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Biochemistry

DNA unwinding induced by photoaddition of psoralen derivatives and determination of dark-binding equilibrium constants by gel electrophoresis

(nicking-closing enzyme/ethidium bromide unwinding angle/agarose)

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ABSTRACT **Derivatives of furo[3,2-*g*]coumarin (psoralen) can bind to the DNA double helix and, in the presence of long-wavelength UV light, the bound psoralen may react covalently with pyrimidine residues on one or both strands of the helix. By using agarose gel electrophoresis, we have determined the unwinding angle associated with each of four different psoralen derivatives to be 28° ± 4°. For 4,5′,8-trimethylpsoralen (trioxsalen) the unwinding angle was found to be independent of the initial DNA superhelix density in the range that is accessible to agarose gel electrophoresis.**

Also by using agarose gel electrophoresis, we have determined the unwinding angle for ethidium intercalation. This was done by the total relaxation of supercoiled DNA in the presence of a series of ethidium concentrations. By using published values for the association constant for ethidium binding to DNA and evaluating the final superhelix density (after removal of ethidium) of the DNA on gels, we calculated an unwinding angle of 29° ± 3°.

Assuming an unwinding angle of 28° for the noncovalent intercalation of psoralen derivatives, we used the same procedure to determine intercalation binding constants. The association constants for 4′-aminomethyltrioxsalen were 300–1400 M^{-1} in NaCl at 0.2–0.05 M and 300–2500 M^{-1} in Mg^{2+} at 4–0.5 mM. The association constant for 4′-hydroxymethyltrioxsalen in 0.5 mM Mg^{2+} was determined to be 70 M^{-1}.

The structure of psoralen is shown in Fig. 1. Upon irradiation with long-wavelength UV light, psoralen and its derivatives can react with adjacent pyrimidines at either the 4′,5′ or the 3,4 double bond to form a putative cyclobutane ring with the 5,6 bond of the pyrimidine (1–4). We refer to this as a monoadduct. When another pyrimidine is situated on the opposite strand of the DNA helix adjacent to the psoralen molecule, the unreacted psoralen double bond can form a second cyclobutane ring. This second photoreaction, diadduct formation, causes the two strands of the DNA helix to be covalently crosslinked (5–7).

There are a number of useful applications for psoralen derivatives. Medically, the psoralens, in combination with long-wavelength UV light, have a tanning effect on the skin. The drugs are used in the treatment of vitiligo and are being clinically tested in the treatment of psoriasis (8, 9). The psoralens have been used in the study of bacterial repair mechanisms (10, 11). They have been shown to inactivate viruses and tumor cells (12–15,†) and psoralen metabolites have been characterized (16). Psoralen derivatives crosslink DNA in living cells and in isolated nuclei at specific sites that appear to be the same as, or a subset of, the sites that are susceptible to micrococcal nuclease digestion (refs. 17, 18; L. Hallick and J. Hearst, unpublished data). The crosslinking reaction has also been used on purified DNA to probe for self-complementary regions along one strand and to identify the size of pyrimidine runs in satellite DNA (19–21).

The experiments described here were designed to develop an assay for two aspects of the chemistry of the reaction between DNA and psoralen derivatives. The first question relates to how the structure of the DNA helix is affected by the covalent addition of the derivatives. Is the helix unwound or overwound, and can any difference be detected between derivatives? The second question relates to the fraction of the derivative in solution that is intercalated in the DNA helix. Because intercalation is presumably necessary before photoreaction is possible, an assay that measures only intercalative binding would be useful in screening new psoralen derivatives.

MATERIALS AND METHODS

Psoralen Derivatives. 4,5′,8-Trimethylpsoralen (trioxsalen) was purchased from the Paul B. Elder Co. (Bryan, OH). [^{3}H]-Trioxsalen, 4′-amino[^{3}H]trioxsalen, 4′-[^{3}H]hydroxymethyltrioxsalen, and 4′-[^{3}H]methoxymethyltrioxsalen were synthesized by S. Isaacs in this laboratory (22).

DNA. Supercoiled colicin E1 (ColE1) DNA was a gift from L. Hallick and C. Chun. It was isolated from *Escherichia coli* JC411 *thy*(ColE1) (obtained from P. Modrich) after chloramphenicol amplification of the plasmid (23), according to the procedure of Modrich and Zabel (24). The molecular weight of ColE1 DNA was taken to be 4.2×10^6 (25).

Nicked ColE1 DNA was produced by using DNase I (Sigma) at 0°C (26). ColE1 DNA (0.5 ml) at 400 μg/ml in 0.01 M sodium phosphate and 1 mM EDTA (pH 6.8) was mixed with 14 μl of 70 mM $MgCl_2$ and 75 μl of DNase I at 10 μg/ml [dissolved in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/2 mM $MgCl_2$/bovine serum albumin at 1 mg/ml (Calbiochem, A grade)]. The reaction was continued for 10 min at 0°C and was stopped by the addition of 50 μl of 0.25 M EDTA.

Partially relaxed ColE1 DNA was produced by mixing 300 μl of ColE1 DNA at 650 μg/ml (in 10 mM sodium phosphate, pH 6.8/1 mM EDTA) with 50 μl of 0.5 M K_2HPO_4, 10 μl of 0.1 M $MgCl_2$, and 4 μl of *E. coli* Ω protein (a gift from R. Depew). The protein concentration of the enzyme stock was approximately 0.5 mg/ml. The relaxation reaction was allowed to proceed for 1–60 min at 37°C and was stopped by the addition of 50 μl of 0.25 M EDTA.

Nicking-closing enzyme isolated from HeLa cells (a gift from W. Bauer) was used to relax ColE1 DNA in the presence of ethidium bromide (Calbiochem), 4′-aminomethyltrioxsalen,

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Abbreviation: ColE1, colicin E1.

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† Hanson, C. V., Riggs, J. L. Arnstein, P., Hearst, J. E., & Lennette, E. H. (1977) *Proceedings of American Society for Microbiology Annual Meeting*, Abstr. E107.

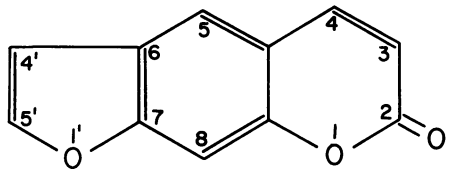

FIG. 1. The structure of psoralen.

or 4'-hydroxymethyltrioxsalen. Reactions were carried out at DNA concentrations of 3.5–40 μg/ml in gel electrophoresis buffer. NaCl was added to obtain the final desired Na^+ concentration and 2 μl of nicking-closing enzyme solution was then added. Drug and water (or ethanol when the drug was in an ethanolic solution) were added to give a final volume of 50 μl and the reaction was continued for 12–24 hr at 37°C. The reaction was stopped by the addition of sodium dodecyl sulfate to a final concentration of 1%. Concentrations of psoralen derivatives were determined by radioactive labeling (17). Concentrations of ethidium were determined spectrophotometrically using a molar extinction coefficient of 5850 $(M\ cm)^{-1}$ at 480 nm (27).

The ligase reaction was carried out at a DNA concentration of 3.5 μg/ml in electrophoresis buffer with different concentrations of drug and enough ethanol to make the final ethanol concentration 10%. $MgCl_2$/ATP, 2:1 (mol/mol) was added to give the final desired Mg^{2+} concentration (0.5–4 mM). The reaction mix was made 100 μg/ml in bovine serum albumin and 3 mM in dithiothreitol, and the reaction was started by the addition of 3 μl of a 1:20 dilution of T4 DNA ligase (Miles Research). The final reaction volume was 50 μl. After 2 hr at 20°C, 10 μl of 0.25 M EDTA was added to stop the reaction.

Irradiation. Irradiation was performed by using a light source consisting of two 400 W General Electric mercury vapor lamps (H 400 A 33-1/T16) mounted on either side of a double-walled sample holder. The holder was cooled to 6°C by continuous circulation of a precooled 40% (wt/wt) cobaltous nitrate solution. The cobalt solution served as an UV filter, allowing transmittance of light at 340–380 nm. All irradiations were for 10 min at a light intensity at the wall of the sample tube of approximately 100 mW/cm^2 (22). Unbound drug was removed by adding sufficient NaCl to make to 1.0 M and extracting three times with chloroform/isoamyl alcohol, 24:1 (vol/vol). The DNA was then dialyzed against three changes of 0.25 M NaCl/10 mM sodium phosphate, pH 6.8/1 mM EDTA and two changes of 10 mM sodium phosphate, pH 6.8/1 mM EDTA.

Gel Electrophoresis. Vertical 1% agarose (Bio-Rad) gels with 0.04 M Tris·HCl, pH 8.0/5 mM NaOAc/0.5 mM EDTA electrophoresis buffer were run at 3 V/cm for 13–17 hr. The gels were stained in electrophoresis buffer containing ethidium bromide at 0.5 μg/ml for at least 1 hr and photographed with Polaroid type 105 film, using a Mineralight C-51 UV light source (UltraViolet Products, Inc., San Gabriel, CA) and an orange filter. The shift in band position was evaluated by enlarging the photograph 20-fold, drawing a grid from the partially relaxed standards, and then measuring the displacement of the photoreacted samples relative to this standard grid. In all experiments the band shifts were also evaluated by using densitometer tracings. Both techniques were found to give band shifts that agreed within the stated uncertainty.

RESULTS

DNA Helix Perturbation Due to Covalent Drug Addition. The method used to determine the helix perturbation associated with covalent drug addition was agarose gel electrophoresis of partially relaxed supercoiled ColE1 DNA. Partially relaxed DNA provides a standard series of bands, each differing by one superhelical turn from the next (28–30). Any change in the superhelical density resulting from drug binding is detected as a change in the mobility of the DNA bands. If covalent addition causes overwinding of the helix, then the number of negative superhelical turns increases and the DNA migrates more rapidly. Similarly, unwinding of the helix relaxes the negative superhelical turns and causes the DNA to migrate more slowly. The amount of drug covalently bound was monitored by using ^3H-labeled psoralen derivatives.

As shown in Fig. 2, covalent addition of increasing amounts of drug caused the DNA to migrate more slowly upon electrophoresis. We interpret this change in mobility to be primarily due to unwinding of the DNA helix by the photoreacted trioxsalen (see *Discussion*). The amount of unwinding was determined by plotting the number of turns relaxed as a function of the number of drug molecules covalently bound per ColE1 DNA molecule (Fig. 3). The unwinding angles determined for trioxsalen, 4'-aminomethyltrioxsalen, 4'-hydroxy-

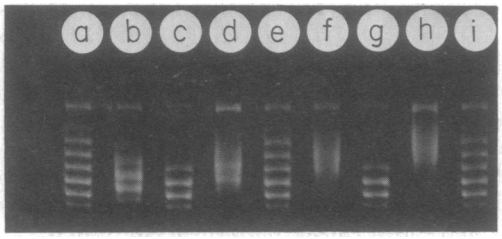

FIG. 2. Agarose slab gel of partially relaxed ColE1 DNA treated with trioxsalen. Lanes: a, e, and i, mixture of ColE1 DNA relaxed for 15 min with *E. coli* Ω protein and ColE1 DNA relaxed for 60 min with *E. coli* Ω protein; c and g, ColE1 DNA relaxed for 15 min with *E. coli* Ω protein; b, d, f, and h, ColE1 DNA relaxed for 15 min with *E. coli* Ω protein and then treated with trioxsalen at 7, 15, 22, and 29 molecules of trioxsalen per molecule of ColE1 DNA, respectively. Note the increased intensity of the nicked ColE1 band (top band in each well) in the trioxsalen-treated samples. We have observed nicking associated with the photoaddition of all of the psoralen derivatives to ColE1 DNA. Under our irradiation conditions, a ratio of 100 adducts per ColE1 DNA molecule, for example, will result in approximately 12% nicked ColE1 DNA molecules. Nicking does not occur when ColE1 DNA is irradiated in the absence of psoralen derivatives.

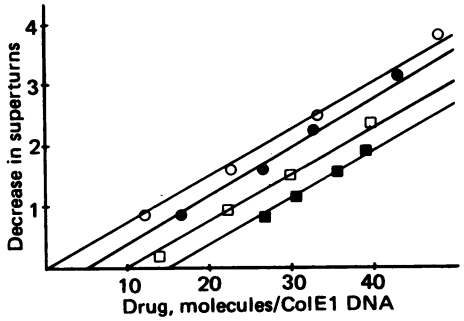

FIG. 3. Unwinding angles for covalent addition of four different psoralen derivatives to ColE1 DNA. ○, 4'-Hydroxymethyltrioxsalen; the least squares slope corresponds to 28° ± 2° unwinding per molecule reacted. ●, 4'-Methoxymethyltrioxsalen; the least squares slope corresponds to 30° ± 3° unwinding per molecule reacted. The plots of this and the following two derivatives have been offset horizontally for clarity. □, Trioxsalen; the least squares slope corresponds to 27° ± 4° unwinding per covalently bound trioxsalen molecule. ■, 4'-Aminomethyltrioxsalen; the least squares slope corresponds to 27° ± 2° unwinding per molecule reacted.

methyltrioxsalen, and 4'-methoxymethyltrioxsalen were 27° ± 4°, 27° ± 2°, 28° ± 2°, and 30° ± 3°, respectively.

Fig. 2 also shows that the band structure of the partially relaxed ColE1 DNA became more diffuse as more drug was bound. This is presumably due to the DNA helix's being unwound by fractional increments of 360°. That is, just as the original partially relaxed sample contained a distribution of molecules differing by 360°, the sample with drug covalently bound had a distribution of molecules differing by 28° around each of the partially relaxed bands. The covalent addition of an intercalated psoralen is analogous to the addition of a base pair to the DNA. As discussed by Depew and Wang (29) the superhelical density of the DNA changes, causing a shift in the mobility of the DNA of 0.1 band when a base pair is added to, or deleted from, a DNA molecule. Similarly, molecules of ColE1 DNA that differ by one trioxsalen adduct should differ in mobility by 0.078 band. The resolution of these gels was not sufficient to distinguish these finer bands.

We next determined the unwinding induced in linear (or nicked-circular) DNA by photoaddition of psoralens. The data in Fig. 3 pertain to negatively supercoiled DNA. In order to test whether or not the unwinding angle depends upon the number and/or sense of the superhelical turns, ColE1 DNA samples with a range of superhelical densities were prepared. Fig. 4 presents the number of bands shifted versus the number of trioxsalen molecules bound per ColE1 DNA molecule. Included are two ColE1 DNA samples of sufficiently low initial superhelical density so that the covalently bound trioxsalen induced net positive superhelical turns. The relative winding of these DNA samples was determined by taking one of the samples as a standard and using partially relaxed ColE1 DNA containing residual negative superhelical turns to measure differences in the number of turns between each sample and the standard. This procedure is justified by the observation that the separation between bands does not depend on the sense of the superhelical turns in the DNA when the absolute number of superhelical turns is small (R. Depew, personal communication). The conclusion drawn from Fig. 4 is that no dependence of the unwinding angle on the number or sense of the superhelical turns in the DNA is found over the range studied. It should be noted that control experiments with linear and nicked-circular DNA showed that high levels of covalently bound drug cause DNA to be retarded in agarose gels (data not shown). This effect is presumably due to stiffening of the DNA helix. The effect is negligible at low trioxsalen levels but becomes noticeable with 150 trioxsalen adducts per ColE1 DNA molecule. It should also be noted that the samples used in the above studies contained a monoadduct:diadduct ≃1 (L. Hallick and J. Hearst, unpublished data).

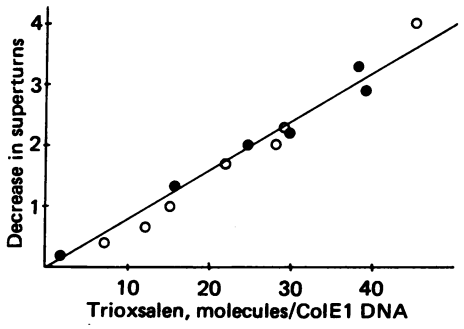

FIG. 4. Unwinding angles for covalent addition of trioxsalen to ColE1 DNA samples with different initial superhelical densities. ○, DNA samples with residual negative superhelical turns after the covalent addition of trioxsalen. For these samples the abscissa refers to the absolute number of trioxsalen molecules per ColE1 DNA molecule (i.e., the band shifts were measured relative to unreacted, partially relaxed ColE1 DNA). The least squares slope calculated from these points corresponds to 29° ± 6° unwinding per covalently bound trioxsalen molecule. ●, DNA samples with positive superhelical turns after the covalent addition of trioxsalen. For these samples, the abscissa refers to the increased number of trioxsalen molecules per ColE1 DNA molecule relative to the "standard" sample (i.e., the band shifts were measured relative to ColE1 DNA that was reacted with enough trioxsalen to produce positive supercoils. The relative shift was evaluated by using bands from an unreacted, negatively supercoiled DNA sample). The absolute number of trioxsalen molecules per ColE1 DNA molecule in these samples ranged from 142 to 190. The least squares slope of the line through these points corresponds to 29° ± 7° unwinding per covalently bound trioxsalen molecule.

Gel Electrophoresis Can Be Used to Determine Unwinding Angles or Binding-Constants. This assay involves either ligating nicked-circular DNA or relaxing supercoiled DNA in the presence of psoralen derivatives (in the absence of long-wavelength UV light) or ethidium bromide. In either case, the result is a complex between drug and covalently closed DNA with the DNA in a relaxed conformation. Upon removal of the drug the DNA becomes superhelical, and the number and sense of the superhelical turns can be determined by gel electrophoresis. In contrast to the covalent binding experiments, the DNA is relaxed or ligated by an integral number of turns, so the band pattern is still present after the reaction.

Fig. 5 shows such an experiment with ethidium bromide and ColE1 DNA. As the concentration of ethidium bromide in the reaction mixture was increased, the DNA (after removal of ethidium) migrated faster on the gel. The change in the number of superhelical turns appears to be linear with increasing concentration of ethidium. Assuming that each intercalated ethidium molecule unwinds the DNA helix by the same amount, the change in the number of superhelical turns cannot be linear with increasing concentrations of ethidium because the concentration of available binding sites decreased as more ethidium was added. All the samples of Fig. 5, however, contained such low ethidium-binding ratios that the number of available sites was essentially unchanged during the experiment. The nonlinear change of superhelical turns was therefore not detectable in this experiment.

When the samples in Fig. 5 were used, addition of 95 ethidium molecules per ColE1 DNA molecule resulted in a change of six superhelical turns. If all of the added ethidium molecules were intercalated, then the unwinding angle per ethidium molecule was 22.7°. Knowledge of the binding constant therefore allows independent calculation of the unwinding angle.

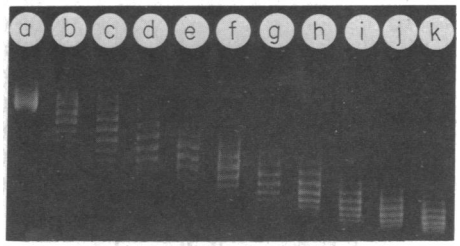

FIG. 5. Agarose slab gel of ColE1 DNA relaxed with nicking-closing enzyme in 0.125 M NaCl with increasing amounts of ethidium bromide. Lanes: a, no ethidium bromide present; b, 76 molecules of ethidium per ColE1 DNA molecule (r = 0.012); c, 95 (r = 0.015); d, 114 (r = 0.018); e, 133 (r = 0.021); f, 152 (r = 0.024); g, 171 (r = 0.027); h, 190 (r = 0.03); i, 209 (r = 0.033); j, 228 (r = 0.036); k, 247 molecules of ethidium per ColE1 DNA molecule present during the nicking-closing reaction (r = 0.039). After the reaction was complete, the samples were placed directly on the gel. Electrophoresis was sufficient to remove the ethidium from the DNA.

If it is assumed that the binding sites are independent then Scatchard analysis (31) results in the following expression:

$$\frac{r}{P} = K_{AP} \cdot (B_{AP} - r).$$

Following the nomenclature of Bresloff and Crothers (27), r is the concentration of bound drug divided by the total concentration of DNA base pairs, K_{AP} is the apparent association constant for the interaction of DNA and drug, B_{AP} is the number of apparent drug binding sites per base pair, and P is the concentration of free drug.

A plot of r/P as a function of r, therefore, should be a line with slope = $-K_{AP}$, abscissa intercept = B_{AP}, and ordinate intercept = $K_{AP} \cdot B_{AP}$. Since these experiments are done at low values of r, r/P is very nearly equal to $K_{AP} \cdot B_{AP}$. In 0.125 M NaCl, $K_{AP}(37°C) = 7.6 \times 10^5$ and $B_{AP} = 0.4$ (32). Therefore, $r/P = 3 \times 10^5$, and for a DNA concentration of 1.8×10^{-4} M the ratio of bound drug to free drug is 55. That is, essentially all of the ethidium was bound to the DNA, and the apparent unwinding angle per ethidium molecule was found to be 23.1°. By using the result of Bresloff and Crothers (27) that 80% of bound ethidium is intercalated, the unwinding angle per intercalated ethidium molecule was 28.9°. This is in reasonable agreement with previously published values of 26° and 28° (33, 34).

Just as an unwinding angle can be calculated if the drug association constant is known, an association constant can be calculated if the unwinding angle is known. Because we have demonstrated a covalent unwinding angle of 28° for psoralen derivatives and because noncovalent intercalation of ethidium has been determined to be essentially the same, we have assumed that the unwinding angle for the noncovalent binding of psoralen derivatives to DNA is 28°.

Using this technique, we have determined association constants for binding of 4′-aminomethyltrioxsalen to DNA at a series of salt concentrations (Table 1). Due to the lower association constants and solubility limits (22), the binding of the other derivatives to DNA was not detectable by this method.

The same type of experiment can be done by using T4 DNA ligase to seal nicked ColE1 DNA. This technique was used to determine binding constants for 4′-aminomethyltrioxsalen and 4′-hydroxymethyltrioxsalen (Table 1). Control experiments were done to ensure that the enzyme/DNA ratios were low enough so that changing the amount of enzyme did not change the amount of unwinding. The results of the above experiments were also the same when the DNA was first completely relaxed with nicking-closing enzyme and then the drug was added and the relaxation continued (data not shown). Thus, the observed bands reflected equilibrium distributions and were not an artifact caused by inhibition of the nicking-closing enzyme by the drug.

Table 1. Association constants for binding of psoralen derivatives to DNA*

$[Na^+]$, M	$[Mg^{2+}]$, mM	$K = K_{AP} \cdot B_{AP}$
4′-Aminomethyltrioxsalen		
0.2 (10% EtOH)		3×10^2 (37°C)
0.2 (18% Me_2SO)		2×10^2 (37°C)
0.2		7×10^2 (37°C)
0.15 (10% EtOH)		3×10^2 (37°C)
0.1 (10% EtOH)		5×10^2 (37°C)
0.05 (10% EtOH)		1.4×10^3 (37°C)
	4 (10% EtOH)	3×10^2 (20°C)
	2 (10% EtOH)	5×10^2 (20°C)
	0.5 (10% EtOH)	3×10^3 (20°C)
	0.5 (18% Me_2SO)	1.2×10^3 (20°C)
4′-Hydroxymethyltrioxsalen		
	0.5 (18% Me_2SO)	70 (20°C)

* The reactions were carried out in gel electrophoresis buffer with NaCl (for reactions with nicking-closing enzyme) or $MgCl_2$ (for reactions using T4 DNA ligase) added to give the final concentrations indicated and in the indicated amounts of ethanol (EtOH) or dimethyl sulfoxide (Me_2SO). Comparison of these data with association constants determined by equilibrium dialysis experiments done at comparable salt concentrations and r values indicates that 50–100% of the bound 4′-aminomethyltrioxsalen was intercalated (35).

DISCUSSION

The conclusion that covalently bound psoralen derivatives unwind the DNA helix by 28° depends on the assumption that the major effect of psoralen derivatives on DNA migration is DNA unwinding. It has been observed that the sedimentation of superhelical DNA with bound ethidium is different from the sedimentation of DNA of the same superhelical density without ethidium (36). Mickel *et al.* (37) observed that nicked-circular, linear, and supercoiled DNAs respond differently to changes in electrophoresis voltage and gel composition. The question therefore arises as to whether or not the migration differences observed after photoreaction are really due only to unwinding. The gel electrophoresis experiments (37) also raise a question as to whether or not the control experiments with nicked-circular and linear DNA are meaningful when applied to unwinding experiments done with partially relaxed DNA.

The unwinding angle of 28° was deduced from comparisons of partially relaxed DNA samples with relatively low degrees of superhelicity to identical samples with drug covalently bound at low values of r. Hsieh and Wang (36) found good agreement between the sedimentation of supercoiled DNA and supercoiled DNA with ethidium bound when the DNA was of low superhelical density. We interpret the partially relaxed samples as being in this low-density region. The partially relaxed samples correspond to neither the nicked-circular nor the highly supercoiled species studied by Mickel *et al.* (37), but we would expect that partially relaxed DNA samples such as used in these studies might respond most nearly like nicked-circular DNA to changes in electrophoresis voltage and gel composition. Thus, we would expect the control experiments on linear and nicked-circular DNA to be useful in predicting when effects other than unwinding might become important. It should be remembered that the r values at which these experiments were done (<0.01) were low enough so that < 0.5% of the charge of the DNA was neutralized by 4′-aminomethyltrioxsalen (the only one of the derivatives that is charged). Therefore, in view of the low r values and low superhelical densities employed in these experiments, and lacking a reasonable alternative explanation, we interpret the migration differences to be due to DNA unwinding.

Another possible objection to the unwinding experiment stems from the nicking during irradiation. One might expect the preferential removal of DNA molecules with higher numbers of drug bound. This effect would cause the unwinding angle to be underestimated, and the effect should increase as the number of adducts increases (i.e., the plots in Fig. 3 would tend to plateau). As stated in the legend to Fig. 2, at 130 adducts per DNA molecule, approximately 30% of the DNA molecules are nicked. The samples in Fig. 3 have as many as 40 adducts per DNA molecule. If 10% of these molecules have 50 adducts (or more) and 10% have 30 adducts (or less), then about 12% of the high-adduct population will be nicked while only 7% of the

low-adduct population will be nicked. This will change the position of the mean by less than 0.05 band. This is a very small effect.

It could also be assumed that the molecules in the original superhelical distribution with the higher superhelical densities bind more drug (35) and are therefore preferentially removed. This is also a small effect, and it is in the opposite direction of the above effect.

The nicking-closing enzyme assay appears to be a simple method of measuring drug intercalation into DNA. The assay can be difficult to interpret, however. When one knows the binding constant *and* that a drug is essentially all intercalated, then one can calculate an unwinding angle (as was done for ethidium). If only one-half of the bound ethidium were intercalated, an unwinding angle of 14.5° would presumably have been found.

It is exactly this complication which makes the assay so useful for psoralen derivatives. If a second mode of binding were to exist that does not unwind the helix, it will not be detected when the binding constant is measured by using this assay. Thus, we are only measuring those molecules that are in a position to photoreact. Clearly, combinations of binding modes that unwind or overwind by different amounts will be difficult to separate from each other with this method. The fact that these experiments are done at low r values and in 0.2 M NaCl should tend to minimize effects of secondary binding sites, however.

The salt concentrations necessary for enzymatic activity were found to have profound effects on the binding of psoralen derivatives to DNA. 4′-Aminomethyltrioxsalen, with a K_{AP} in low-salt buffer nearly as large as that of ethidium (35) is the only derivative with enough binding in 0.2 M Na^+ to be measurable by this technique. These drugs are thus very sensitive to tightening of the DNA helix. This selectivity of binding most probably explains why trioxsalen, for example, binds preferentially to internucleosomal DNA in nuclei (17).

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