Volume 3 no.2 February 1976 Nucleic Acids Research

Studies on the mechanism of DNA cleavage by ethidium.

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Received 10 September 1975

ABSTRACT

Ethidium causes the cleavage of DNA via a light and oxygen dependent process. Using covalently closed circular DNA as a substrate, the saturation kinetics and the dependence on superhelical density of the cleavage indicate that intercalated ethidium is mainly responsible for nicking DNA. Superoxide dismutase has little effect on the reaction and catalase none. Lowering the pH inhibited the reaction. The reaction mechanism and its use in determining superhelical densities of covalently closed circular DNAs are discussed.

INTRODUCTION

Ethidium bromide, a trypanocidal drug, has been widely used in the study of nucleic acids (1,2). Ethidium interacts with DNA in at least two different ways, both of which are probably important in understanding its in vivo toxicity. (a) It can intercalate between the base pairs of DNA, causing a partial unwinding of the DNA primary helix (3,4,5) and a lengthening of the molecule (6). For DNA in which topological restrictions to rotation exist, e. g. covalently closed circular (ccc) DNA or the E. Coli nucleoid (7), the unwinding of the primary helix by ethidium causes positive supercoiling of the DNA, with many alterations to the biochemistry of DNA (8). (b) Ethidium can cause a light induced cleavage of DNA (9). This reaction has become important to us since we use PM2 ccc-DNA and ethidium for a variety of sensitive assays (10). The ethidium induced cleavage is a liability when interfering with the assays but can be an asset for determining superhelical densities by a simple fluorometric procedure (see discussion). We, therefore, investigated the cleavage mechanism with the hope of being able to control it.

Recently we have shown that the superoxide radical ($O_2^{\cdot-}$) cleaves DNA by a mechanism which is sensitive both to superoxide dismutase and catalase (11). $O_2^{\cdot-}$ dismutates to O_2 and H_2O_2 and H_2O_2 can react with $O_2^{\cdot-}$ to produce hydroxyl radicals ($OH^{\bullet}$) which damage DNA (11,12). Many autoxidation reactions give rise to $O_2^{\cdot-}$ (13,14) and more recently we have shown that streptonigrin, an antibiotic known to cleave DNA via an oxygen dependent pathway, also produces

$O_2^{\cdot}$ by autoxidation of the reduced form (15). Mitomycin C was also shown to degrade DNA apart from its well-known cross-linking reaction (16) and this also occurs via $O_2^{\cdot}$ (16). Certain aromatic molecules, such as the dye fluorescein, produce $O_2^{\cdot}$ only on photosensitisation (17). It was, therefore, not so surprising to find the light-catalyzed cleavage of DNA by ethidium could be completely suppressed by purging the solutions with helium. However, a simple mechanism mediated by $O_2^{\cdot}$ was ruled out since DNA cleavage was hardly affected by superoxide dismutase or catalase.

METHODS

DNAs. PM2 covalently closed circular (ccc) DNA was isolated as before (10) and was contaminated with about 20% open circular (oc) DNA. PM2 ccc-DNA was relaxed with the calf thymus omega protein (18) as follows: PM2 DNA (4 A_{260}) was incubated at 37° for one hour in 1 ml of 10 mM pH 7.5 K^+ phosphate, 2 mM EDTA, 0.2 M NaCl and sufficient partially purified calf thymus omega protein (high salt extract of chromatin and polyethylene glycol precipitation of DNA (18)) to relax the DNA. Using the ethidium assay procedure (10) the 30% drop in the fluorescence enhancement for ccc-DNA and the renaturability confirmed complete relaxation. The reaction mixture was then made 2 M in NaCl to dissociate omega protein from the DNA and heated for 10 minutes at 50° to insure inactivation, and finally the DNA was excluded from a 15 M (200 - 400 mesh) Agarose column (10 ml) in 10 mM Tris HCl pH 8, 0.1 mM EDTA, concentrated by dialysis versus polyethylene glycol (6000) and finally dialyzed in Tris/EDTA to give 2.25 A_{260}.

Fluorescence Assays. The assays for nicking and relaxation of ccc-DNA have been described (10). The main features of the fluorescence assays are that when ethidium intercalates there is a 25-fold enhancement of its fluorescence specific for base-paired structures. At pHs around 11.7 denatured DNA no longer contains short intramolecular base-pairs and therefore shows no fluorescence enhancement. The unique topological properties associated with ccc-DNA restrict the uptake of intercalated ethidium and cause the immediate renaturation of ccc-DNA after a heat denaturation step. In contrast oc-DNA has no topological restrictions to ethidium intercalation and is completely denatured after heat treatment. The % loss of fluorescence after the heat denaturation step has been used to calculate % nicking of ccc-DNA.

Irradiation of DNA. The reaction mixtures were irradiated either in sunlight through a double-glazed window or 6-8 cm from a 100 watt tungsten bulb mounted in a silvered reflector. For the latter case the reactions were

cooled by an air jet to room temperature. The standard reaction mixture contained 5 mM K^+ phosphate pH 7.5, 0.5 mM EDTA and PM2 DNA and ethidium as indicated in the legends to figures. Although the incident light varied slightly from one set of experiments to another, for a given set the flux was identical for the reaction mixtures.

Enzymes. Superoxide dismutase was kindly donated by Dr. Allan Davison and was assayed for activity using xanthine-xanthine oxidase to generate $O_2^{\cdot}$ which reduced cytochrome C (19). Bovine catalase was from Sigma Chemical Co.

RESULTS

The ethidium fluorescence assay for measuring DNA cleavage. Although previously (10) we have shown the advantages of using the fluorescence enhancement of intercalated ethidium and ccc-DNA for detecting scissions in DNA, irradiation of ethidium and DNA might lead to alterations in ethidium or DNA which affect the assay. The following controls show that within the time course of the reactions followed no problems arose. In Figure 1 is shown the increase in fluorescence as supercoiled and relaxed

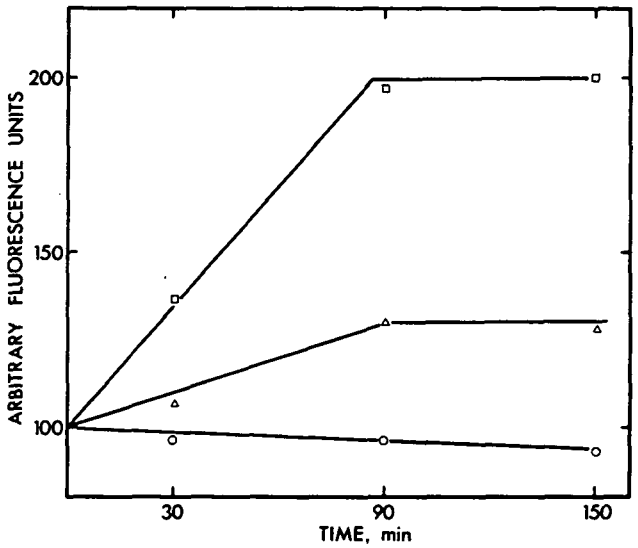

Figure 1. The reaction mixtures irradiated in sunlight contained 5 mM Tris pH 8, 0.5 mM EDTA, 0.5 µg/ml ethidium bromide, 0.005 A_{260} PM2 DNA, supercoiled Δ—Δ or relaxed □—□ or 0.005 A_{260} calf thymus DNA o—o. The zero time fluorescence is arbitrarily taken as 100 for each sample.

ccc-DNA are nicked in sunlight. For supercoiled PM2 DNA showing 100 arbitrary fluorescence units nicking leads to 130 units (30% increase (10)) whereas relaxation leads to 67 units (33% loss (18)). Relaxed ccc-DNA on

being nicked must also necessarily give 130 fluorescence units, i.e., an increase of about 100% in fluorescence. This is borne out by the data in Figure 1. Calf thymus DNA was used as a control and a slow but real loss in fluorescence was observed, probably due to a radiation-induced reaction of ethidium. When ethidium was irradiated alone the UV spectrum showed a shoulder developing on the short wave-length side of the 480 nm absorption band. This was not investigated further since it does not seriously affect any of the subsequent data. There is an even greater change in fluorescence after a heat denaturation step with oc-DNA giving zero fluorescence in contrast to ccc-DNA which shows 100% return of fluorescence. The loss of fluorescence after the heat step is therefore a more sensitive assay for cleavage and gives essentially the same results (see Table I). This table

Table I Comparison of changes in fluorescence before and after heating

Time of irradiation minutes	Fluorescence before heat	% ccc-DNA nicked	Fluorescence after heat	% ccc-DNA nicked
0	100	0	100	0
27	114	47	54	46
55	130	100	10	90
80	126	87	8	92

also shows that there are no light-induced changes giving rise to a thermolabile DNA species which are only cleaved during the brief heat treatment. This is not always the case. Most irradiations were at pH 8 and 10-20 µl samples were added to the pH 11.7 fluorescence assay mixture (10). At both pH 8 and on raising the pH to 11.7 the expected increase in fluorescence was observed for nicking of DNA. However if the DNA and ethidium were irradiated at pH 11.7, no increase in fluorescence was observed although after heating the expected drop in fluorescence occurred. This suggests that irradiation led to a thermolabile structure in the DNA which did not result in any strand breaks. For this reason wherever irradiation was at pH 11.7 the amount of cleavage is determined from the samples after heating in the pH 11.7 assay mixture.

Oxygen and light dependence of DNA cleavage by ethidium. PM2 DNA was added to the high pH (∿11.7) ethidium assay solution, and the solution was purged either with helium or oxygen for various times and then the solution was irradiated for various times with a 100 watt bulb. Table II shows that passage of helium prior to illumination for 3 minutes drastically reduced the amount of cleavage and for 15 minutes essentially inhibited it com-

Table II Effect of helium and oxygen on ethidium-induced cleavage of DNA

Minutes of purging with He	% ccc-DNA remaining at 30 minutes	Minutes of purging with O_2	% ccc-DNA remaining at 15 minutes
0	45	0	69
3	86	2	46
6	93	5	41
15	100	10	39

pletely. On the other hand oxygen-purging increased the rate by nearly two-fold. In the dark there was no nicking of DNA by ethidium within 2 days. Although an attempt was made to determine the wave-length at which maximum nicking occurred, the light intensity in the Gilford Spectrometer was insufficient to cause nicking at an appreciable rate. Presumably visible light only is required since little UV light was expected from the 100 watt tungsten bulb and the solutions were housed in borosilicate glass fluorescence cuvettes, opaque to UV light.

Dependence of cleavage rate on ethidium concentration and superhelical density. In Figure 2 is shown the kinetics of nicking of supercoiled and relaxed PM2

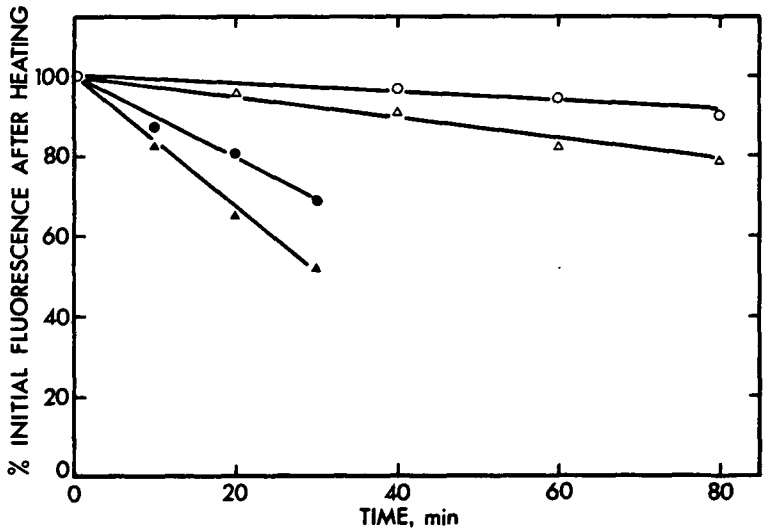

Figure 2. Kinetics of PM2 DNA cleavage and the effect of ethidium concentration. The conditions were as for Figure 1 but with DNA at 0.5 A_{260} in the presence of 0.07 µg/ml (open symbols) or 0.5 µg/ml (closed symbols) of ethidium bromide. Supercoiled DNA Δ—Δ and relaxed DNA o—o. 20 µl samples of the irradiated and oxygenated solutions were added to 2 ml of alkaline ethidium assay mixture (10) and the % loss in fluorescence was determined after heating relative to the fluorescence of the zero time heated samples.

DNAs at two different ethidium concentrations. At the lower level of ethidium (0.07 μg/ml), the supercoiled DNA is nicked two times as fast as the relaxed DNA. However at 0.5 μg/ml of ethidium the supercoiled DNA is nicked only 1.75 times as fast. For these experiments a preparation of L cell omega protein was used to relax the DNA and it was heat inactivated prior to the nicking experiments. Although the crude omega protein preparation had been diluted 1,000 fold into the reaction mixture, there was a possibility that trace contaminants might alter the course of a free radical reaction. In Figure 3 in which the rates of nicking were obtained from plots similar to Figure 2, the relaxed DNA was prepared as in the Methods but free of any possible contaminants in the relaxation mixture. Again the supercoiled DNA was nicked more rapidly than the relaxed DNA and at high concentrations of ethidium both rates of nicking tended to plateau, with the negatively supercoiled DNA being nicked about 50% faster. The negatively supercoiled DNA will of course be positively supercoiled in the presence of excess ethidium but will bind more ethidium than the relaxed DNA. With saturating ethidium naturally occurring PM2 ccc-DNA with negative supercoils shows about 50% more fluorescence enhancement than relaxed PM2 ccc-DNA (18), suggesting 50% more ethidium is bound. Considering the errors in measuring the rates of nicking for high ethidium concentrations, this is in surprising agreement with the relative plateau levels. Also at low levels of ethidium, a greater differential rate is expected between supercoiled and relaxed DNAs if intercalated ethidium is the species responsible for nicking as is observed in Figure 2.

The effects of superoxide dismutase and catalase. In control experiments it was shown that superoxide dismutase was unaffected by ethidium in its activity on $O_2^{\cdot}$ produced by xanthine/xanthine oxidase. At levels of 4 μg/ml, superoxide dismutase completely inhibited vitamin C and streptonigrin-induced DNA degradation (11,15) but even at 40 μg/ml as shown in Figure 4, there was only a slight initial inhibition of ethidium-induced cleavage and then no apparent further effect. This was reproducible, so that possibly there is a very minor side reaction involving $O_2^{\cdot}$ initially. Catalase had no effect at all at 12.5 μg/ml whereas previously at 1 μg/ml it had completely suppressed $O_2^{\cdot}$ cleavage (11). Even when the two enzymes were added together the synergystic effect observed with mitomycin C (16) was absent. Further the hydroxyl radical scavenger, mannitol, also previously found to affect $O_2^{\cdot}$ cleavage had no observable effect at 1 mM.

The effects of pH. In Table III reducing the pH reduces the rate of the DNA cleavage. $O_2^{\cdot}$ dismutates orders of magnitude more slowly than $HO_2^{\cdot}$. The pKa

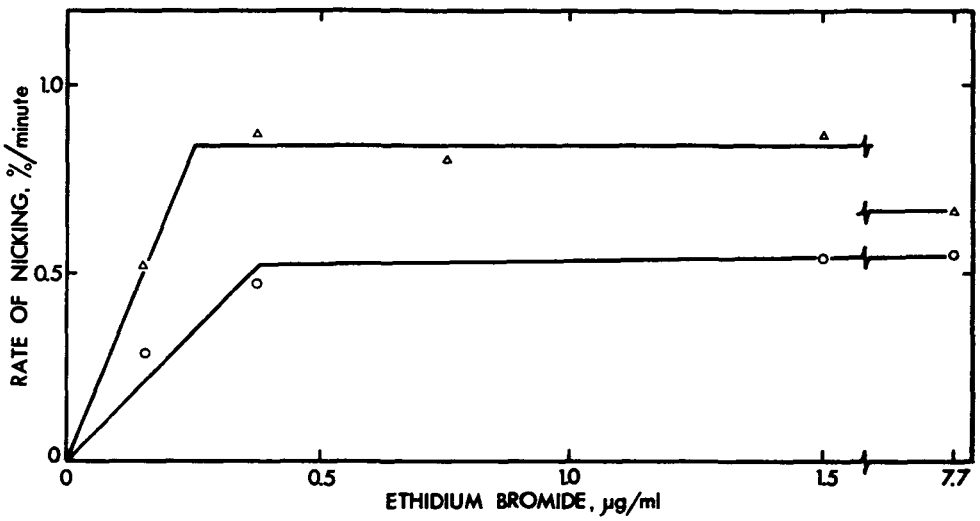

Figure 3. The effect of ethidium concentration on the rate of nicking of supercoiled and relaxed PM2 DNA. The rate of nicking was determined from the initial slope of plots similar to Figure 2 but at various concentrations of ethidium. Otherwise the assays were identical. Supercoiled DNA Δ—Δ and relaxed DNA o—o.

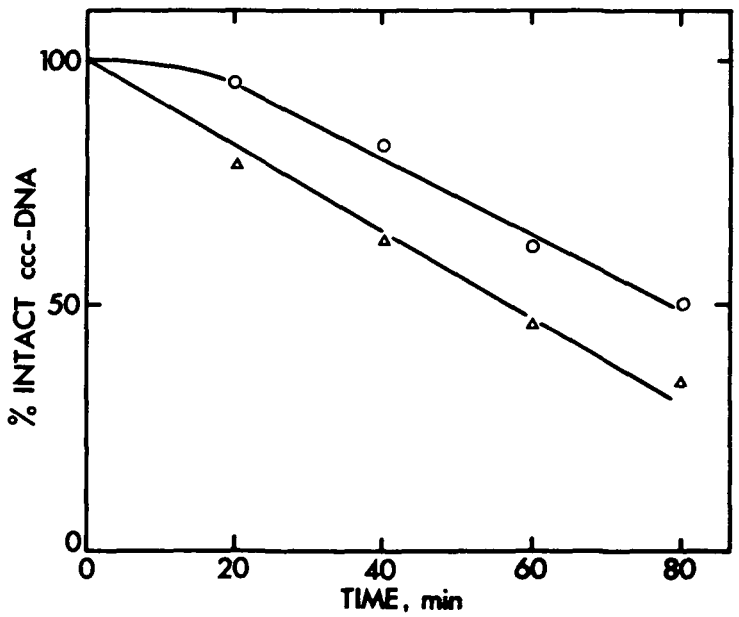

Figure 4. The effect of superoxide dismutase on DNA cleavage by ethidium. The irradiation was in 5 mM Tris pH 8, 0.5 mM EDTA, 0.5 µg/ml ethidium bromide and 0.5 A_{260} PM2 DNA, with o—o and without x—x 10 µg/ml of superoxide dismutase. Irradiation was by sunlight.

Table III Effect of pH on the cleavage of DNA by ethidium*

Buffer (25 mM)	Rate of cleavage (%/minute) sunlight	100 watt tungsten bulb
NaOAc pH 5	0.8	0.4
NaOAc pH 6	1.4	0.85
KP_i pH 6	1.4	0.7
KP_i pH 7	1.4	1.2
KP_i pH 8	2	1.4

*The PM2 DNA was 0.33 A_{260} and ethidium 3.5 µg/ml. 20 µl samples were added to pH 11.7 ethidium assay mixture.

for $HO_2^{\cdot}$ is 4.8 (13) and the pH effect is not related to it.

DISCUSSION

The mechanism by which ethidium induces scissions in DNA although light and oxygen dependent is still obscure. It appears that in the range of ethidium concentrations studied, intercalated ethidium is responsible for cleavage. If $O_2^{\cdot-}$ were produced in some initial step it could not be liberated into solution but must act *in situ* since superoxide dismutase has little effect. Since $O_2^{\cdot-}$ cleavage of DNA is also inhibited by catalase, but catalase does not affect the ethidium-induced cleavage, it seems highly unlikely that $O_2^{\cdot-}$ has any role or is even produced *in situ*. The finding that irradiation at high pHs gives rise to a thermolabile product, not observed at pH 8, probably suggests some addition of an ethidium radical. The disposition of the two amino groups of ethidium suggest analogies with hydroquinones with the formation of intermediate diimino-products (analogues of quinones) on autoxidation. However aromatic amino functions can show very complex free radical oxidation pathways as exemplified by aniline and the simple picture found with the quinoline quinones such as streptonigrin and mitomycin C cleaving DNA is not applicable.

As regards the fluorescence assays, the nicking by ethidium can be abolished by minimum exposure to light. Under normal laboratory lighting the cleavage takes 4 hours to produce a detectable conversion of ccc-DNA. On the other hand it would be very useful to quantitatively nick ccc-DNA in the pH 11.7 ethidium assay mixture since as already noted the difference in fluorescence increase on nicking for relaxed PM2 ccc-DNA (100%) and naturally occurring negatively supercoiled PM2 DNA (30%) is pronounced. Hence the superhelical density of a topologically restricted DNA could be readily

determined by two fluorometric readings. The rise in fluorescence on nicking is a simple function of superhelical density (18). Many procedures have been attempted for quantitatively breaking DNA in the ethidium assay mixture (e.g. sonication, X-rays, vitamin C, etc.) but all suffer from various disadvantages. Unfortunately, the high pH precludes the use of enzymes. Nicking by ethidium itself on irradiation with visible light seems the best solution although some care must be exercised since as shown in Figure 1 at excessive times ethidium is significantly altered.

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