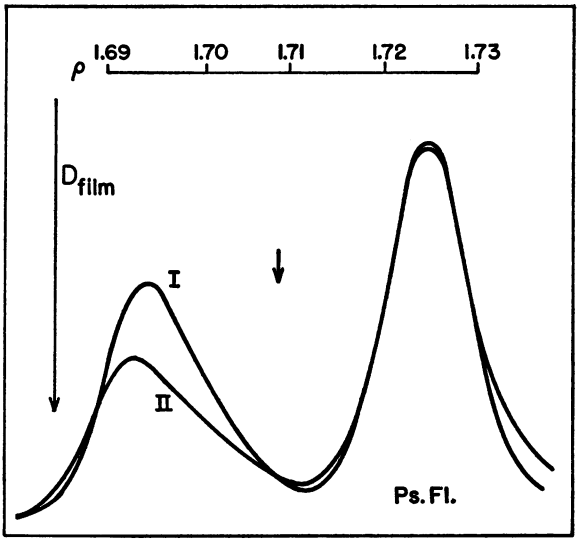

FIGURE 3 Density gradient centrifugation of DNA (ST-1) in 7.5 M CsCl. Comparison of irradiated (II) (90 minutes at 8 × 10^5erg/cm²/sec; $\eta_{sp}/\eta_{sp,0} = 0.73$) and unirradiated (I) DNA. Reference standard is *Ps. fluorescens* DNA. Apparent densities interpolated from data of Rolfe and Meselson (12).

↓: Apparent density of denatured salmon DNA.

3. *Requisites for Degradation.* At high ionic strength, binding of AO to DNA is markedly decreased (10). If 0.5 M NaCl is the solvent at irradiation, the effect for a given dose is markedly reduced; in 2 M NaCl no effect is evident even for extremely large doses. It seems clear that, at these concentrations, the dye is a photosensitizer only when bound to the DNA.

In the course of work on the AO-sensitized photoinactivation of yeast, evidence for an oxygen effect was obtained (3); *i.e.,* irradiation in the absence of atmospheric oxygen (in so far as oxygen can be removed from suspensions of living cells) has little or no effect on viability. Consequently, we examined the effect of deoxygenation on the photolytic degradation of AO-DNA and found that oxygen is required. Indeed, if oxygen is scrupulously removed, no effect on the sedimentation or viscosity properties of DNA is observed, even after prolonged irradiation. That the oxygen is not catalytic and is actually consumed was shown by irradiation of normal (not deoxygenated) solutions in sealed vessels without an airspace. The degradation effect was reduced.

4. *Mechanism of Degradation: Single- versus Double-Strand Scission.* The multi-hit kinetics of the decrease in intrinsic viscosity with time of DNA digested by DNAase has been given as evidence that molecular scission occurs when breaks in the individual strands of the double-stranded helix are matched (13). On the contrary, sonic degradation of DNA proceeds by simultaneous breakage of

both strands; *i. e.*, double-strand scission (14). A reliable kinetic analysis cannot, however, be made for the degradation of AO-DNA even though the curve of Fig. 2 seems to lack a multi-hit shoulder.[4] The principal difficulty is the bleaching of the dye because: (*a*) the number of absorbing molecules progressively decreases; (*b*) the binding and degradative efficiency of the AO will vary as the concentration of the AO changes; and (*c*) the binding and interaction of the bleached product are unknown. These effects result in, among other things, a progressive expansion of the time scale of the degradation curve which could easily obscure an initial shoulder expected from a two-hit curve. Consequently attention is primarily focused on the use of a general method developed for detecting single-strand breaks (15).

The technique consists of separating the two strands of degraded DNA and examining the sedimentation properties of the then single-strand material. In the case of degradation by double-strand scission, when the molecules are separated into single strands the molecular weight will be halved. However, when the molecular weight of the original molecules is lowered by the juxtaposition of two of numerous random single-strand breaks, the separation of the strands will result in a large number of molecules of low molecular weight. This type of relationship between single- and double-chain breaks is to be anticipated even if not all the strands are completely separated. The data of Table I are consistent with this model but could not, by themselves, be considered as proof. Doty *et al.* (16) have given evidence that during heat denaturation of DNA dissociation occurs. To be useful, however, the dissociation procedure should not break any undamaged fragments and reaggregation must be prevented. These effects unfortunately occur with ordinary thermal denaturation. However, the addition of formaldehyde (*a*) lowers the denaturation temperature so that hydrolysis of phosphoester links does not occur, and (*b*) prevents reaggregation by titrating the amino groups of adenine and guanine (17, 18).

Table I compares the changes in sedimentation coefficient for DNA degraded by sonic irradiation (double-strand scission), and DNAase digestion (matching of single-strand breaks), and for visible light irradiation of the AO-DNA complex. It is clear that the marked decrease in $s_{20,w}$ upon denaturation for photolyzed AO-DNA matches that for DNAase digestion. We conclude that many single-strand breaks are "hidden" in undenatured AO-photolyzed DNA. It should be pointed out that the identity of the broken bonds is not known.

It must, however, be remarked that double-strand scission has not been excluded and the sedimentation and viscosity data on undenatured DNA suggest that both types of action do, in fact, occur. However, since the kinetic analysis has not been made, this evidence must be considered imperfect.

5. *Evidence for Other Damage to DNA.* The thermal denaturation

[4] It was thought at first that the absence of the shoulder might have resulted from the presence of prior single-strand breaks in the DNA, especially in view of the dry storage of the salmon testis DNA. However, a similar curve was obtained for freshly prepared calf thymus DNA.

temperature of polynucleotides of high molecular weight is not strongly dependent on chain length (19). Changes in secondary structure stability of AO-DNA upon irradiation should therefore serve as an indication of radiation damage other than that already evident from the sedimentation experiments described above.

However, AO-DNA binding presents one minor complication. At low ionic strength, DNA-bound dye strongly influences the thermal stability of DNA. For instance, 1×10^{-5} M AO raises the denaturation temperature, $T_{1/2,d}$, of 7.4×10^{-5} M (P) DNA from 61 to 72°C (in 4×10^{-3} M aqueous NaCl, 1×10^{-3} M tris, pH 7.1). Probably only part of this stabilization is due to electrostatic effects since only 13 per cent of the phosphates have their charge neutralized by the AO. In this situation, changes in $T_{1/2,d}$ reflect AO photolysis as well as changes in DNA.

In order to eliminate this interference one must dissociate the AO-DNA complex after irradiation, either by raising the ionic strength, or by other changes in the solvent medium. In the former case one is forced to follow denaturation at rather elevated temperatures (85-95°C). We have, instead, used the following medium for our denaturation experiments: 44 weight per cent methanol (1 volume water + 1 volume methanol), 4×10^{-3} M NaCl, 1×10^{-3} M tris, pH 7. That AO is almost completely dissociated from DNA in this solvent is shown by (*a*) the absorption spectrum of the dye, which has λ_{max} at 493 mμ and only a very small shoulder at 465 mμ, and (*b*) the relative insensitivity of $T_{1/2,d}$ to the presence of AO. DNA, on the other hand, has the same intrinsic viscosity in 44 weight per cent methanol as in aqueous salt solution.

Results of the thermal denaturation experiments on irradiated DNA are sum-

TABLE I

DECREASE IN $S_{20,w}$ UPON DENATURATION OF SALMON TESTIS DNA PRETREATED BY SONIC IRRADIATION, DNAASE, OR ACRIDINE ORANGE + VISIBLE LIGHT

	S_{native}	$S_{denatured}$
Sonically degraded	18.2	17.2
	13.6	10.8
	12.3	10.4
DNAase	13.0	4.2
	11.3	3.5
	7.7	1.4
AO–visible light	18.2	6.6
	13.7	3.7
	11.3	3.6

Denaturation conditions: 20 minutes at 70°C in 2 per cent HCHO, 0.01 M phosphate buffer, pH 7.6.
Control experiments: (1) Hyperchromicity of each solution at least 37 per cent. (2) Once hyperchromicity is complete, there is no further drop in $S_{20,w}$ even with heating up to 3 hours.

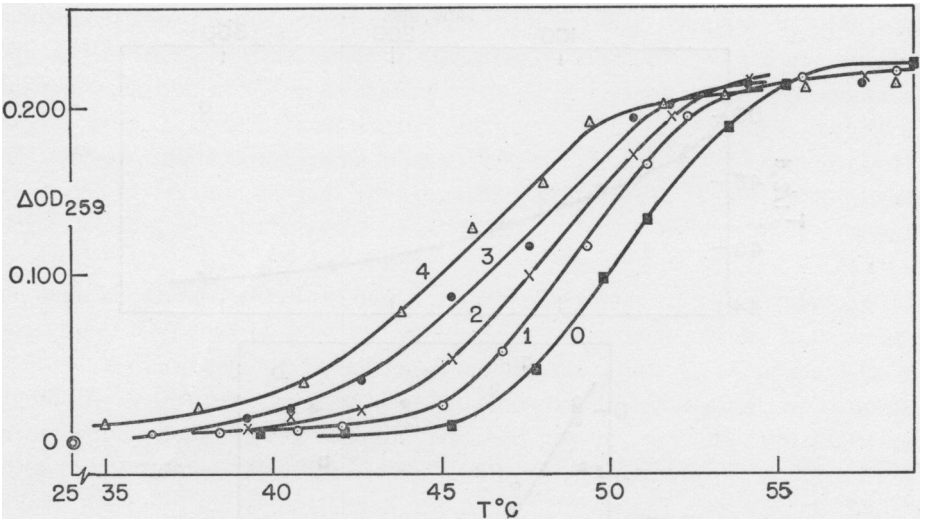

FIGURE 4 Spectrophotometric thermal denaturation curves of DNA + AO after varying periods of irradiation. ΔOD is the change in optical density (relative to 25°C) at 259 mμ, measured at the ambient temperature. Optical densities are corrected for volume changes.

Curves: 0—no irradiation; 1—25 minutes;
2—55 minutes; 3—133 minutes;
4—313 minutes, at 10^5 erg/cm²/sec.

Materials: 7.4×10^{-5} M DNA (ST-1, $OD_{259} = 0.48$), 1×10^{-5} M AO, 10^{-3} M NaCl, 10^{-3} M tris, pH 7.1; 44 weight per cent methanol.

marized in Figs. 4 and 5. The total range of thermal denaturation temperatures (5.6°C) covered by the irradiation experiments is much greater than the maximum effect due to AO binding in this medium (1°C). It is therefore clear that we are observing changes in the stability of DNA secondary structure. Curve 4 of Fig. 4 also shows that at longer irradiation times, not only is $T_{1/2,d}$ lowered substantially, but the transition curves are noticeably broadened at the low temperature end.

It is clear from the appropriate control experiments on enzymatically and sonically degraded DNA that this change in $T_{1/2,d}$ is not merely due to phosphate-ester bond breakage of either the double- or single-stranded variety. Denaturation of enzymatically and sonically degraded DNA was studied in slightly different media. While the methanol concentrations were still 44 weight per cent, the ionic constituents differed: (*a*) 10^{-3} M NaCl, 10^{-3} M tris, pH 7.1 for the sonic series, and (*b*) 8×10^{-4} M NaCl, 8×10^{-5} M $MgCl_2$, 10^{-3} M tris, 8×10^{-4} M versene, pH 5.7 for the enzyme series. In NaCl-tris (*a*) $T_{1/2,d}$ of undegraded DNA-C is 45.2°C, while in the NaCl–$MgCl_2$–versene–tris medium (*b*) $T_{1/2,d}$ of undegraded DNA ST-1 is 40.7°C. The change in $T_{1/2,d}$ with degradation for both types of treatment is shown to be small over the range of degradations appropriate to our experiments.

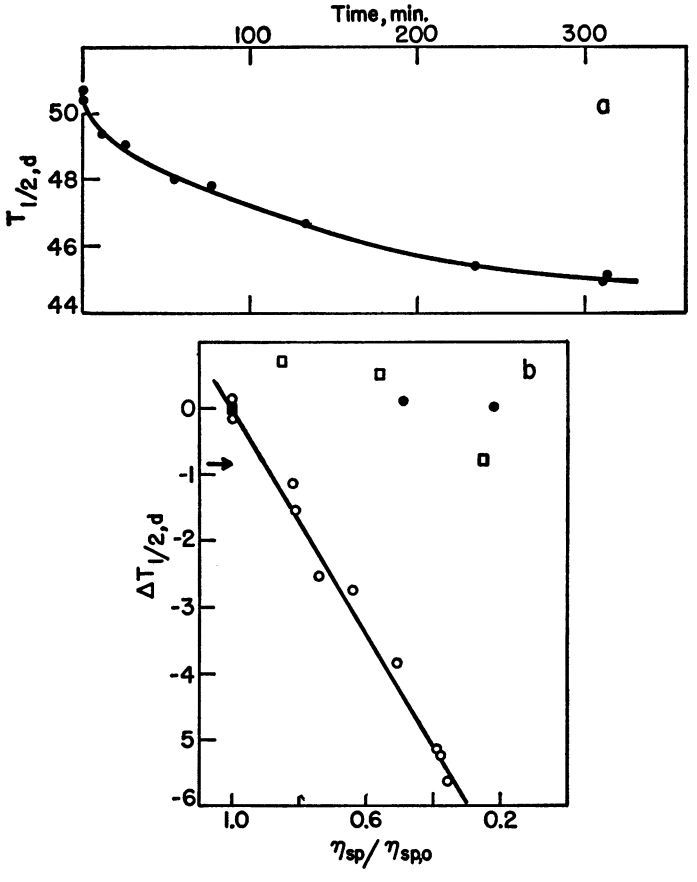

FIGURE 5

(*a*) $T_{1/2,d}$ as a function of irradiation time. $T_{1/2,d}$ is the midpoint of denaturation curves, such as those of Fig. 4.

Materials: Same as Fig. 4.

(*b*) Comparison of the effects of irradiation on viscosity and $T_{1/2,d}$.

$\Delta T_{1/2,d}$: Change in "denaturation temperature" relative to unirradiated or unreacted DNA.

η_{sp}: Specific viscosity (extrapolated to zero shear gradient) of irradiated or reacted DNA.

$\eta_{sp,0}$: Specific viscosity of unreacted control at the same concentration.

o: AO-DNA (DNA ST-1) in solvent 1.

→: DNA ST-1 in solvent 1 in absence of AO.

●: Sonically degraded DNA-C in solvent 2.

□: Enzymatically degraded DNA ST-1 in solvent 3.

Solvent: (1) 4×10^{-3} M NaCl, 10^{-3} M tris, pH 7.1; 44 weight per cent methanol.

(2) 10^{-3} M NaCl, 10^{-3} M tris, pH 7.1; 44 weight per cent methanol.

(3) 8×10^{-4} M NaCl, 10^{-3} M tris, 8×10^{-4} M versene, 8×10^{-5} M $MgCl_2$, pH 5.7; 44 weight per cent methanol.

The observed decrease of secondary structure stability must therefore be due to other reactions. Oxidation of deoxyribose, or of amino groups on adenine, guanine, and cytosine are two of several possibilities. Both these changes would tend to lower secondary structure stability—the former because of increased flexibility in the phosphate sugar backbone, and the latter because of decreased hydrogen bond stabilization.[5] However, we have not yet identified photolysis products.

COMMENTS

The data presented above are consistent with the view that, when AO molecules bound to DNA absorb visible light, the energy of the light quanta can ultimately be used to break chemical bonds in DNA. At least two different reactions can occur. These lead to reduction of the molecular weight of the DNA on the one hand, and destabilization of secondary structure on the other. The requirements for base damage and depolymerization are: (*a*) oxygen, and (*b*) binding of dye to DNA. This last condition probably reflects the formation of some unstable intermediate which has a high probability of reacting with DNA only if formed in its immediate vicinity and held there during its lifetime.

A number of obstacles prevent quantitative comparison of the doses required for DNA photolysis with those which lead to mutagenic and lethal action in living cells. The main problem is that, at present, one knows little about *in vivo* binding of AO to DNA-containing structures, relative to other cellular constituents. However, as expected, the survival of cells (yeast or *E. coli*) is decreased by radiation doses one or two orders of magnitude smaller than those to which DNA must be subjected to produce substantial effects on viscosity or thermal denaturation temperature.

The obvious (though not yet established) possibility that DNA is the site of the photosensitizing action of acridine orange on cells alerted us to the necessity for studying the action of visible light on the AO-DNA complex. We have found effects, *in vitro,* which could, in principle, correspond to the *in vivo* inactivation (depolymerization) and mutagenesis (damage to deoxyribose, purines, and pyrimidines). It will be interesting to see whether acridine orange can, indeed, be used as an *in vitro* mutagenic agent for transforming principle.

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[5] Partial deamination of DNA by HNO_2 does, in fact, lower its thermal stability (20).

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