

# Single- and double-strand photocleavage of DNA by YO, YOYO and TOTO

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Received November 29, 1995; Revised and Accepted January 29, 1996

## ABSTRACT

Photocleavage of dsDNA by the fluorescent DNA stains oxazole yellow (YO), its dimer (YOYO) and the dimer TOTO of thiazole orange (TO) has been investigated as a function of binding ratio. On visible illumination, both YO and YOYO cause single-strand cleavage, with an efficiency that varies with the dye/DNA binding ratio in a manner which can be rationalized in terms of free dye being an inefficient photocleavage reagent and externally bound dye being more efficient than intercalated dye. Moreover, the photocleavage mechanism changes with binding mode. Photocleavage by externally bound dye is, at least partly, oxygen dependent with scavenger studies implicating singlet oxygen as the activated oxygen intermediate. Photocleavage by intercalated dye is essentially oxygen-independent but can be inhibited by moderate concentrations of  $\beta$ -mercaptoethanol—direct attack on the phosphoribose backbone is a possible mechanism. TOTO causes single-strand cleavage approximately five times less efficiently than YOYO. No direct double-strand breaks (dsb) are detected with YO or YOYO, but in both cases single-strand breaks (ssb) are observed to accumulate to eventually produce double-strand cleavage. With intercalated YO the accumulation occurs in a manner consistent with random generation of strand lesions, while with bisintercalated YOYO the yield of double-strand cleavage (per ssb) is 5-fold higher. A contributing factor is the slow dissociation of the bis-intercalated dimer, which allows for repeated strand-attack at the same binding site, but the observation that the dsb/ssb yield is considerably lower for externally bound than for bis-intercalated YOYO at low dye/DNA ratios indicates that the binding geometry and/or the cleavage mechanism are also important for the high dsb-efficiency. In fact, double-strand cleavage yields with bis-intercalated YOYO are higher than those predicted by simple models, implying a greater than statistical probability for a second cleavage event to occur adjacent to the first (i.e. to be induced by the same YOYO molecule). With TOTO the efficiency of the ssb-accumulation is comparable to that observed with YOYO.

## INTRODUCTION

The dimers (YOYO and TOTO, Fig. 1) of the asymmetric cyanine dyes oxazole yellow (YO) and thiazole orange (TO) bind strongly to double-stranded DNA (dsDNA), and the fluorescence quantum yields of the bound dyes are typically 1000-fold higher than when free in solution (1). As a result, the background fluorescence from free dye is extremely low which makes these dyes excellent probes for high sensitivity quantification of DNA (2), and for imaging of individual DNA molecules (3–5). YOYO can also be used for imaging of single-stranded DNA (ssDNA) (6).

The use of these dimeric dyes for such applications is not without problems, however. First, upon mixing, the strong binding results in a long-lived bimodal distribution of dye molecules between the DNA molecules in the sample (7). Such inhomogeneous staining may produce serious artefacts in fluorescence-based DNA quantification, since the quantum yield is strongly dependent on binding ratio (8). This dye distribution problem can be overcome, however, by a simple equilibration protocol (7). A potentially more serious side-effect in many applications is the tendency of these dyes to photocleave the stained DNA, especially under the intense illumination used for imaging of individual molecules by fluorescence microscopy. By trial and error it has been found that the cleavage problem can be minimized by exclusion of oxygen, either by purging with argon (5) or by enzymatic methods (3), and usually  $\beta$ -mercaptoethanol is also added, as is common in fluorescence microscopy in order to minimize dye bleaching (9). However, the mechanisms of photocleavage by YOYO and TOTO and the manner in which these agents provide protection are not known.

The present work is the first step in a systematic study of the photochemical behaviour of these dyes, with the aim of rationally devising the most effective protocols for reduction or prevention not only of strand cleavage, but also of other photochemical reactions whose effects may not be so dramatically manifest in imaging studies, e.g. abasic site or adduct formation. The strongest binding mode for YOYO is bisintercalation, but at higher dye loading the dye also binds externally to the helix (10). Since high dye loading is commonly used in applications of these dyes, our starting point has been the investigation of how DNA photocleavage depends on the mode of binding of YOYO, by studying the effect of binding ratio on the rates of strand cleavage. The dye binding mode is also of interest from a mechanistic point of view, since the binding geometry determines not only the contact points between the helix and the dye, but also the

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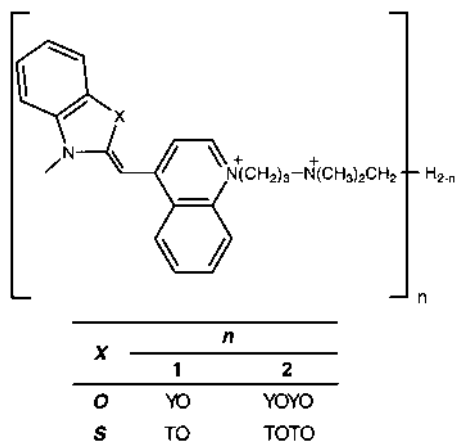


Figure 1. Structural diagram of the cyanine dyes.

accessibility of the dye to potential intermediate species in the surrounding solution, such as oxygen.

When a DNA molecule is imaged with YOYO in the microscope only double-strand breaks (dsb) are observed. However, few DNA cleaving agents directly cause dsb. Most induce single strand breaks (ssb) (11), which result in dsb only when two of the ssb occur by chance on opposite strands and separated typically by <15 bp (12). A DNA molecule might thus have sustained considerable damage via ssb, before a break is observed in the imaged polymer. It was therefore important to assess both the ssb and dsb efficiencies of YOYO. Supercoiled DNA was used to sensitively assay for ssb and dsb, which are detected as conversion of the supercoiled DNA into the relaxed circular and linear forms, respectively, both of which can be separated from the initial supercoiled form by gel electrophoresis. We show here that the monomer YO behaves as a classical ssb-agent, whereas the fact that the two chromophores in YOYO are forced into proximity results in a high dsb activity because of non-random distribution of ssb breaks.

## MATERIALS AND METHODS

### Chemicals and samples

Supercoiled and nicked circular form  $\Phi$ X174 DNA were from Pharmacia and New England Biolabs, respectively. The negatively supercoiled sample contained 5–10% nicked circles. Linear form was produced by cleavage of a supercoiled sample with restriction enzyme *Xho*I (Pharmacia). YO-PRO-1, YOYO-1 and TOTO-1 (referred to here as YO, YOYO and TOTO) were from Molecular Probes, (Eugene, OR, USA) and were used as received. Methylene blue (puriss) was from Fluka, and was purified by column chromatography (13). Concentrations were determined by optical absorption, using the following extinction coefficients:  $\epsilon(260 \text{ nm}) = 6600 \text{ M}^{-1} \text{ cm}^{-1}$  for DNA bases (14),  $\epsilon(481 \text{ nm}) = 66\,000 \text{ M}^{-1} \text{ cm}^{-1}$  for YO,  $\epsilon(457 \text{ nm}) = 96\,100 \text{ M}^{-1} \text{ cm}^{-1}$  for YOYO,  $\epsilon(480 \text{ nm}) = 97\,900 \text{ M}^{-1} \text{ cm}^{-1}$  for TOTO (15) and  $\epsilon(664 \text{ nm}) = 81\,600 \text{ M}^{-1} \text{ cm}^{-1}$  for methylene blue (16). Unless otherwise stated, the dye loading is given as the N/C ratio between concentrations of DNA nucleotide and added dye chromophores (one for monomer and two for dimer).

### Sample preparation

All samples contained 40  $\mu\text{M}$  DNA nucleotide and a dye concentration corresponding to the stated N/C, except for the lowest N/C of 1.5 (of YO or YOYO) which contained 20  $\mu\text{M}$  DNA nucleotide. The lower DNA concentration was chosen to keep the maximum absorption of the dye in the samples (at 480 nm) below  $\sim 0.1$ . All samples had a final total volume of 20  $\mu\text{l}$  in 50 mM TBE-buffer (pH = 8.3), and were prepared by adding 5  $\mu\text{l}$  DNA stock to the dye diluted in the buffer, and subsequently incubating at 50°C for 2 h to reach an equilibrium in the dye distribution (7). For deoxygenation, 100  $\mu\text{l}$  (i.e. five aliquots) was bubbled vigorously with water-saturated argon for 1 min. The efficiency of this degassing procedure was confirmed by the observation of a 64% reduction compared to air-saturated solution of the ssb induced in dsDNA by methylene blue (N/C = 15 and 50 mM ionic strength) (17). The rate of ssb formation with YO at different N/C was also investigated in the presence of 15–50 mM concentrations of  $\text{NaN}_3$ , in  $\text{D}_2\text{O}$ , urea, thiourea or glycerol, and in the presence of 0.5–2.5  $10^3$  U/ml of superoxide dismutase and/or catalase.

### Illumination

Illumination was performed using a 150 W xenon lamp, a lens and a long-pass (320 nm) filter, with the sample forming a droplet (maximum path length 0.1 cm) in the closed end of a horizontal Eppendorf tube sealed with a glass lid. In the absence of dye no DNA cleavage was detected during the longest illumination time used in this study (45 min). The stability of the lamp over a 7 h period was confirmed by a  $\pm 3\%$  variation in the relative concentrations of nicked and supercoiled forms obtained by illumination of an air-saturated sample containing YOYO at N/C = 3 for 5 s.

The main experimental parameter in this study was the dye binding ratio. For each binding ratio the time profiles for the conversion of the supercoiled species (form I) into nicked circles (form II), linear DNA (form III), and linear fragments (form IV) were determined. As the N/C was increased the reaction rates decreased, and the range of illumination times therefore had to be increased in order to monitor the formation of all DNA forms. At the highest N/C of 40 some illuminations extended over 15 min, and in those cases evaporated solvent that had condensed on the walls of the tube was recovered by spinning the samples every 15 min in a bench-top microcentrifuge for 2 min.

### Electrophoretic analysis

Photocleavage products were analysed on 1% agarose gels (in cleavage buffer) at 3 V/cm for 4.5 h. The positions of the zones of the separated products in each lane were determined from the fluorescence intensity profiles of YO (or YOYO) remaining bound, measured by scanning the gel on the stage of a Zeiss Axioplan epifluorescence microscope (excitation filter: 450–490 nm, dichroic mirror: 510 nm, emission long-pass filter: 520 nm, 5 $\times$  objective, numerical aperture = 0.15), equipped with a Hamamatsu photomultiplier. The epifluorescence illumination provided excellent signal-to-noise ratio, even for the highest N/C value (= 40), because of the very low quantum yield of fluorescence for free compared with bound dye (1). As evidenced by comparison with non-illuminated samples stained at the same N/C, the order of migration was always nicked circle, linear and supercoil forms. The DNA forms were baseline separated except for a slight overlap

of the zones of the supercoiled and nicked circles at binding ratios close to  $N/C = 12$  (due to unwinding effects from the intercalated dyes), which were resolved on the basis of the concentration profiles of the pure DNA forms. The zone of the intact linear form (III) and the tailing smear of linear fragments (form IV) were resolved on the basis of the observation that zones of pure form III are symmetric.

### Quantification of product concentrations

The quantity of each form of DNA was obtained by integrating the scanned intensity over each zone. Repetitive scans along the same lane showed that no detectable bleaching occurred as a result of the scanning. Two different approaches were used for staining the DNA. Mainly we have exploited the intrinsic fluorescence of the YO or YOYO that remains bound after electrophoresis, in order to make use of the very good signal-to-noise ratio for those dyes. This protocol also avoids the procedures of removing these strongly bound dyes and subsequently purifying and restaining the DNA, steps which could introduce artificial ssb, cleavage at alkali-labile sites or other damage. Some bound dye was probably lost during electrophoresis, but the quantification of ssb and dsb cleavage rates only requires knowledge of the relative concentrations of the different DNA forms (see eq. 2 below), which were obtained by normalizing with respect to the total intensity of the pertinent lane. For any experiment (i.e. at a certain  $N/C$ ), as long as the fraction of dye lost is the same for the different DNA forms, the relative concentrations and the evaluated rates should not be affected. As with conventional post-electrophoresis staining, corrections for the differential binding to supercoiled and nicked circular/linear DNA have to be made if an intercalating dye is employed. Prior to illumination the dye could be considered bound to the supercoiled form at the nominal  $N/C$ , since the contamination of relaxed circles in the supercoiled sample could be neglected. However, since large amounts of relaxed circles were produced in the course of the cleavage experiments, dye may have transferred between the newly created nicked circles and molecules that remained supercoiled, before the two forms were electrophoretically separated. (No dye transfer will occur between nicked circular and linear DNA since the affinity for an intercalator is the same.) This possibility was tested by removal of the dye and post-electrophoresis staining with the groove-binder DAPI, the affinity of which is negligibly affected by DNA topology. To keep DNA handling simple and gentle, destaining was performed after the illuminated samples had been migrated a few millimeters into the gel (at 3 V/cm for 0.5 h). The gel (150 ml) was soaked for 5 h in 1000 ml of 0.1 M NaCl containing 10 mM SDS (CMC under these conditions is 2 mM), a step which removed all the YO or YOYO from the DNA, as evidenced by no detectable fluorescence from the zones. The gel was washed twice in electrophoresis buffer for 4 h, electrophoresis was resumed for 4 h at 3 V/cm, and finally the gel was soaked in 500 ml of 10  $\mu$ M DAPI in electrophoresis buffer for 4 h and washed in electrophoresis buffer for 1 h. The DNA zones were again detectable, by the blue-white fluorescence characteristic of DAPI bound to DNA, and the gels were scanned using a 365 nm excitation band-pass filter, a 395 nm dichroic mirror and a 420 nm emission long-pass filter. [The fluorescence is from groove-bound DAPI, because of the quenching of the fluorescence of the GC-intercalated DAPI (20), present in a minor fraction at the staining conditions employed; 18,19.] The separation was better than with YO, where

unwinding slows down and broadens the zone of the supercoiled form compared with form II, but the  $S/N$  ratio is considerably lower with the DAPI protocol. Therefore DAPI was used for quantification only to investigate effects of differential staining by YO or YOYO on apparent cleavage kinetics, by illuminating two sets of samples and analysing them with both methods.

### Relative quantum yields of nicking

The consumption of form I DNA was analysed in terms of single exponential kinetics with a time constant  $\tau$ . The actual absorbance of each sample was not measured, so absolute quantum yields have not been obtained. The rate of nicking per DNA molecule ( $1/\tau$ ) was normalized with respect to the number of absorbed photons by dividing by the number of dye molecules per DNA molecule

$$k_1 = (1/\tau) \times (N/C) \quad (1)$$

where  $k_1$  is the relative nicking efficiency per YO chromophore (in units of  $s^{-1}$ ) whether in a monomer or a dimer. Since cleavage rates are normalized with respect to added chromophore,  $k_1$  is the average over intercalated, externally bound and free dye, which have slightly different absorption cross-sections. Except at the highest  $N/C$  values used, where all dye chromophores can be considered intercalated, there are potential effects on the relative rates due to this simplification which are discussed further in the main text.

### Calculation of number of ssb and dsb

The average numbers of ssb ( $n_1$ ) and dsb ( $n_2$ ) per DNA molecule were calculated from the equations of Povirk and co-workers (21,22). If the supercoiled form is consumed by both ssb and dsb which are randomly distributed among the DNA molecules, the fraction  $f_I$  of surviving supercoiled circles is related to the total number of breaks,  $n_{tot} = n_1 + n_2$ , via

$$n_{tot} = -\ln f_I \quad (2a)$$

However, we will argue that in the case of YO and YOYO form I is consumed only by ssb since no true dsb (i.e. as a result of a single absorption event) occurs. In such a case  $f_I$  is given by

$$n_1 = -\ln f_I \quad (2b)$$

If the supercoiled fraction  $f_I$  decreases monoexponentially with time, eq. (2b) implies the number of ssb increases linearly in time.

For a random distribution of dsb over all the DNA molecules, the total number of dsb can be calculated from the fraction  $f_{III}$  of the molecules which are in the linear form

$$f_{III} = n_2 \exp(-n_2) \quad (2c)$$

which can be expressed in a more useful form (22)

$$n_2 = \frac{1}{(f_I + f_{II} + f_{III})/f_{III} - 1} \quad (2d)$$

where  $f_{II}$  is the fraction of the molecules which are found as form II. The presented values of  $n_1$  and  $n_2$  are based on data comprising <6% of form IV. In the case of YO at  $N/C = 40$  it is only over a very limited range of illumination times that forms I and III occur simultaneously, which is necessary for eq. 2b and 2d to be applicable to the same sample. In this case the number of ssb for longer times is estimated by extrapolating the average number of ssb per DNA molecule linearly in time, since the supercoil nicking rates were mono-exponential.

## RESULTS

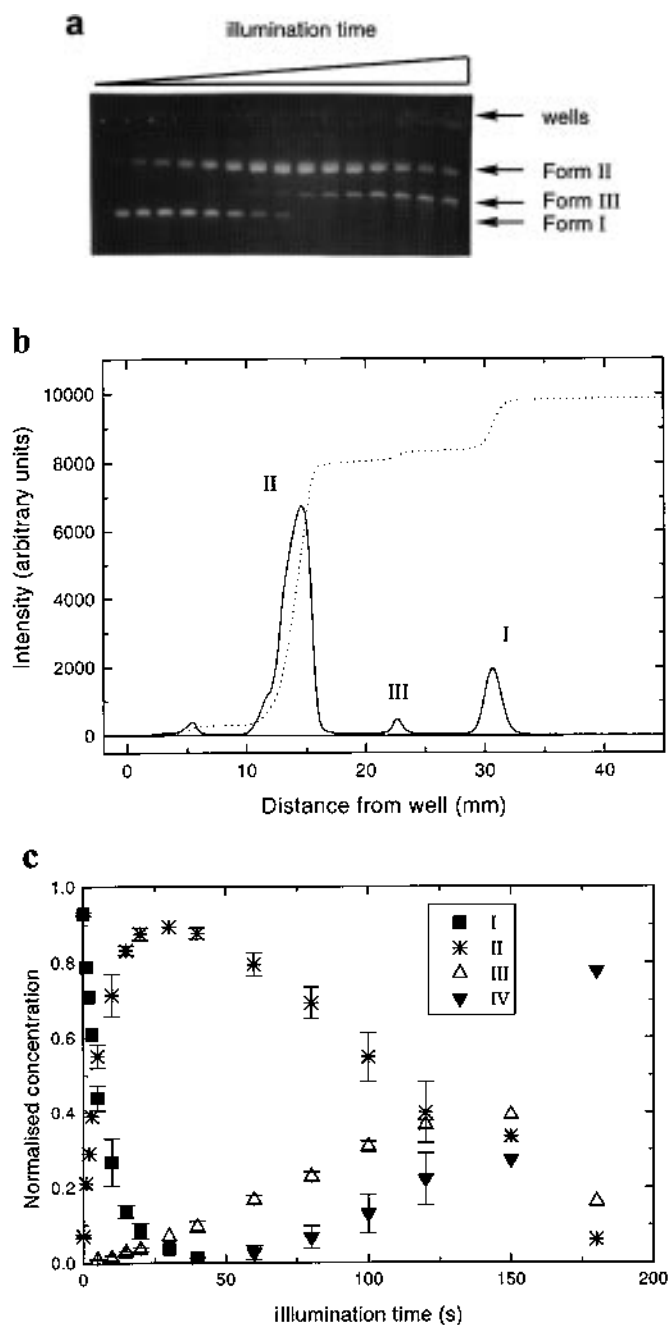
### Time profiles of cleavage products

The results of a typical cleavage experiment, in this case with YOYO at  $N/C = 3$ , are presented in Figure 2. The overall band pattern for the illumination time course (Fig. 2a) is that expected if the first cleavage event is a single-strand break. Initially the amount of nicked circles (II) increases, at the expense of the supercoil. With longer illuminations, II is consumed by accumulation of ssb producing dsb, which results in the appearance and growth of an intermediately positioned zone of linear molecules (III). Ultimately these linear molecules are analogously degraded by accumulated ssb to form linear fragments, manifest as a smear of weak intensity extending from the band of intact linear molecules in the direction of migration. After normalization with respect to the total lane intensity (Fig. 2b), the relative concentrations of each component can be plotted versus illumination time (Fig. 2c). The error bars demonstrate that the variation between experiments is within 5% except when there is an appreciable fraction of fragmented linear molecules (IV), the quantification of which requires very accurate baseline determinations since the intensity is dispersed over a long distance on the gel. All quantification of ssb and dsb has therefore been limited to data containing <6% of form IV. With YO at  $N/C = 3$  (results not shown) the product profiles of all DNA forms are very similar to those observed with YOYO. Also TOTO at  $N/C = 3$  exhibits product profiles similar to YOYO but cleaves about five times slower than YOYO. This is exemplified by the maximum concentration of form II occurring much later (125 s) than with YOYO (30 s, Fig. 2c), but with similar amounts of nicked circular (85%) and linear DNA (10%) at this point.

At a higher  $N/C$  of 40 the overall cleavage rates with YOYO and YO (Fig. 3) are considerably lower than at  $N/C = 3$ , as expected, since there is less dye per DNA molecule. The concentration profiles were similar to those observed at  $N/C = 3$  (Fig. 2c), however there were some differences between the YO and YOYO profiles. The rates of consumption of form I and the initial appearance of II are very similar in the two cases, but the linear form clearly appears (and form II disappears) considerably faster with YOYO than with YO.

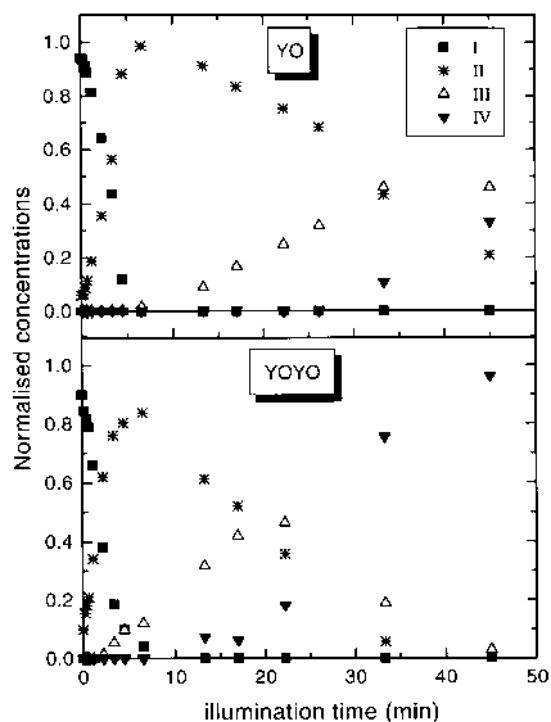
### Quantification of the formation of single-strand breaks

Nicking of the supercoil exhibited monoexponential kinetics in most cases (Fig. 4). However, with YO at  $N/C = 40$  (Fig. 4, inset) an apparent lag in the disappearance of supercoiled circles gave rise to non-monoexponential kinetics if YO was used for quantification (open symbol). By contrast, mono-exponential kinetics (and no lag) were observed if the DAPI-protocol was employed for the same sample (closed symbols). With the YO-protocol (but not with DAPI post-staining) an apparent lag is expected if YO is transferred from form II to form I in the period between illumination and electrophoretic separation of the two components. This can occur with YO because the negatively supercoiled DNA binds an intercalator more strongly than the nicked circle, if the  $N/C$  is higher than the equivalence binding ratio needed for complete removal of the supercoiled turns of the DNA (23). The equivalence point of our DNA sample is  $N/C = 10$ –11 for YO because the mobility of the supercoiled form has a minimum at  $-0.09$ – $0.1$  YO per nucleotide, at which point the mobility is close to that for the nicked circle (data not shown). The apparent lag was pronounced only at  $N/C = 40$ , and as expected from the proposed



**Figure 2.** Cleavage of  $\Phi$ X174 supercoiled DNA by YOYO at  $N/C = 3$ . (a) Gel photograph showing separation pattern after illumination for 0, 1, 2, 3, 5, 10, 15, 20, 40, 60, 80, 100, 120, 150 and 200 s. Positions of wells, supercoiled (I), nicked circular (II) and linear (III) forms are indicated. (b) Gel scan of lane corresponding to 15 s illumination time, showing band profile (—) and integrated dye fluorescence intensity (.....). (c) Concentration profiles of forms I, II, III and IV obtained from integrated zone intensities normalized to the total fluorescence intensity of each lane. Error bars indicate the deviations between two separate experiments.

mechanism disappeared completely at  $N/C = 12$  which is close to the equivalence point of our sample. Below  $N/C = 10$ –11 transfer in the opposite direction should occur, but the semi-logarithmic plots remained linear, presumably since the driving force for transfer becomes smaller as saturation of intercalation sites is



**Figure 3.** Cleavage of  $\Phi$ X174 supercoiled DNA by YO (top) and YOYO (bottom) at  $N/C = 40$ . Concentration profiles of forms I (■), II (\*), III ( $\Delta$ ) and IV ( $\blacktriangledown$ ) obtained as in Figure 2c.

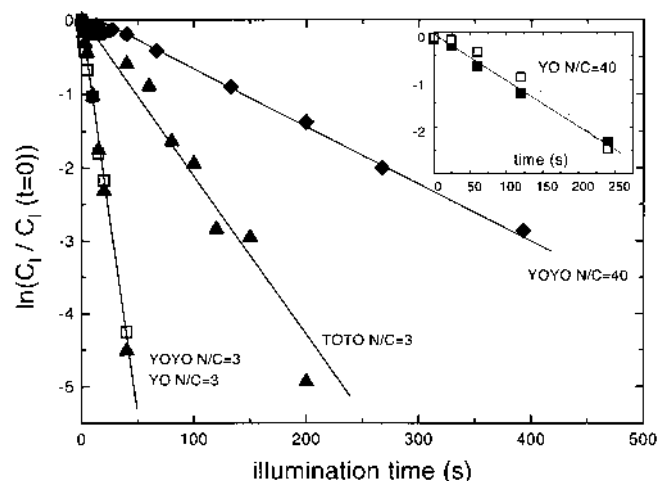
approached, and since externally bound dye present at low  $N/C$  should exhibit no DNA-topology dependence of binding.

The apparent fraction of form I was lower with DAPI by a magnitude which was reasonable in view of an estimated (23) ratio of 1.5 for the binding constants of YO at  $N/C = 40$  (assuming an unwinding angle of  $20^\circ$ ) (10), which in equilibrium corresponds to a transfer of 10% of dye from II to I when half of the circles have been converted. For YO such a transfer is likely to have enough time to occur before the DNA forms are separated by electrophoresis ( $\geq 10$  min), since the dissociation time constant of the similar intercalator propidium (divalent, with a side chain similar to that of YO-PRO) is typically in the range of seconds or faster (24). With bis-intercalating YOYO (which did not exhibit a lag) the off-rate is on the order of hours (7,25), and control experiments on a mixture of stained and non-stained DNA indeed showed that no detectable transfer of YOYO occurred on the time scale of the electrophoretic analysis (not shown).

The accumulated evidence shows that the lag is due to dye-transfer effects which can be eliminated by using DAPI post-staining. Because of superior signal-to-noise ratios, the rate constants for supercoil nicking were obtained from the YO-scans except when these exhibited a significant lag (YO at  $N/C = 40$ ), in which cases the DAPI-scans were employed.

### Mechanistic studies of the single-strand cleavage

The effect of binding mode on the single-strand cleavage was investigated by evaluating the rate constant (per chromophore) for supercoil nicking (eq. 1) at different binding ratios of nucleotide to added dye chromophore (Fig. 5). At high  $N/C$ , YO (top) and YOYO (bottom) exhibit a nicking efficiency that is

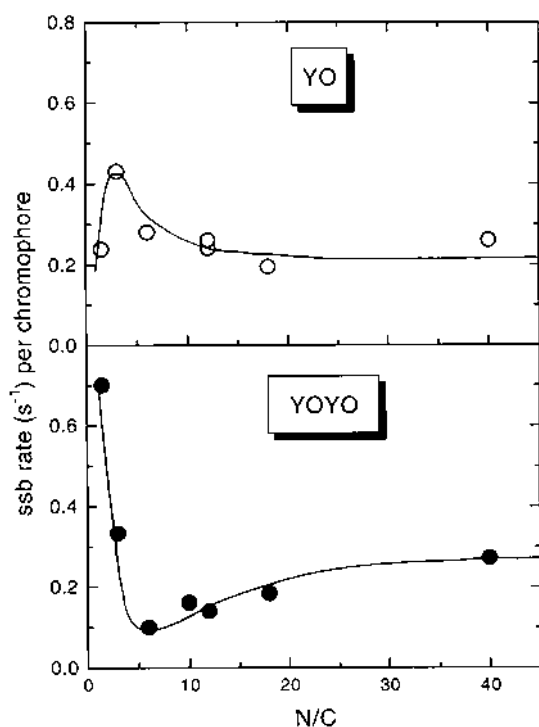


**Figure 4.** Semilogarithmic plot of the fraction of supercoiled DNA versus illumination time, at the conditions of  $N/C$  indicated. Inset: semilogarithmic plots of the fraction of supercoiled DNA versus illumination time for YO at  $N/C = 40$ . Gel scans based on the fluorescence of YO remaining bound (open symbols) or of DAPI added after electrophoresis (closed symbols) (see Materials and Methods).

essentially the same per chromophore for the monomer and the dimer. As  $N/C$  decreases the rate increases gradually with YO, and goes through a maximum around  $N/YO$  of 3, from which it falls rapidly for the lowest  $N/C$ . By contrast, with YOYO (bottom) the nicking rate decreases gradually as  $N/C$  decreases from 40 down to  $N/C = 10$ , where from a shallow minimum, the rate grows rapidly to a value per chromophore almost twice as large as the maximum level observed with YO. No decrease is observed with YOYO even at the lowest  $N/C$ . It is clear that the photo-cleavage by YO and YOYO cannot be explained in terms of a single process for one binding mode, since the rate constants for supercoil nicking vary strongly with the binding ratio.

Further insight into the cleavage mechanism can be found by probing for intermediate species. It is common to remove oxygen and add  $\beta$ -mercaptoethanol ( $\beta$ MeSH) in microscopy and other applications of YOYO and related dyes, and we have therefore examined the effects of such conditions on the rate of supercoil conversion. With YOYO, removal of oxygen by argon-bubbling reduces the cleavage rate by  $45 \pm 5\%$  at  $N/C = 1.5$ , but has a much smaller effect ( $10 \pm 5\%$ ) at  $N/C = 10$ . By contrast, with 5%  $\beta$ MeSH (which is an efficient scavenger of both singlet oxygen  $^1O_2$  and radical species; 26) the cleavage rate is dramatically reduced (by 90–95%) at both binding ratios.

The results evidence the participation of oxygen at low  $N/C$  and since the most common photocleavage mechanism involving oxygen is via photosensitized production of  $^1O_2$ , this possibility was tested in the case of YO by carrying out the reaction with added sodium azide,  $NaN_3$  (a  $^1O_2$  quencher, although non-specific) (11) and in  $D_2O$  (which increases the lifetime of  $^1O_2$  and hence its potential to cause damage) (11). At  $N/C < 5$ ,  $NaN_3$  inhibited cleavage and  $D_2O$  enhanced it, suggesting that  $^1O_2$  is indeed the damaging species. By contrast at  $N/C > 5$ ,  $D_2O$  had no significant effect on the ssb yield, consistent with the observed minor role of  $O_2$  while  $NaN_3$ , which may also scavenge radicals, had a small inhibiting effect. At  $N/C < 5$ , addition of catalase and/or superoxide dismutase had no significant effect on the ssb yield, indicating that



**Figure 5.** Rate of nicking of supercoiled DNA per chromophore for YO (top) and YOYO (bottom) versus the ratio  $N/C$  between DNA nucleotide and added chromophore concentrations. Rate constants evaluated according to eq. 1.

neither  $O_2^-$  nor  $H_2O_2$  were involved in the cleavage by either intercalated or externally bound dye. Neither did hydroxyl radical scavengers such as urea, thiourea or glycerol (27) significantly affect the ssb yield.

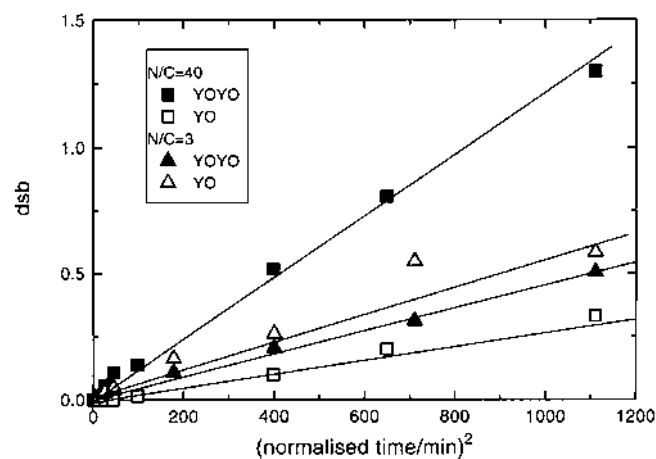
## DISCUSSION

### Modes of binding of YO and YOYO under our conditions

The binding of YO and YOYO to double-stranded DNA has been studied (8,10) under the the same buffer and DNA conditions as employed here, except T2 DNA was used. The population of the different binding modes should be very similar with  $\Phi X174$  DNA, since YO exhibits very little sequence dependence of binding (8). Many of the results presented here can be understood in terms of YO and YOYO having two binding modes: (i) at high  $N/C$ , intercalated (bis-intercalated for YOYO) and strongly fluorescent, and (ii) at low  $N/C$ , externally bound and more weakly fluorescent (8,10). Being structurally very similar to YOYO (Fig. 1) a similar binding behaviour for TOTO is indicated by very similar spectral changes in absorption and fluorescence (1) upon binding to dsDNA, and NMR studies (28) support bis-intercalation as the most stable binding mode.

### Neither YO nor YOYO cause direct double-strand breaks

With YOYO at  $N/C = 40$  form III appears when 50% of the supercoil has been converted (Fig. 3). By contrast, with YO the linear form appears only when >95% of the supercoiled form has



**Figure 6.** Yield of dsb plotted versus the square of time normalized for the binding ratio,  $[time \times (N/C)]^2$ , for YO and YOYO at  $N/C = 40$  (data from Fig. 3), and  $N/C = 3$  (data for YOYO from Fig. 2c).

been consumed, which is expected for random accumulation of ssb created by a true single strand cleaving agent (29). We will return below to the enhanced efficiency of linearization with the dimer, but at this stage we simply demonstrate that YOYO is not a true double-strand cleaving agent, in the sense that a dsb is not created as a result of a single excitation event. The number of dsb per DNA molecule (Fig. 6) grows quadratically with time (normalized to binding ratio by multiplication by  $N/C$ ), both for YOYO and YO. A linear growth in the number of dsb with time is expected for true dsb (21,30), but inclusion of a linear term did not improve the quality of the fits. Thus, the data for the photocleavage of DNA by YOYO and YO can be analysed assuming that the first damaging event is always nicking of one strand.

After equilibration (7) the sites of bound YOYO molecules are expected to be approximately randomly distributed, since YOYO does not exhibit any significant sequence specificity (8). The sites of the first nick should therefore occur randomly over the supercoiled molecules, leading to Poisson statistics for the distribution of the ssb (21), and to monoexponential kinetics for the supercoil conversion (12) as we observe. The rate constant for single-strand cleavage of form I evaluated from semilogarithmic plots was used to assess the nicking activity of YO and YOYO under various conditions.

### Single-strand breaks

*Bound dye cleaves more efficiently than free.* The drop of ssb activity for YO below  $N/C = 3$  (Fig. 5, top) indicates that the free dye does not cause photocleavage, since spectroscopic studies (10) show that free YO is present at these binding ratios. This interpretation is further supported by the observation that release of bound dye by addition of  $Mg^{2+}$  decreases the photo-cleavage yield (results not shown). By contrast, with YOYO no decrease in supercoil-cleavage efficiency is observed even at very low  $N/C$  (Fig. 5, bottom). No data are available for the amount of free dye at  $N/C = 1.5$ , but no free dye could be detected at  $N/C = 2.5$  (10), where a significant fraction of the more weakly bound YO was free. We therefore suggest that the absence of a decrease in ssb activity for YOYO at low  $N/C$  is due to a low free dye concentration as a result of the strong dimer binding. Attempts to create free dye by

increasing the YOYO concentration even further, resulted in aggregation of the DNA.

An apparent decrease in cleavage rate at low N/C ratios (e.g. 1.5 with YO) could potentially arise from strong light absorption at the comparatively high dye concentrations. However, the cleavage rate increases with YOYO but decreases with YO when N/C is reduced from 3 to 1.5, even though the dye absorption is similar in all four samples, so this is not a dominant effect.

*Externally bound dye cleaves more efficiently than intercalated.*

The decrease of the average cleavage rate per YO above N/C = 3, towards an approximately constant level at high N/C (Fig. 5, top) indicates that externally bound YO, present at low N/C, induces ssb more efficiently than intercalated dye, which is the only bound form at high N/C. For YOYO (Fig. 5, bottom), the supercoil conversion rate is also considerably higher when externally bound dye is present (N/C < 4) than when all dye molecules are bis-intercalated (N/C >> 4), consistent with intercalated chromophores being less efficient at creating ssb also as dimers.

There are clear differences in the behaviour of YO and YOYO, however. YOYO exhibits a shallow minimum in the cleavage rate (at N/C = 6), before the rapid increase at lower N/C. By contrast, with YO there is a monotonous and more gradual increase in cleavage rate as N/C is decreased. We tentatively ascribe these differences in the cleavage profiles to YO and YOYO being differently partitioned between the two binding modes at intermediate N/C values. With YOYO, intercalation sites are essentially saturated (at N/C = 4) before external binding occurs (10), which explains the abrupt increase in cleavage efficiency for the dimer below N/C < 4. For YO, the two binding modes overlap (10) and the transition to higher average cleavage rates due to the presence of externally bound dye therefore occurs more gradually. The question, why with YOYO there is an increase in the cleavage efficiency with increasing N/C above N/C = 4, although all molecules should be in the same bis-intercalated binding mode, is returned to after discussion of the results of mechanistic studies.

*Mechanistic studies of the rate of ssb by YOYO.* As a first step towards understanding the chemistry underlying the cleavage, we have investigated some key elements of the reaction mechanism, including the role of binding geometry. The fact that deoxygenation had a stronger inhibiting effect on cleavage at N/C = 1.5 (both intercalated and externally bound dye) than at N/C = 10 (intercalated dye only), shows that O<sub>2</sub> plays a more important role in cleavage by externally bound than by intercalated dye. This observation suggests different cleavage pathways for the different YOYO binding geometries, and it is thus likely that the large difference in the rates of ssb formation in the two binding modes is due to the existence of two different cleavage mechanisms, rather than only one mechanism which is less efficient for the intercalated mode.

The accumulated evidence from the quencher experiments with NaN<sub>3</sub>, D<sub>2</sub>O, catalase and superoxide dismutase suggests that while externally bound dye effects cleavage at least in part via photosensitized <sup>1</sup>O<sub>2</sub> production, intercalated dye reacts primarily via a pathway that does not involve an activated oxygen species. (Some quencher experiments were performed with YO rather than YOYO, but it is reasonable to assume that the YO chromophore cleaves by the same mechanism in the monomer and the dimer for a given binding mode.) Lack of oxygen involvement in cleavage by intercalated dye is not surprising, since it is well known that the intercalation pocket usually protects fluorescent dyes from quench-

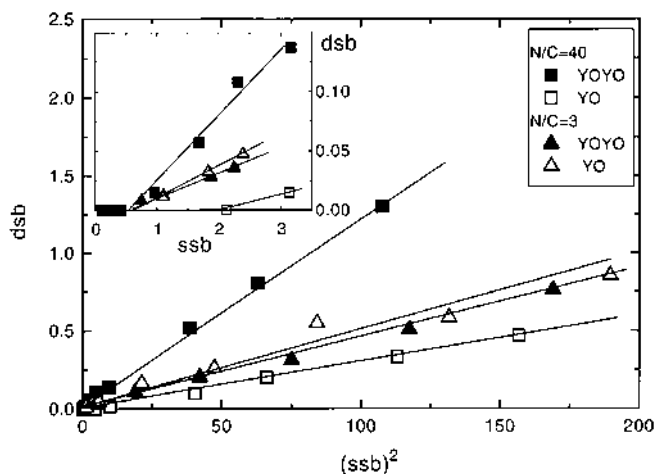
ing agents in the solution, such as dissolved oxygen (31). No evidence was found for involvement of hydroxyl radical species (either at high or low N/C), which suggests that intercalated dye reacts directly with the DNA, perhaps via hydrogen-abstraction from the sugar, but more detailed studies of the cleavage mechanism by the intercalated YO chromophore are needed to clarify this point.

*Cleavage efficiency at intermediate N/C of intercalated chromophore.* With YOYO there was an increase in cleavage efficiency with increasing N/C (>4), which was surprising because in this range the dimer is reported to bind only by bis-intercalation (10). YOYO may bind in a third mode as monointercalated dimer, which is expected to occur when the intercalation sites are almost saturated (32) i.e. just above N/C = 4. However, this seems an unlikely explanation for the partial quenching of the cleavage in this range of binding ratios, since externally bound YO-chromophores cleave more efficiently than intercalated ones.

Variations with N/C of the quantum yields for fluorescence of fully intercalated dye have been reported for both YO and YOYO (10). These observations suggest interactions between intercalated chromophores that might also modulate other photophysical properties of the DNA-dye complex, such as cleavage efficiency. A notable similarity is that for both fluorescence quantum yield (8) and the cleavage rate (Fig. 5), the same value per chromophore is obtained for YO and YOYO, when measured at such high N/C (= 40) that interactions between different dye molecules should be negligible. Hence, the chromophores seem to act independently when constrained in a dimer, which apparently contradicts the argument that interactions between dyes are responsible for the variation of fluorescence quantum yield and cleavage rates with N/C, i.e. the effect of interactions between intercalated YO chromophores in close proximity would be expected to be permanently manifest in YOYO.

To explain the variation of the fluorescence quantum yield with N/C, Larsson *et al.* (10) have suggested that the dye molecules interact either directly, by electronic coupling of the chromophores, or indirectly, through alterations in the DNA helix due to unwinding. In solution the absorption spectrum of YOYO is different from that of YO, most likely because of electronic interactions between stacked chromophores in the dimer (33). Bis-intercalation of YOYO with DNA ruptures chromophore-chromophore stacking, which essentially removes the electronic interactions between the chromophores as evidenced by the YO and YOYO absorption spectra being almost identical when the dyes are intercalated (10). This suggests that the interactions between intercalated chromophores are not electronically coupled but mediated by a helix perturbation. Possibly, the demands of accommodating the YOYO-linker by the extended and unwound DNA result in less pronounced perturbation of the helix between two YO chromophores within a dimer than between adjacent monomers or dimers. Hence, helix-mediated interactions within isolated dimers may be reduced compared with interactions between neighbouring dimers or monomers, consistent with fluorescence, cleavage and absorption observations.

At intermediate N/C, where interactions between different intercalated dye molecules are observed to occur (8), there is less consistency between fluorescence quantum yield and cleavage. It is only with YOYO that we observe an increase in cleavage rate with increasing N/C above N/C = 4 (Fig. 5), and in fluorescence it is instead the quantum yield of YO which increases most strongly with increasing N/C (8). However, direct comparison is not trivial



**Figure 7.** Plot of number of dsb versus the square of the number of ssb, for the samples of Figure 6. Inset: plot of number of dsb versus the number of ssb, for the samples of Figure 6.

since with YO there is overlapping of binding modes, and further studies of the excited state properties of YO and YOYO in both binding modes are clearly needed in order to understand the effects of N/C on single-strand cleavage.

### Double-strand cleavage

*Enhanced double-strand cleavage with the dimer.* Since with both YO and YOYO dsb occur as a consequence of ssb-accumulation, comparison of the dsb rates must take the nicking efficiencies of YO and YOYO into account. Figure 5 shows that the rates of ssb formation per chromophore by YO and YOYO are indeed very similar at N/C = 40 ( $0.35 \text{ s}^{-1}$ ), and it is thus clear from Figure 3 that the dimer induces dsb considerably more efficiently (per chromophore) than the monomer. This effect is better demonstrated by a plot of the number of dsb versus the number of ssb (Fig. 7, inset), from which it is clear that at N/C = 40 the number of dsb induced per ssb is much higher with YOYO than YO.

The intuitive interpretation is that linkage forces the two YO chromophores of YOYO into close proximity, thus significantly increasing the probability that two ssb will occur within the 15 bp separation typically required to produce a dsb (12). This interpretation is reinforced by the observation that the dsb efficiency is approximately the same for YO and YOYO at N/C = 3 (Fig. 7, inset), where the dye density is so high that the YO chromophores bind close together, whether linked or not. An enhanced probability for ssb-proximity is anticipated if the two YOYO chromophores are excited and create ssb on opposite strands in succession, without the dimer changing binding site between excitation events. However, at this stage we cannot exclude the possibility that two ssb are induced by the same chromophore, and the only role of dimerization is to enhance the binding constant of the (single) ssb agent. This is clearly a possibility if the ultimate cleaving agent is an intermediate diffusible species, such as  $^1\text{O}_2$ , but may be unlikely if the excited dye molecules attack the backbone directly and are chemically altered by the photo-cleavage reaction.

Attempts to attach an intercalating (34) or groove-bound (35) anchoring-group to a repetitively cleaving monomeric ssb-agent has resulted in enhanced rates of ssb, but to date no significant increase in dsb yields (34,35). By contrast, dimerization of

chemical (as opposed to photochemical) ss cleaving agents has been shown to enhance the dsb efficiency compared to the corresponding monomer (36,37). However, in both cases the extent of reaction was controlled by varying the cleaving agent concentration (rather than time), so there are potential effects due to variations in the occupancy of different binding modes during the titration. Here, by direct comparison of the monomer and the dimer under conditions where all dye molecules can be considered intercalated, we have shown that the dimer of YO does exhibit a considerably enhanced dsb efficiency.

The observation that the dsb rate per ssb for YOYO is lower at N/C = 3 than at N/C = 40 indicates that bis-intercalated YOYO induces dsb more efficiently than the externally bound form. However, since the ssb mechanism is different in the two binding modes, we cannot say if the enhanced dsb activity is an inherent property of the bisintercalative binding geometry, or a result of the cleavage mechanism for the intercalated chromophore being more favourable for generation of dsb. (We also note that the dsb efficiency per ssb of TOTO is similar to that of YOYO, although the overall cleavage efficiency is lower with TOTO.) The dsb efficiency of YOYO can be compared with that of true dsb agents, such as enediynes (38,39), for which the slope of the number of dsb versus the number of ssb is a convenient measure of dsb activity. At N/C = 40 a slope of  $\sim 0.05$  dsb per ssb is obtained for YOYO, which is a factor of four lower than optimal yields obtained with neocarzinostatin (40), while YO produces only about 0.004 dsb per ssb at N/C = 40. Although useful for comparison with other dsb agents, the analysis of dsb generation based on the plot in the inset of Figure 7 is not entirely correct since a strict proportionality between dsb and ssb is not observed, reflecting that YOYO is not a true dsb agent.

*The dsb yield increases quadratically with ssb for both YO and YOYO.* With both YO and YOYO, the number of ssb increases linearly with time (as evidenced, in view of eq. 2a, by the linearity of the plots such as those in Fig. 4). The quadratic time dependence of the number of dsb (Fig. 6) therefore implies that the relationship between the number of dsb and ssb is more correctly described as quadratic than as linear. This is borne out by Figure 7 which reveals a quadratic dependence on ssb, explaining the absence of linearity in the inset of the same Figure.

A quadratic dependence on the number of ssb is expected for dsb caused by accumulation of randomly distributed ssb, according to the equation

$$n_2 = \frac{n_1^2}{4L}(2h + 1) \quad (3)$$

where  $L$  is the length of the DNA in bp (5386 bp for  $\Phi\text{X174}$ ) and  $h$  the maximum distance (in bp) between two ssb that will lead to a dsb if they are on opposite strands (12). For YO at N/C = 40 the slope in Figure 7 corresponds to a value for  $h$  of 32.7. This is about twice as large as the value of 15.8 obtained at a somewhat lower ionic strength (10 mM) by Freifelder and Trumbo (12), who used X-rays to induce ssb in linear B3-DNA (30 kbp), and ultracentrifugation to analyse the number of ssb and dsb. Povirk *et al.* (21) also employed ionizing ( $\gamma$ ) radiation to induce ssb, but used supercoiled ColE1 DNA (10 900 bp) and an electrophoretic analysis similar to ours. At 100 mM ionic strength they obtained dsb efficiencies which were  $\sim 2$ -fold higher than predicted for random accumulation of ssb (12), assuming  $h = 15$ . Their data was too limited to establish a quadratic dependence, but an average



value for  $h$  of ~34 can be calculated from their Figure 2. The reason for the discrepancy between the two studies employing ionizing radiation is not fully clear. Since the higher ionic strength used by Povirk *et al.* (similar to ours) should decrease  $h$  (12), it appears that DNA topology or the fact that B3-DNA naturally contains nicks (corrected for by Freifelder and Trumbo) may be of importance in the process of ssb-generation. Values of  $h$  as large as 40–80 have been observed when ssb are induced by UV irradiation, although in these cases enhanced helix-instability due to other types of photo-induced damage may contribute to apparently high values of  $h$  (41).

We conclude that using similar techniques of analysis, YO has approximately the same dsb/ssb yield as ionizing radiation (20), which is known to induce ssb (12). In this sense YO acts as a true ssb agent which nicks DNA randomly, as concluded above (by comparison with theory; 29) from the small amount of form I remaining when the linear form is first detected. Importantly, a quadratic dependence is in itself not evidence that a ssb agent creates nicks randomly; only by comparing the value of the slope with those of known ssb-agents can this be established. For example, for YOYO at N/C = 40 the slope corresponds to an apparent value of  $h = 142$ , which is considerably higher than expected for accumulation of randomly distributed ssb. This is not surprising, since the basic assumption (underlying eq. 3) of random distribution of the ssb over the nicked circular DNA cannot be fulfilled when the ssb agents are coupled in a dimer which is tightly bound. Nonetheless, a quadratic relation is still expected for this case of non-random distribution of the ssb (as long as no true dsb are created), which is apparent from the following simple model for isolated dimers.

*Model for dsb generated by non-random accumulation of ssb by dimer.* Consider the limiting case of such low densities of bound dimer, that the average distance between different molecules is much greater than that required to produce a dsb from two ssb generated on opposite strands. Furthermore, assume that each chromophore is capable of creating only one ssb (for example because it is consumed in the cleavage reaction), and that the dye dissociation time constant is much longer than the average time between two cleavage events. Hence, there will be a finite probability that a second ssb will be generated close to an extant ssb, since two successive ssb are created while the molecule is bound at the same site. Assuming there are  $M$  dimers per DNA molecule, the probability that ssb ( $j + 1$ ) will result in a dsb, given that none of the  $j$  earlier ssb has done so, is

$$P_{j+1} = \frac{(1 - \alpha)j}{2M - j} \quad (4)$$

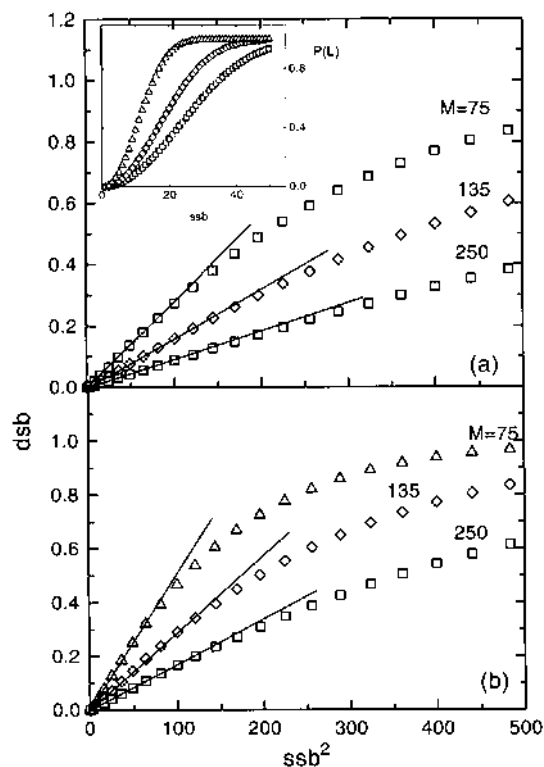
where  $\alpha$  is the probability that the second ssb is on the same strand as the first ssb caused by the other chromophore of the same dimer. The total probability of linearization  $P_L$  after  $j + 1$  ssb will be

$$P_L = 1 - P_{\text{surv}} \quad (5)$$

where  $P_{\text{surv}}$  is the probability that the molecule remains circular after  $j + 1$  ssb. Thus  $P_{\text{surv}}$  is the accumulated probability for survival of all  $j + 1$  ssb

$$P_{\text{surv}} = \prod_{i=1}^{j+1} (1 - P_i) \quad (6)$$

The probability of linearization ( $P_L$ ) is equal to the probability for generation of dsb per DNA molecule and  $P_L$  will therefore



**Figure 8.** The number of dsb versus the number of ssb for low number of ssb, calculated from the model of  $M$  isolated dimeric cleavers per DNA molecule, under the assumption that (a) each chromophore is destroyed after the first ssb (eq. 4–6) or (b) each chromophore is capable of repetitive cleavage (eq. 5–7). Inset shows the probability of linearization,  $P_L$ , for the whole range of ssb.

approximate the average number of dsb per DNA molecule well (i.e.  $n_2 \approx P_L$ ), as long as  $n_2 \ll 1$ . When there is an appreciable probability for more than one dsb per original DNA molecule, the probability that ssb will accumulate to give two or more dsb per molecule (that will lead to ds-cleavage of the linear form) has to be taken into account. Therefore we consider the predictions of the model only in the limit of the number of ssb per DNA molecule approaching zero, where the average number of dsb equals  $P_L$ .

Figure 8a shows that initially the predicted number of dsb (calculated for different values of  $M$ ) is a quadratic function of the number of ssb. This model thus demonstrates that a quadratic dependence is possible even if accumulation of ssb is non-statistical and the model may thus be applied to the data we obtain with the dimer. With YOYO at N/C = 40 an average of 1 dsb/molecule is created after ~15 min (Fig. 6), which is considerably shorter than the typical dissociation-time for similar dimeric dyes (25). The assumption of a long-lived dye–DNA complex is thus reasonably fulfilled in this particular example.

For YOYO at N/C = 40, which corresponds to  $M = 135$  for  $\Phi X174$  DNA, the observed value for the slope (0.0135, filled squares in Fig. 7) is considerably higher than that predicted (0.00156, Fig. 8a,  $M = 135$ ) despite assuming a perfect dsb yield for a given dimer (i.e. setting  $\alpha = 0$ , which leaves the model with no adjustable parameters). This could be due to the assumption of isolated dimers not being fulfilled. At N/C = 40 the average distance between the dimers is 40 bp, which is larger than the generally accepted value for the critical distance of 16 bp, but not

much larger than the value of 33 deduced from YO-data under our conditions. Thus dsb should result also from combination of ssb caused by different dimers, but since this contribution also is quadratic in the number of ssb, the combined number of dsb will still exhibit this dependence. An estimate of the contribution to the slope from intermolecular dsb can be obtained from the number of dsb created by YO at N/C = 40 (Fig. 7, open squares), which is actually an upper limit because the average distance between the YO monomers is only 20 bp. However, even after including this contribution of 0.0031 to the theoretical slope, the predicted value (0.0047) remains considerably lower than that observed.

The deviation between experiment and theory could in principle be due to the YO chromophore not fulfilling the assumption of only one cleavage event per chromophore. This possibility was tested by altering the model to allow repetitive generation of ssb by the same chromophore. Equation 4 is then replaced by

$$P_{j+1} = \frac{2j}{2M} \quad (7)$$

assuming that the second ssb caused by a certain dimer always occur on the opposite strand and results in a dsb. Again the number of dsb is proportional to ssb squared initially (Fig. 8b) and, as expected, this model gives higher dsb-yields per ssb than the model where the chromophore is destroyed. However, even in the limiting case of maximum dsb-efficiency for the second ssb, the observed slope (0.0135) is still considerably higher than the predicted slope (0.00283, Fig. 8b,  $M = 135$ ), also after including the contribution of intermolecular events to give a total predicted slope of 0.0069.

The failure of the presented models to explain the observed slope with YOYO at N/C = 40 shows that additional effects must be included in order to explain the dsb efficiency of YOYO. In our continuing investigations of enhanced ds-cleavage by dimers we will focus on two possible explanations. We have recently observed long-lived inhomogenous distribution of dimeric dyes upon mixing with DNA (7), suggestive of cooperative contributions to the initial events of the binding process. Although in this study we have used protocols that eliminate uneven distribution of dye molecules between different DNA molecules, electrophoretic analysis of equilibrated samples (undertaken before sample illumination) would not detect uneven distribution of dye within the molecules. Tracts of cooperatively bound dimers would concentrate the inflicted ssb into a small region of each DNA molecule and enhance the dsb probability, essentially by enhancing the trend expected when going from monomers to dimers. Another possibility which has to be considered is that the quantum yield for generation of a second ssb by a given YO-chromophore may be higher than that for generation of the first ssb, as a result of altered DNA structure or possible changes in the dye itself as a consequence of the first ssb-event. This would enhance the dsb efficiency compared with present models, where all ssb caused by intercalated dye are assumed to be created by the same mechanism with equal efficiency.

## Conclusions

Both YO and YOYO cleave DNA by formation of ssb.

Free dye causes negligible cleavage of DNA, while both intercalated and externally bound dye generate ssb, but by different cleavage mechanisms.

Externally bound dye cleaves comparatively more efficiently, in an oxygen-dependent manner.

Intercalated dye cleaves less efficiently, and by an essentially oxygen-insensitive pathway.

YOYO does not cause direct dsb, but the dsb-yield is still in clear excess of that expected (and observed with the monomer YO) from accumulation of randomly distributed ssb, because the coupling of the chromophores in YOYO leads to an enhanced accumulation of ssb compared to the monomer.

Models for permanently bound and isolated dimers of an ssb-inducing agent predict an initially quadratic variation of the number of dsb with the number of ssb, in agreement with observations for YOYO, but the predicted dsb yields are considerably lower than those observed even if repetitive cleavage by each chromophore is allowed.

YOYO gives higher yields of dsb/ssb at high N/C than at low N/C, which indicates that bis-intercalation is important for realization of the dimer's potential for double-strand cleavage.

## ACKNOWLEDGEMENTS

The Magn. Bergwall (B.Å.) and Carl Trygger (E.T.) foundations are thanked for support.

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