Binding of Psoralen Derivatives to DNA and Chromatin: Influence of the Ionic Environment on Dark Binding and Photoreactivity†

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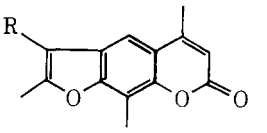
ABSTRACT: The binding of two psoralen derivatives to DNA and to chromatin has been compared in some detail, particularly with respect to the influence of the ionic environment. The compounds used were the extremely hydrophobic 4,5′,8-trimethylpsoralen (TMP) and its extremely hydrophilic derivative 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT). Both the dark noncovalent interaction and the UV light induced covalent interaction with DNA were strongly reduced by increasing concentrations of Na^+ and Mg^{2+} ions, particularly in the range up to the physiological region. For any given ionic conditions, higher binding levels were observed for supercoiled relative to linear DNA and at 4 °C relative to 20 °C. About 28% of the DNA in chromatin was accessible to either drug in low ionic strength, but nearer 20% in physiological NaCl. In addition to the dramatic increase in aqueous solubility, the introduction of the 4′-aminomethyl group into TMP brought about an increase in the sensitivity of DNA binding to ionic environment and a decrease in the quantum efficiency of its photoreactions. In particular, the susceptibility of AMT to photobreakdown relative to photoaddition was increased over that of TMP. Overall, the results are compatible with a principally intercalative mode of binding for both derivatives that is strongly dependent upon the stabilization of the DNA duplex by counterions and which, in turn, has a corresponding influence on the subsequent photoreaction.

Derivatives of the furocoumarin, psoralen, have been used for many years in biological experiments relating to skin photosensitization (see Scott et al., 1976) and thus in the treatment of skin diseases such as psoriasis (Parrish et al., 1974). Only recently have they been used as molecular probes for chromatin structure (Hanson et al., 1976; Wiesehahn et al., 1977), hairpin regions in viral DNA (Shen & Hearst, 1976), satellite DNA structure (Shen & Hearst, 1977), and bacterial repair mechanisms (Cole et al., 1976).

The basis for all of these recent studies lies in the ability of psoralen derivatives to penetrate intact cells or isolated nuclei and covalently bind to the DNA upon irradiation with UV[1] light in the region of 320–380 nm, to form monoadducts or cross-links (Musajo & Rodighiero, 1970; Cole, 1970). However, a wide variety of solution conditions were employed in these experiments and, up to now, no systematic study of the effects of ionic environment on the dark binding and photoreaction of these drugs has been undertaken. In this work the influence of monovalent and divalent cations over a broad concentration range on psoralen binding to DNA has been examined, specifically to linear and supercoiled DNA and to DNA in chromatin where the effect of chromosomal protein dissociation at higher ionic strengths is superimposed upon the intrinsic effect of the metal ions on the DNA.

Experiments have been carried out in parallel, using two psoralen derivatives of the same series which represent extreme types and thus may be regarded as model compounds: (1) 4,5′,8-trimethylpsoralen (trioxsalen or TMP) which shows pronounced hydrophobicity and hence low aqueous solubility (0.8 μg/mL) (Dall'Acqua et al., 1971); and (2) 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride (AMT), one of several modifications of **1** synthesized in this laboratory (Isaacs et al., 1977). This molecule carries the strongly functional $-CH_2NH_3^+$ group and is the most hydrophilic of the TMP derivatives synthesized so far (aqueous solubility at least 10 mg/mL). This type of study should form a basis for experiments involving derivatives of other psoralen series currently being produced.



1, R = H
2, R = $CH_2NH_3^+Cl^-$

Materials and Methods

DNA. Either Sigma type 1 calf thymus DNA or *Drosophila melanogaster* embryo DNA isolated as described previously (Wiesehahn et al., 1977) was used in experiments requiring only linear DNA. Supercoiled colicin E1 (Col E1) plasmid DNA, a kind gift from L. Hallick and C. Chun, was isolated from *Escherichia coli* JC411 thy (Col E1) (obtained from P. Modrich) after chloramphenicol amplification of the plasmid (Clewell & Helinski, 1972) according to the method of Modrich & Zabel (1976).

For experiments directly comparing supercoiled and linear DNA, the latter was produced from the former by sonication on ice under an atmosphere of N_2 using two 30-s bursts at 35 W separated by 1 min on a Sonifier Cell Disruptor W185 (Heat systems: Ultrasonics Inc., Plainview, Long Island, N.Y.). In addition, sonicated calf thymus or *Drosophila* DNA was found to yield more reproducible results in equilibrium

† From the Department of Chemistry, University of California, Berkeley, California 94720. *Received August 16, 1977; revised manuscript received December 15, 1977.* This work was supported by U.S. Public Health Service Grant No. 11180 and by a Harkness Fellowship from the Commonwealth Fund of New York to J. E. Hyde.

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[1] Abbreviations used: TMP, 4,5′,8-trimethylpsoralen; AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; TMKS, 25 mM KCl–1 mM $MgCl_2$–0.25 M sucrose–15 mM mercaptoethanol–50 mM Tris-HCl (pH 7.4).

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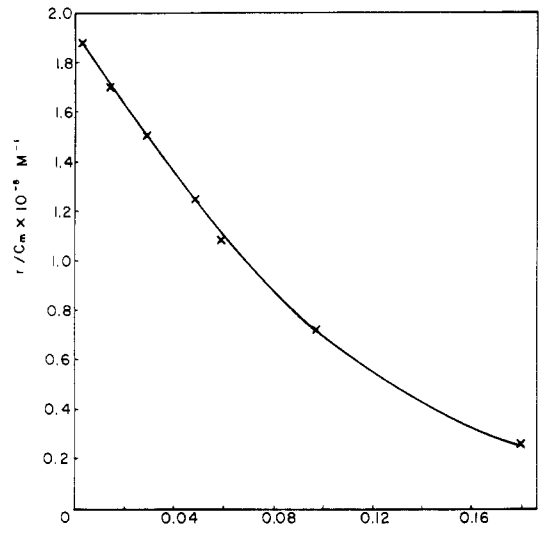

FIGURE 1: Scatchard plot of the binding of AMT to linear (calf thymus) DNA at 4 °C. [DNA] = 75.8 μM in base pairs. Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.4.

dialysis experiments than the very high molecular weight material.

Chromatin, a kind gift of D. Ring, was isolated as follows: nuclei were isolated from fresh steer thymus glands by homogenization in 0.25 M sucrose, 0.01 M Tris-HCl, 0.05 M $NaHSO_3$, 0.01 M $MgCl_2$, pH 8.0. After washing, pelleted nuclei were resuspended in TMKS buffer (Honda et al., 1974), $CaCl_2$ was added to 1 mM, and the chromatin was released by the method of Noll et al. (1975) using mild micrococcal nuclease digestion. A concentrated stock solution of chromatin oligomers (weight average DNA size about 9000 base pairs [Noll et al., 1975]) in 0.5 mM EDTA and saturated with $PhCH_2SO_2F$ (Sigma) (to inhibit histone proteolysis) was diluted into the various reaction mixtures as required.

Psoralen Derivatives. 4,5′,8-Trimethylpsoralen (trioxsalen, TMP) was obtained from Paul B. Elder Co. (Bryan, Ohio) and tritiated in this laboratory (Isaacs et al., 1977). Tritiated 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride (AMT) was synthesized from the parent compound as described previously (Isaacs et al., 1977). Stock solutions of TMP were made up in 100% ethanol to a concentration of approximately 0.5 mg/mL (ϵ_{249} = 31 008 L M^{-1} cm^{-1} [Wiesehahn et al., 1977]), while AMT stock solutions of similar concentration were made up in 50% ethanol/50% water (the alcohol being present to maintain sterility). For AMT in this solvent, ϵ_{249} = 27 590 L M^{-1} cm^{-1}. The specific activities of these solutions were generally in the range of 10^5 to 10^6 cpm/μg.

Dark Binding of Psoralen Derivatives to DNA. Equilibrium constants for dark binding were obtained by the equilibrium dialysis method. One-milliliter aliquots of DNA solution (25–50 μg/mL) in the relevant solvent (10 mM Tris-HCl, 1 mM EDTA, pH 7.4, together with various concentrations of other ionic species) were placed in a dialysis bag together with the psoralen derivative at an initial concentration equal to that outside the dialysis bag (20 mL total volume). The dialysis bags were made from Spectrapor membrane type 2 (Spectrum Medical Industries Inc., Los Angeles) pretreated by boiling in $NaHCO_3$ and rinsing thoroughly with double-distilled H_2O. The drug was allowed to redistribute as the system was vigorously stirred for at least 72 h, after which the concentrations of the drug inside and outside the membrane were determined by radioactivity measurements and that of the DNA inside the bag from its A_{260} value. The association constant for noncovalent binding (K) for the particular solution conditions used was then calculated from the expression

$$K = \frac{[PS]}{[P][S]} \quad (1)$$

where [P] is the concentration of free drug, [PS] is the concentration of bound sites, and [S] is the concentration of unoccupied binding sites (expressed in base pairs) (Isaacs et al., 1977).

Because this method of calculating the concentration of bound drug involves taking a difference between counts inside and outside of the dialysis membrane, the error increases quite steeply as this difference diminishes. Thus for the levels of drug and DNA used in this work, a 1% error in the estimation of free and bound drug concentrations leads to approximately a 2% error in the estimate of K, if K is approximately 10^5 M^{-1}. For Ks of about 10^4 M^{-1}, this figure becomes 5%, but at Ks of about 10^3 M^{-1}, the figure jumps to about 30%.

Photoreaction of Psoralen Derivatives to DNA. Mixtures of the derivatives and DNA (or chromatin) were irradiated while stirring in a water cooled cuvette of 1-cm path length placed in the beam of a 500-W Varian xenon arc lamp (VIX-500). The incident light was passed through a 5-mm glass filter which allowed passage of light between 320 and 410 nm (maximum about 370 nm). The intensity incident on the cuvette was approximately 10 mW/cm^2. After irradiation, DNA samples were extracted three times with chloroform:isoamyl alcohol (24:1) to remove excess noncovalently bound TMP and organic-soluble photobreakdown products of both TMP and AMT. Chromatin samples were extensively pronased (1 mg/mL, 50 °C, 6–8 h) prior to this step. The aqueous phases from the extraction were removed and dialyzed extensively into 10 mM Tris-HCl, 1 mM EDTA, 0.25 M NaCl, pH 7.4, which efficiently removes excess noncovalently bound AMT plus aqueous-soluble photobreakdown products of both drugs. Finally, the samples were dialyzed into 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, and the amounts of drug covalently bound to the DNA determined as described previously (Wiesehahn et al., 1977).

Results

Dark Binding of AMT and TMP to DNA. Equilibrium dialysis experiments were performed at low ionic strength on mixtures of AMT and sheared calf thymus DNA where the concentration of the latter was held constant at 50 μg/mL (75.8 μM), while the total drug concentration was varied over a 250-fold range (0.034–8.5 μM). The results are shown in Figure 1 as a conventional Scatchard plot (Scatchard, 1949) where r = (concentration of bound drug)/(total concentration of DNA in base pairs) and C_m = concentration of free drug. In the nomenclature of eq 1, $r = [PS]/[S_0]$ where $[S_0]$ is the total concentration of DNA base pairs, and $C_m = [P]$.

Following the nomenclature of Bresloff & Crothers (1975), the Scatchard plot for independent binding sites is represented by the equation

$$\frac{r}{C_m} = K_{ap}(B_{ap} - r) \quad (2)$$

where B_{ap} is the apparent number of binding sites per base pair. In this convention, our association constant, K, is given by eq 3

$$K = \frac{r}{C_m}\left(\frac{1}{1-r}\right) \quad (3)$$

TABLE I: Association Constants (M^{-1}) for AMT and TMP Binding to Various DNAs; NaCl Concentration and Temperature Dependences.

	Temp (°C)	AMT		TMP	
		[NaCl] = 0	[NaCl] = 0.15 M	[NaCl] = 0	[NaCl] = 0.15 M
Supercoiled Col E1 DNA	4	1.1×10^5	7×10^3	3.0×10^4	4×10^3
Linear Col E1 DNA	4	1.0×10^5	4×10^3	1.1×10^4	2×10^3
Linear *D. melanogaster* DNA	4	1.3×10^5	6×10^3	1.7×10^4	5×10^3
Linear *D. melanogaster* DNA	20	8.2×10^4	1×10^3	4×10^3	5×10^2

and the "intrinsic" association constant, $K(0)$, described in Bresloff & Crothers (1975) is equal to K at $r = 0$

$$K(0) = \lim_{r \to 0} \frac{r}{C_m}$$

The observed convexity toward the r axis suggests that there is more than one type of binding site on the DNA, lower affinity sites becoming occupied as r increases. Curvature in the Scatchard plot due to neighboring site exclusion does not become significant until $r = 0.3$ (Bauer & Vinograd, 1970; Bresloff & Crothers, 1975) and probably does not contribute here. It is possible that analogous to the cases of the dye molecules ethidium bromide (Bauer & Vinograd, 1970; Bresloff & Crothers, 1975) and proflavin (Peacocke & Skerrett, 1956; Li & Crothers, 1969) some type of weaker outside binding mode is superimposed upon intercalation, although curvature due to a sequence dependent intercalation is also quite possible (Gellert et al., 1965).

The intercept of the nearly linear portion of the curve on the r/C_m axis yields an intrinsic binding constant, $K(0)$, of 1.9×10^5 M^{-1} with the intercept on the r axis occurring at $B_{ap} = 0.13$. As pointed out by Crothers (1968), however, the interpretation that 13% of the available sites have an apparent binding constant, K_{ap}, of 1.5×10^6 ($K_{ap} = K(0)/B_{ap} = (1.9 \times 10^5)/0.13$) may be an oversimplification (a corresponding K_{ap} for ethidium bromide is $3\text{-}5 \times 10^6$; Lepecq & Paoletti, 1967; Waring, 1965). The low aqueous solubility of TMP precludes a comparable plot being made as r values only as large as 0.02 can be attained.

In the experiments that follow, the DNA and total drug concentrations are held constant at approximately 35–75 μM and 1.75 μM, respectively, unless otherwise indicated. (The solubility limit of TMP in H_2O is about 3.5 μM (Dall'Acqua et al., 1971).) The conditions ensure a fairly low occupation of binding sites, corresponding at least in the case of AMT to the more nearly linear end of the curve in Figure 1, although, in the following experiments, no extrapolations are performed and association constants, K, in Table I are calculated at finite r values rather than at $r = 0$.

Salt Dependence of Dark Binding. The dependence of the association constant as defined in Materials and Methods upon NaCl concentration up to 0.15 M is shown in Figure 2 for the case of superhelical Col E1 DNA and the two psoralen derivatives. In 10 mM Tris-HCl, 1 mM EDTA alone (4 °C) K for AMT was found to be 1.1×10^5 M^{-1} and for TMP 3.0×10^4 M^{-1}.

As NaCl is added to the psoralen–DNA mixtures (Figure 2), the association constants of both drugs are strongly depressed, the principal effect occurring up to 0.05 M NaCl and leveling off by 0.15 M NaCl at which point K for AMT has fallen about 15-fold while K for TMP has fallen only about 7-fold. Thus, at 0.15 M NaCl, the Ks for both drugs are about 5×10^3 M^{-1}.

Also shown in Figure 2 are data points for the effect of Mg^{2+} and the dicationic pentane derivative cadaverine (Sigma,

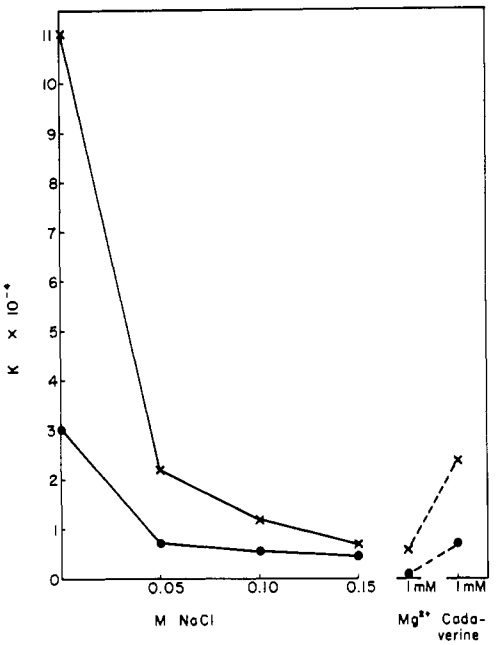

FIGURE 2: The effect of increasing concentrations of NaCl, $MgCl_2$, and cadaverine upon the association constants of AMT (X—X) and TMP (●—●) for dark binding to supercoiled Col E1 DNA at 4 °C, as determined by equilibrium dialysis.

1,5-diaminopentane, bought as the hydrochloride). A 1 mM level of cadaverine reduces the binding of both drugs by about 4-fold. Mg^{2+} (1 mM) is an even more potent inhibitor, reducing the binding about 20–30-fold, to levels below those induced by 0.15 M NaCl. Qualitatively similar curves were obtained if linear, rather than supercoiled DNA was used in these experiments. The quantitative differences are examined in the next section.

Dark Binding to Supercoiled and Linear DNA. In order to investigate the putative role of intercalation, a comparison was made between the binding of the two psoralens to supercoiled and to linear DNA. Intercalation of a drug into a negatively supercoiled DNA such as Col E1 reduces torsional strain by unwinding the base pairs to some extent; thus there is an extra favorable free energy term associated with the binding of the drug relative to the case of linear DNA (Bauer & Vinograd, 1970).

To avoid variations in base composition, linear fragments of Col E1 were produced from the supercoiled species by sonication. The observed binding constants are summarized in Table I. Consistent with an intercalative mode, the binding in all cases is somewhat stronger to the superhelical DNA, the difference being most obvious for the case of TMP, especially in low salt. This may reflect a greater predominance of intercalative binding relative to an outside binding mode compared to AMT at these conditions.

NaCl appears to have a greater effect on the binding to linear DNA than it does to supercoiled DNA in the case of

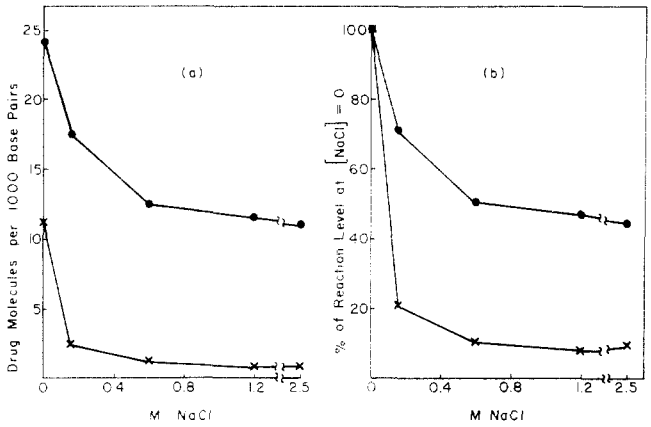

FIGURE 3: (a) The effect of increasing concentrations of NaCl upon the photobinding of AMT (X—X) and TMP (●—●) to supercoiled Col E1 DNA at 4 °C. (b) The data of a normalized to show the reaction level at each NaCl concentration relative to that observed in the absence of NaCl for the same drug. [Drug] = 1.74 μM; [DNA] = 37.9 μM.

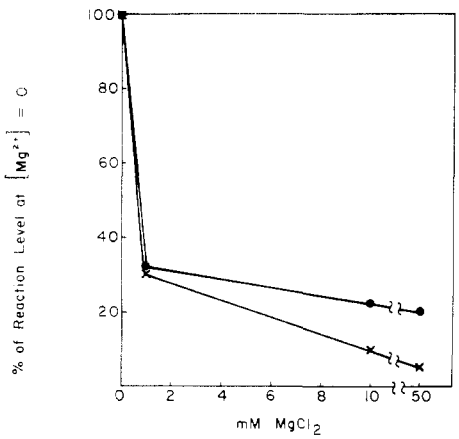

FIGURE 4: The effect of increasing concentrations of $MgCl_2$ upon the photobinding of AMT (X—X) and TMP (●—●) to supercoiled Col E1 DNA. Conditions as for Figure 3.

AMT. Similar experiments with cadaverine revealed a more pronounced effect, K values for linear DNA and both drugs being diminished at least four times more than those for supercoiled DNA. If it is assumed that any outside binding mode will be affected roughly equally by NaCl whether the DNA is supercoiled or not, the important factor may then be the tendency of these counterionic species to induce a tightening (overwinding) of the double helix by reduction of electrostatic repulsion between DNA phosphate groups. This would increase the energy barrier to intercalation, something that would be more strongly resisted in the torsionally constrained superhelical DNA, since overwinding increases the degree of negative superhelicity.

The UV-Induced Photoreaction of AMT and TMP with DNA. Irradiation of psoralen-DNA mixtures with UV light in the region of 365 nm converts noncovalently dark-bound drug into covalent adducts (see introductory section). The following experiments measured the overall level of covalent binding of AMT and TMP to DNA as a function of the ionic conditions.

Aliquots of Col E1 superhelical DNA (25 μg/mL = 37.9 μM) were irradiated for 5 min at 4 °C as described in Materials and Methods with a constant concentration (1.75 μM) of either TMP or AMT in increasing amounts of NaCl. Figure 3a shows the actual levels of binding obtained under these conditions. As in the case of dark binding, the photobinding

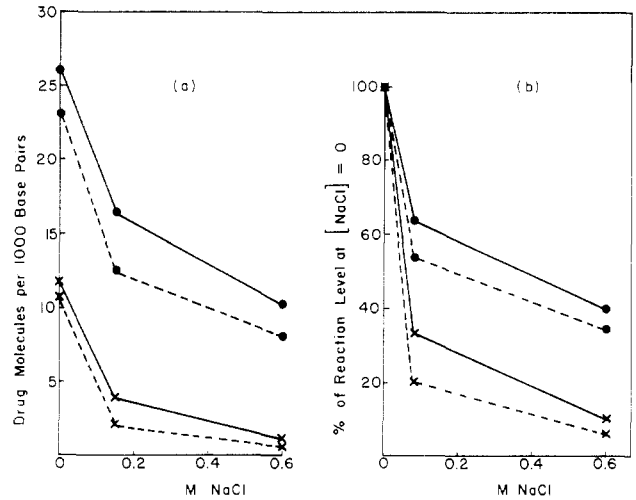

FIGURE 5: A comparison of the photobinding of AMT (X—X) and TMP (●—●) to supercoiled Col E1 DNA (solid line) and to linear Col E1 DNA (dashed line) at different NaCl concentrations; (a) absolute levels of drug bound; (b) levels normalized to the value of [NaCl] = 0 for each drug. Conditions as for Figure 3.

of both drugs is strongly diminished by increasing ionic strength, the effect being considerably more pronounced in the case of AMT. This is more clearly shown in Figure 3b, where the curves of Figure 3a are normalized. From the association constants in Table I (which pertain to the same experimental conditions) we can calculate using eq 1 the concentration of drug bound in the initial dark equilibrium mixture before irradiation for the particular drug to DNA ratio used here. These show that this concentration is reduced about 4-fold in both cases by addition of 0.15 M NaCl. The relative reductions in photobinding caused by this concentration of NaCl are 4.8-fold and 1.4-fold, respectively for AMT and TMP. Thus a reasonably close parallel exists between the effects of NaCl on both the dark and photoinduced binding processes.

A similar phenomenon occurs when Mg^{2+} is used (Figure 4) except that the binding of both drugs is now affected to a more similar extent. Analogous again to the case of dark binding, 1 mM $MgCl_2$ reduces the levels of the photoreaction to a degree similar to that observed for 0.15 M NaCl, although the ionic strength increment (relative to the background buffer) is about 50-fold greater in the latter case. This parallels well the known markedly greater stabilization of the double helix by Mg^{2+} relative to Na^+ (Dove & Davidson, 1962) and again suggests that conformational changes induced in the DNA by the particular ionic species are of primary importance in influencing the efficiency of the photoreaction.

Figures 5a and 5b show the results of an experiment comparing levels of photobinding to supercoiled and linear Col E1 DNA under identical conditions. At all salt concentrations, the absolute level of binding is somewhat higher to supercoiled DNA (Figure 5a) following the general pattern of relative association constants for dark binding given in Table I. Moreover, increasing sodium chloride concentrations repress the binding levels to linear DNA to a greater degree than to superhelical DNA (Figure 5b) consistent again with the picture of the latter being more resistant to conformational overwinding by positive ions.

Figures 3a and 5a show that AMT gives rise to a lower level of photoreaction than TMP despite its higher association constant in low salt, when irradiated with DNA for the same time (5 min) under identical conditions as an equivalent mixture of TMP and DNA. It is instructive to consider whether the level of photoreaction will exceed or fall short of the equi-

librium dark binding value if the irradiation is continued until no further covalent addition occurs.

The results of a time-course experiment dealing with this question are shown in Figure 6. The conditions were identical with those pertaining to the dark binding data of Table I, with [NaCl] = 0. The equilibrium binding value for AMT from that data is 37.2 drug molecules per 1000 base pairs and for TMP, 24.4 drug molecules per 1000 base pairs. The maximal photobinding levels of both drugs exceed these dark binding levels, by a factor of 1.1 with AMT and 1.5 with TMP. Similar experiments using linear DNA yielded factors of 1.0 and 2.5, respectively. Thus, not only is the photobinding of TMP to DNA initially about 3-fold faster than that of AMT (its dark equilibrium value being exceeded in the first 5 min, compared with nearly 50 min in the latter case) but its final level is considerably in excess of the dark binding value, which is not the case for AMT. The great advantage of AMT, of course, is the fact that it is more than 1000 times more soluble than TMP, so while its photochemistry is slower, the absolute rates of addition to DNA which can be achieved with it are much higher than with TMP.

The difference in photochemical kinetics may be understood in terms of the competing processes of photoaddition of adduct and photodestruction of unbound drug. A discussion of the equilibria involved has been presented previously (Isaacs et al., 1977) where it was shown that to a good approximation, for low levels of binding

$$\frac{\text{rate of photobreakdown}}{\text{rate of photoaddition}} = \frac{k_3}{k_2[\mathrm{S}]K} + \frac{k_3}{k_1}\frac{I}{[\mathrm{S}]} \qquad (4)$$

where k_1, k_2, and k_3 are the rate constants for the processes:

$$\mathrm{P} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{PS} \qquad (5)$$

$$\mathrm{PS} + h\nu \xrightarrow{k_2} \text{adduct} \qquad (6)$$

$$\mathrm{P} + h\nu \xrightarrow{k_3} \text{breakdown product} \qquad (7)$$

and I is the intensity of incident radiation. If I is low (as in the present experiments), the second term on the right-hand side of eq 4 may be ignored and we can write:

$$\frac{\text{rate of photobreakdown}}{\text{rate of photoaddition}} = \frac{k_3}{k_2[\mathrm{S}]K} = \frac{k_3}{k_2}\frac{[\mathrm{P}]}{[\mathrm{PS}]} \qquad (8)$$

from eq 1 in Materials and Methods. The underlying assumption in the derivation of eq 8, that the expression is time independent, means that we can conveniently substitute the ratio of the final levels of photobreakdown to photoaddition for the left-hand side of eq 8 and thus derive:

$$\frac{\text{rate constant for photoaddition}}{\text{rate constant for photobreakdown}} = \frac{\text{final level of photoaddition}}{\text{final level of photobreakdown}}\frac{[\mathrm{P}]}{[\mathrm{PS}]} \qquad (9)$$

Inserting the data of Figure 6 into the right-hand side of eq 9, we obtain a figure for the ratio of rate constants of 1.9 for AMT and 3.3 for TMP. Thus the presence of the $-CH_2NH_3^+$ in AMT reduces the quantum efficiency of the two photochemical pathways to differing degrees, having a more detrimental effect on the photoaddition step.

Binding of the Psoralen Derivatives to Chromatin. As an example of the application of ionic strength dependent studies of psoralen–DNA interactions, the binding of the two psoralen

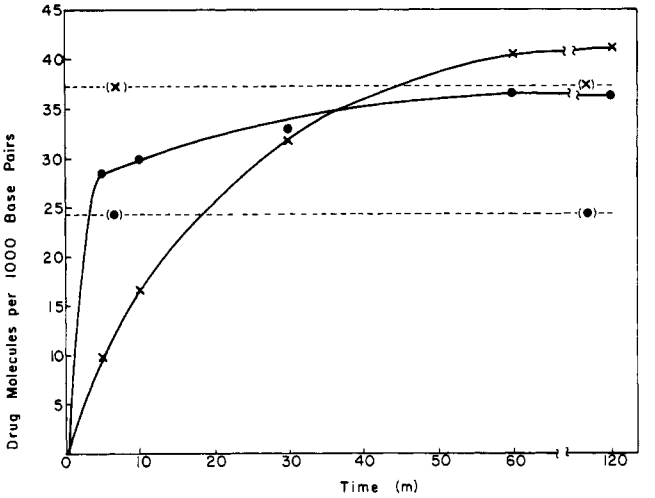

FIGURE 6: Time course of the photoreaction of AMT (X—X) and TMP (●—●) with supercoiled Col E1 DNA. The upper and lower dashed horizontal lines represent the equilibrium levels of dark binding for AMT and TMP, respectively, calculated from the data of Table I. Conditions as for Figure 3.

derivatives to chromatin has been considered. In this system, as the ionic strength is progressively increased, the histones are selectively removed; first H1 is completely dissociated in 0.6 M NaCl (Ohlenbusch et al., 1967; Henson & Walker, 1970); H2A and H2B then dissociate together between 0.7 and 1.2 M followed by the H3, H4 pair above 1.2 M (Burton et al., 1975). No histones remain bound at 2.0–2.5 M NaCl. To correlate these events with the binding of psoralen derivatives to chromatin, account must be taken of the intrinsic effect observed above of the ions on the DNA–psoralen interaction.

Attempts to study the dark binding of AMT and TMP to chromatin were only partially successful. Binding constants from equilibrium dialysis experiments could not be reproduced to any degree of satisfaction when NaCl was present and, even in the absence of NaCl, reproducibility was poor. The quantitative effect of the histone proteins was thus determined from photobinding studies.

Photobinding Studies of the Psoralen Derivatives and Chromatin. Solutions of steer thymus chromatin in NaCl concentrations from 0 to 2.5 M were irradiated with AMT and TMP in parallel with solutions identical in every respect except that thymus DNA at an equal concentration replaced the chromatin. To demonstrate the difference in behavior of the two drugs, it is necessary to plot the ratio of drug bound in the case of any given chromatin sample to that observed for the equivalent DNA sample, thus taking into account the dissociation of the histones. This is done in Figure 7. In low salt, both drugs react with chromatin to a level of about 28% of that observed for purified DNA. As the NaCl concentration is raised to 0.15 M, the percentage of sites accessible on the chromatin diminishes in the case of both drugs from about 28% to nearer 20%. This effect, though quite small, is very reproducible. It may be due to a simple aggregation effect that reduces accessibility of the psoralen derivatives to the DNA (although there was no visible precipitation) or possibly to an increased internal stabilization of the chromosomal subunit (nucleosome) as ionic conditions approach more closely those of the physiological environment.

Removal of the extra-nucleosomal H1 fraction in 0.6 M NaCl leads to a considerable increase in accessible sites, especially in the case of TMP, where 60% of the DNA is now accessible. Disruption of the nucleosomal subunit structure by

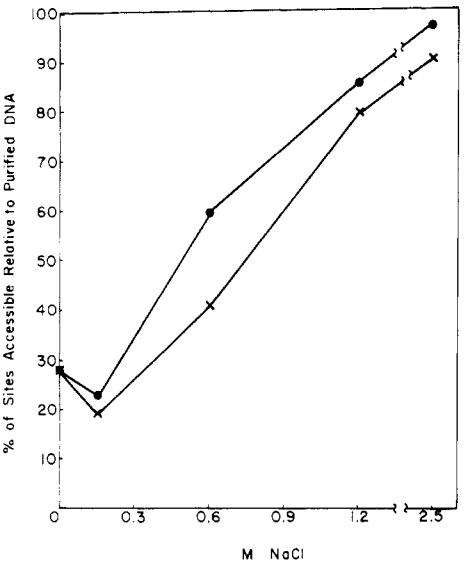

FIGURE 7: Salt dependence of the photobinding of AMT (X—X) and TMP (●—●) to steer thymus chromatin. The results are expressed as the percentage of sites accessible on the chromatin relative to purified DNA under the same conditions, as the chromosomal proteins are dissociated by increasing NaCl concentration. Temperature and drug/DNA concentrations as for Figure 3.

subsequent removal of the core proteins H2A and H2B leads to another sharp increase in accessible sites such that both drugs can now react to about the 80–85% level. Disruption of the nucleosomes appears to be the main step in reducing the protective effect of the core proteins since the removal of the remaining proteins H3 and H4 above 1.2 M NaCl accounts for only the small increase from the 80–85% level to near the theoretical maximum 100% level observed in 2.5 M NaCl, where the DNA is completely devoid of histones. The small degree of protection remaining at this ionic strength may be due to a minor fraction of tightly bound nonhistone protein.

The nucleosome of steer thymus chromatin may be regarded as a stretch of 180–190 base pairs of DNA comprising 140 base pairs of strongly protected DNA and 40–50 base pairs of a less protected region (Compton et al., 1976). The latter thus represents about 22–26% of the total DNA, a figure in close agreement with the level of accessibility found for both AMT and TMP in 0–0.15 M NaCl. The psoralen derivatives differ greatly in this respect from ethidium bromide, which can overcome the tendency of the histones to prevent intercalation and bind to chromatin to a level of 95% of that for purified DNA (Lawrence & Duane, 1976). The psoralen derivatives resemble more closely drugs such as quinacrine and acridine orange (Brodie et al., 1975) which bind more weakly to chromatin with about 30–40% accessibility. The significant differences in the levels of binding for the two drugs after h1 removal may, however, reflect their differing abilities even in high salt to perturb further the chromatin structure already destabilized by the loss of H1.

Discussion

With the increasing use of psoralen derivatives as a probe for nucleic acid structures in both in vivo and in vitro systems, there is now an expanding activity in the synthesis of new compounds, attempting to improve upon the photoreaction properties of the naturally occurring derivatives such as xanthotoxin (8-methoxypsoralen), bergaptan (5-methoxypsoralen), and TMP. More efficient molecular "tailoring" will require a complete understanding of the factors affecting the dark and UV light induced binding. The experiments in this paper have investigated the influence of ionic environment, one of the more important of these factors.

The introduction of the $-CH_2-NH_3^+$ group into the TMP molecule to give AMT leads to a much higher aqueous solubility and association constant to DNA in low ionic strength. Although a weaker binder than ethidium bromide, AMT does show analogies to this positively charged intercalator in its Scatchard plot (Figure 1), and in the dramatic reduction of its association constant (Lepacq & Paoletti, 1967) with increasing NaCl (Figure 2). It is difficult as yet to ascertain what fraction (if any) of the drug bound under dark conditions is due to an outside binding mode resulting from ion-pair formation, but it is significant that the dark binding of TMP, where such an interaction is not likely, shows a very similar salt dependence with Na^+, Mg^{2+}, or cadaverine, although to a less pronounced degree. This suggests that the two drugs share a common principal mode of binding despite the great difference in their relative hydrophilicities. That this principal mode is intercalation is suggested strongly by their enhanced binding to supercoiled DNA relative to linear DNA (Table I), by their ability to covalently cross-link the two strands of the DNA duplex (Musajo & Rodighiero, 1970; Cole, 1970) and from gel electrophoresis studies of supercoiled DNA partially relaxed in their presence (Wiesehahn & Hearst, in preparation).

The close parallels between the detrimental effect on both the dark and photoinduced binding of Na^+ and Mg^{2+} (and in particular the extreme effect of the latter at 1 mM) (Figures 2–5) strongly suggest that the counterion stabilization of the helix conformation, which increases the free energy barrier to intercalation and thus reduces the concentration of intercalated drug, is a primary factor in determining the level of photoreaction attained for a given drug. Thus, the greater the concentration of unbound drug, the more efficiently it can be lost as photobreakdown products. The suggestion (Ben-Hur, 1975) that counterions do not affect the dark equilibrium binding level of the drug, but diminish the level of photoreaction solely by stabilizing against a distortion required *after* intercalation to allow covalent addition of the drug is not supported by the present results.

In addition to the factors mentioned previously, the $CH_2NH_3^+$ group substituted at the 4′ position in AMT does not appear to introduce any steric hindrance to intercalation at low ionic strength. It does, however, reduce substantially the quantum efficiency of its photochemical reactions; moreover, the efficiency of the photoaddition of adduct is reduced to a greater degree relative to that of the photobreakdown of free drug (Figure 6). This suggests that as well as the electronic effect of the substitution, there may be a steric factor involved in the transition from intercalated to covalently bound drug.

The features deemed desirable or necessary in a psoralen molecule may differ considerably depending upon for instance, whether in vitro nucleic acid structure is being probed at low ionic strength or whether skin photosensitization or cellular experiments are being conducted in vivo, and it is clear that the balance of the effects of ring substitution on aqueous solubility, on the electronic energy levels, and on the free energy of intercalation (the latter in turn a strong function of the ionic environment) will determine the utility of any given psoralen derivative in a particular type of study.

Acknowledgments

The authors thank Stephen Isaacs for providing the psoralen derivatives, Gary Wiesehahn for helpful discussions, and Marshall Tuttle for technical assistance.

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High-Pressure Liquid Chromatography in Polynucleotide Synthesis†

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ABSTRACT: Reverse phase high-pressure liquid chromatography (HPLC) using columns containing microparticulate materials with bonded octadecyl groups has been developed as a rapid and efficient method for the separation of nucleosides, nucleotides, and, in particular, of protected oligonucleotides which are standard intermediates in the stepwise synthesis of deoxyribopolynucleotides. Reported are extensive studies of the influence of the different purine and pyrimidine bases, of protecting groups, of the phosphate groups, and of the chain lengths of oligonucleotides on their retention on such columns. Further, the application of HPLC in the stepwise synthesis of an oligonucleotide, d(G-G-A-A-G-C-T-T-A-A-C), has been described. The methods, which are herein described, lend themselves to separations on a preparative scale and effect a marked reduction (up to 50%) in the time required for the synthesis of oligonucleotides.

The current methodology for the total synthesis of biologically specific DNA involves (a) the chemical synthesis of short deoxyribopolynucleotides corresponding to the entire two strands and (b) the end-to-end joining of deoxyribopolynucleotides following enzymatic phosphorylation of the 5′-hydroxyl end groups by the use of the polynucleotide ligase (Khorana et al., 1976). Chemical synthesis has so far remained

† From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received September 12, 1977.* This investigation was supported by the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service (Grant No. CA11981), and the National Science Foundation (Grant No. PCM73-06757). This is paper 146 in the series Studies on Polynucleotides. The preceding paper is by Sekiya et al., 1976.

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0006-2960/78/0417-1257$01.00/0