Photobinding of 8-Methoxypsoralen and 5,7-Dimethoxycoumarin to DNA and Its Effect on Template Activity†

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ABSTRACT: 8-Methoxypsoralen (8MOP) in its ground state intercalates with native double-stranded DNA with the molar binding ratio (8MOP/nucleotide) of 1:50 (or 1:25 per base pair). The sequence d(A-T) is the preferential site for intercalation of 8MOP. The maximum number of photobinding of 8MOP to DNA (calf thymus) is 1 8MOP per 26 nucleotides, with an overall quantum yield of 3.7×10^{-3}, based on chemical actinometry. Spectroscopic data, i.e., fluorescence and lifetimes, suggest that the photobinding is heterogeneous. The enzyme digest of the photoadducts yields four different 8MOP-nucleotide photoadducts. The formation of cross-linked photoproduct(s) which involve both 3,4- and 4′,5′-bifunctional groups on the 8MOP molecule is supported by hypochromicity and hydroxylapatite chromatographic studies. The monofunctional coumarin, 5,7-dimethoxycoumarin (DMC), also intercalates with DNA in the dark, the preferential intercalation site being d(A-T) sequences. The CD minimum at 305 nm of the DMC-DNA photoadduct is suggestive of a photocycloaddition type binding. In contrast to 8MOP, DMC photoadds to DNA via its singlet excited state and no cross-linking of the DNA strands is involved. 8MOP-DNA lost 97% of its DNA polymerase template activity, while DMC-DNA lost 50% of the template activity. The former with the same 8MOP/DNA_p ratio as DMC/P lost 90% of its template activity. Thus, cross-linking of DNA by 8MOP is responsible for the difference in template activity inhibition.

Furocoumarins (psoralens) photosensitize skin erythema, skin cancer in animals, bacterial killing, and mutagenicity (see reviews by Giese, 1971; Pathak et al., 1960; Musajo, 1969; see also Song et al., 1975b). The photobiological activity of psoralens has been described in terms of (a) their photocycloaddition reactivity toward pyrimidine bases of DNA (Musajo, 1969, Musajo et al., 1967a,b; Musajo and Rodighiero, 1970; Krämer and Pathak, 1970; Song et al., 1970, 1971, 1975a; Mantulin and Song, 1973) and (b) the cross-linking of DNA strands (Cole, 1970, 1971, 1973; Chandra et al., 1971; Marciani et al., 1972; Shen and Hearst, 1976; Isaacs et al., 1977; Ou et al., 1977).

The aims of the present study were to quantitatively characterize the photoinduced interactions of psoralen (8-methoxypsoralen; 8MOP) with DNA and to examine the effect of such photomodified DNA on the DNA polymerase activity as a possible criterion for the photobiological activity. In addition, we employed 5,7-dimethoxycoumarin (DMC) as a model to ascertain the differential reactivity and photobiological effects of monoadduct vs. cross-links with DNA. DMC is highly lethal in *Bacillus subtilis rec* and *hcr* strains (Harter et al., 1976). Since DMC lacks the furyl C=C double bond necessary for photocycloaddition to pyrimidine bases of DNA, the covalent photoaddition of DMC to DNA cannot involve interstrand cross-linking. Thus, DMC can be compared with 8MOP in evaluating the photobiological implication of DNA cross-link.

Experimental Section

Materials. Crystalline 8-methoxypsoralen (free of fluorescent impurities) from two sources (Professor G. Rodighiero and Sigma Chemical Co.) and 3,4-dihydropsoralen (from Professor Rodighiero) were used as received. [^{3}H]8MOP was custom tritiated by New England Nuclear and further purified with cellulose TLC (specific radioactivity 3.15 Ci/mmol). The molar extinction coefficientof 8MOP in water was determined as 1.24×10^4 at 301 nm. 5,7-Dimethoxycoumarin (DMC) was purchased from Aldrich (ϵ_{327} 1.2×10^4). Poly[d(A-T)] and poly[d(G-C)] were from Boehringer Mannheim Biochemicals. Calf thymus DNA (sodium salt, type V), obtained from Sigma Chemical Co., was further purified before use according to Marmus (1961). *Bacillus subtilis* DNA (sodium salt, grade A from Calbiochem) was used without further purification. Molar extinction coefficients of polydeoxynucleotides are: calf thymus DNA ϵ_p 6620 at 258 nm; poly[d(A-T)] ϵ_p 6500 at 262 nm (Gill et al., 1974); and poly[d(G-C)] ϵ_p 7100 at 255 nm (Pohl and Jovin, 1972). dATP, dGTP, dCTP, dTTP, and DNA polymerase I (*Escherichia coli* B) were from P-L Biochemicals. [^{3}]TTP was from ICN Pharmaceuticals.

Bio-Gel THP for hydroxylapatite column chromatography was obtained from Bio-Rad Laboratories. Deoxyribonuclease I, micrococcal nuclease, spleen phosphodiesterase, snake venom phosphodiesterase, and alkaline phosphatase were obtained from Worthington Biochemical Corp. Ribonuclease A (type 1A) was from Sigma Chemical Co.

Synthesis of 4′,5′-Dihydro- and 3,4,4′,5′-Tetrahydropsoralen. 4′,5′-Dihydropsoralen was synthesized according to Horning and Reisner (1948) and confirmed by NMR. 3,4,4′,5′-Tetrahydropsoralen was prepared from the dihydropsoralen by hydrogenation (H_2/PtO) in 2.5% methanol at 1 atm, 25 °C, for 20 h.

Synthesis of [^{3}H]DMC. The synthesis of [^{3}H]DMC was carried out in T_2O (5 Ci/mL; New England Nuclear) by the acid-catalyzed tritium-exchange method. [^{3}H]DMC was separated and purified by TLC. The purified [^{3}H]DMC was identified by absorption, corrected fluorescence, and excitation spectroscopy (Song et al., 1970, 1971, 1975a,b; Mantulin and Song, 1973). Prior to [^{3}H]DMC synthesis, deuterium-labeled DMC was prepared by the same method as described above. NMR revealed that the exchangeable protons are located at C_4, C_6, and C_8.

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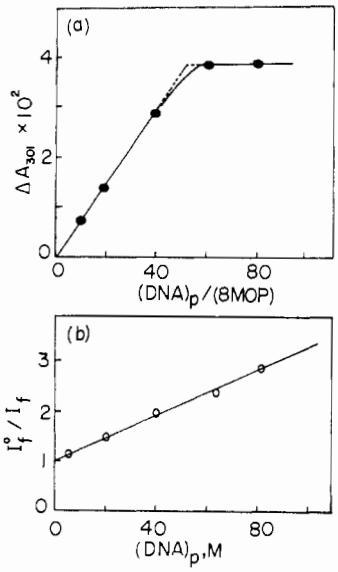

FIGURE 1: (a) The plot of the absorbance decrease at 301 nm against the molar ratio of DNA (p) to 8MOP in various complexes of 8MOP with DNA. (b) The Stern–Volmer plot of fluorescence quenching of DMC by DNA.

Complex Formation between DNA or Polynucleotides and 8MOp or DMC. The complexation studies were carried out spectrophotometrically and spectrofluorometrically as described by Ou et al. (1976).

Determination of Photobinding Ratio. Ten milliliters of 8MOP (230 μM) SSC buffer solution was mixed with 0.2 mL of [^{3}H]8MOP solution (0.2 mCi/mL) and 10 OD units of calf thymus DNA (equimolar concentrations of 8MOP and nucleotides). After deoxygenation by flushing with pure nitrogen for 1 h, the sample was irradiated at 365 nm in a Pyrex container equipped with a filter solution of 10% $CuSO_4$ as described by Ou (1977). The temperature was maintained at 25 °C. The light source was a RUL 3500-Å lamp in a Rayonet preparative photoreactor; actinic intensity was $\sim 6.5 \times 10^{14}$ quanta s^{-1} 10 mL^{-1} or 0.14 W/m^2. After irradiation, the sample was chromatographed on an Ultrogel or a Sephadex G-50 column using 1 M NaCl as the eluting solution. Three-milliliter fractions were collected and each fraction was assayed for its A_{258} and radioactivity. Fractions corresponding to native DNA were collected and added into 2 volumes of cold 95% ethanol (−20 °C) and 0.1 volume of 20% potassium acetate. The photoadduct was spun at 8000 rpm for 10 min and redissolved into 1–3 mL of doubly distilled water or SSC buffer (pH 7.1). The photoadduct was purified by extensive dialysis against doubly distilled water or buffer. Its A_{258} was measured and radioactivity was counted. The same amount of native calf thymus DNA was used as a control for irradiation.

The binding ratio of the 8MOP/nucleotide(p) was calculated from radioactivity data and A_{258} of the purified photoadduct. The amount of bound 8MOP in moles is equal to the radioactivity of the photoadduct (dpm) divided by the specific radioactivity (dpm/mol) of the irradiated sample. The molar concentration of DNA in the photoadduct is obtained from its A_{258} and the molar extinction coefficient.

Preparation of DMC–DNA Adducts. The irradiation and isolation of DMC–DNA adducts[1] were carried out in a manner similar to the irradiation of 8MOP and DNA, as described above.

[1] DMC–DNA and 8MOP–DNA represent photochemically induced covalent adducts.

Optical Measurements of 8MOP–DNA Adducts. All the photoadducts isolated for optical measurements were dialyzed extensively against doubly distilled water at 4 °C. The absorption spectrum and % hypochromicity were measured on a Cary 118C spectrophotometer. Fluorescence emission and excitation spectra were recorded on a Perkin-Elmer spectrofluorometer Model MPF-3. The fluorescence lifetime was measured as described by Fugate and Song (1976). Circular dichroism spectra were recorded on a JASCO J-20 automatic recording spectropolarimeter.

Hypochromicity and Hydroxylapatite Column Chromatography. The photoadduct and control samples were dialyzed against dilute standard saline buffer at 4 °C for 24 h. The solution was diluted to A_{258} ~0.5 with the buffer. The hypochromicity of the photoadduct was calculated on the basis of absorbance measurements.

Hydroxylapatite (0.4 g per column) was suspended in 0.01 M phosphate buffer, pH 6.8, and boiled for 10 min. The column was equilibrated with 0.01 M phosphate buffer at 60 °C in a water-jacketed column for 30 min before use. Heat-denatured *B. subtilis* DNA was used as a reference for single-stranded DNA and native calf thymus DNA for double-stranded DNA. The *B. subtilis* DNA and photoadduct were heat-denatured in 0.01 M phosphate buffer as described previously. All the samples were diluted to 4 mL and applied to the hydroxylapatite column. The samples were eluted with 4 × 4 mL of 0.01–0.30 M phosphate buffer, pH 6.8, in 0.02 M intervals. The A_{258} of each fraction was monitored.

Enzymatic Digest of Photoadducts and TLC. The photoadduct (40 OD_{258}/mL) was dissolved in 1 mL of 0.1 M Tris–0.02 M $MgCl_2$, pH 7.5, containing a mixture of DNase I (2500 units), micrococcal nuclease (5000 units), snake venom phosphodiesterase (0.5 mg), spleen phosphodiesterase (0.5 mg), and alkaline phosphatase (1.5 units) as described by Ho and Gilham (1974). After incubation at 35 °C for 2 h, the enzymatic digest was spotted on a cellulose TLC plate. Both one- and two-dimensional TLC were developed with the solvent mixtures of water–1-propanol (7:3) and water–1-propanol (1:1) vs. water–1-propanol (3:7), respectively. Spots were then visualized on x-ray film by low-temperature fluorography at −70 °C for 1 week.

Assay for DNA Synthesis. Photomodified DNA was assayed for its template activity. The assay measured the incorporation of a labeled nucleotide into an acid-insoluble fraction as described by Fujimura and Roop (1976). The assay mixture (0.3 mL) contained 33 μM dCTP, dATP, dGTP, and dTTP, 20 μCi of [^{3}H]dTTP, 67 mM Tris-HCl (pH 8.6), 67 mM dithiothreitol, 30 nmol of DNA, and 2 units of DNA polymerase I from *E. coli* B (158 units/mg of protein; 1 unit catalyzes the incorporation into acid-insoluble product of 10 nmol of nucleotide under the conditions described by Richardson et al. (1964) using "activated" DNA as the template/primer).

In order to compare the relative importance of monofunctional vs. bifunctional photoadducts, different 8MOP–DNA with different molar binding ratios of 8MOP/nucleotide obtained from various irradiation times were used in this assay. Irradiation of 8MOP and DNA was carried out for 1, 2, 5, 10, and 30 min and 4 h, respectively, for this purpose.

Results

Complex Formation in the Ground State. 8MOP shows hypochromicity at 300 nm as a function of DNA_p, indicative of stacking interactions between DNA base pairs and 8MOP. From Figure 1 showing the absorbance decrease of 8MOP with increasing nucleotide/8MOP ratio, the binding ratio of 1

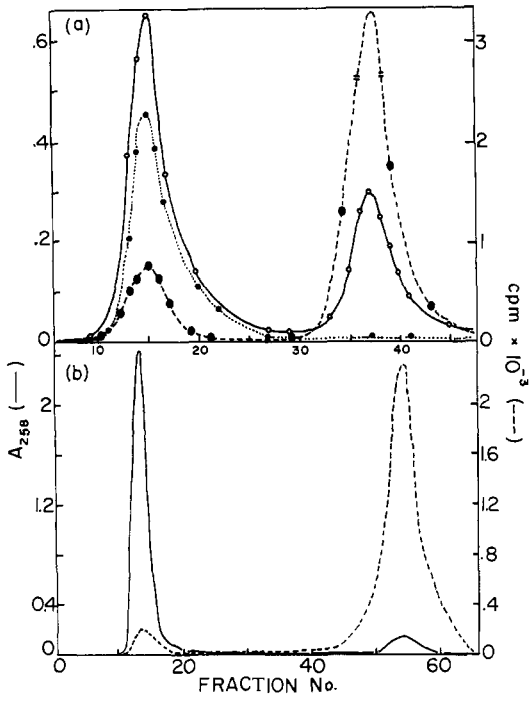

FIGURE 2: (a) Elution profile of the 8MOP-DNA photoadduct on Ultrogel AcA 22 column chromatography. The irradiation time was 8 h. Concentration and condition of irradiation are the same as described under Methods. The flow rate was 18 mL/h and 3-mL fractions were collected and assayed for absorbance at 258 nm (—) and radioactivity (- - -). Native calf thymus DNA was used for reference (• • •). (b) Sephadex G-50 column chromatography of the irradiated solution containing calf thymus DNA and [^{3}H]DMC. The molar ratio of DNA(p)/DMC was 10. Irradiation was carried out as described under Methods. Irradiation time was 8 h. Solvent was SSC buffer (pH 7.1). The concentration of DNA (in moles of nucleotide) was 1.12×10^2 μM. The eluting solvent was 1 M NaCl. The flow rate was 0.5 mL/min and 3-mL fractions were collected and assayed both for radioactivity (• • •) and for absorbance at 258 nm (—).

8MOP per 50 nucleotides has been found. The same ratio of 1:50 was found from the fluorescence measurements, as the fluorescence maximum of 8MOP blue shifts upon complexing with DNA. A Job plot of absorbance change as a function of a fraction of 8MOP also yielded the same value for binding of 1 8MOP/50 nucleotides.

In contrast to native DNA, heat-denatured calf thymus and *B. subtilis* DNA did not show significant complex formation. The structural preference from the intercalation of 8MOP to DNA shows that the d(A-T) sequence is preferred over the d(G-C) sequence (data not shown).

The ground-state complex between DMC and calf thymus DNA was similarly characterized by the absorption and fluorescence spectra (λ_F 453 nm). The λ_{max} at 327 nm shifted to the red and decreased in absorbance with increasing concentration of DNA. A well-defined isosbestic point was found at 351 nm in the series of absorption spectra of DMC at different DNA concentrations. The fluorescence intensity also decreased with the concentration of DNA. The emission λ_{max} did not change, indicating that the complex is nonemitting due to static quenching (i.e., fluorescence lifetime remains unchanged). The % hypochromicity at 327 nm reached a plateau level at $(DMC)/(DNA)_p = 160$. From the Stern-Volmer plot (Figure 1b) the binding constant was calculated to be 2.3×10^{-2} M^{-1}. Complex formation was less favored in higher ionic strength buffers (e.g., standard saline citrate buffer) than in 0.3 mM phosphate buffer. The complex was not detectable between single-stranded DNA and DMC and between DNA and other coumarins.

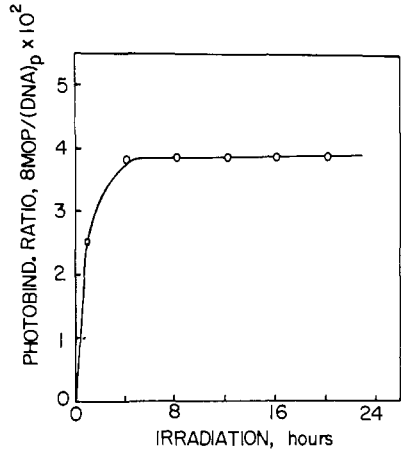

FIGURE 3: Photobinding kinetics of [^{3}H]8MOP to calf thymus DNA. The solutions were prepared as described under Methods. The determination of the molar binding ratio is described therein.

Poly[d(A-T)] and poly[d(G-C)] were also used to characterize the preferential binding site. Complex formation between DMC and poly[d(A-T)] is about twofold greater than between DMC and poly[d(G-C)], as judged from the hypochromicity.

Photobinding of 8MOP and DMC to DNA. Figure 2a shows the elution pattern of 8MOP-DNA from the Ultrogel column. It can be seen that the first band corresponds to DNA and 8MOP-DNA, while the second band is due to free 8MOP and/or its photoproducts. From the [^{3}H]8MOP incorporation in the former, the photobinding to DNA can be estimated, and the photobinding kinetics are shown in Figure 3. The maximum binding (after 4 h of irradiation) corresponds to the 8MOP to nucleotide ratio of 1:26, with a quantum yield of 0.0037. No additional photobinding was observed upon further irradiation of the irradiated solutions after extensive dialysis to remove unreacted 8MOP and 8MOP photoproducts (e.g., dimers) and subsequent resupply of fresh 8MOP to 8MOP-DNA, indicating that the above ratio represents the saturation of photobinding sites in the calf thymus DNA.

The DMC-DNA adduct was similarly isolated. The elution from Sephadex G-50 is shown in Figure 2b. The control samples containing irradiated [^{3}H]DMC or [^{3}H]8MOP plus nonirradiated DNA did not give any radioactivity at the DNA peak. The DNA fraction was further purified by precipitation in ethanol and dialysis against buffer or doubly distilled water, and the radioactivity of the DNA solution was counted.

The photobinding kinetics of DMC to DNA shows a plateau level at 4 h of irradiation time. From the specific radioactivity of [^{3}H]DMC in the reaction mixture, the binding ratio of DMC to DNA nucleotide is 1 molecule of DMC bound per 575 nucleotides in DNA.

Spectroscopic Characterization of 8MOP-DNA and DMC-DNA. The 8MOP-DNA spectra show substantial modifications from the absorption spectrum of free 8MOP, with some absorbance at 300–380 nm (Figure 4). The DMC-DNA also exhibits similar absorption characteristics (spectra not shown).

Table I presents fluorescence characteristics of the photoadducts. Table II lists fluorescence lifetimes of 8MOP, DMC, and their DNA photoadducts. Both 8MOP and DMC show single-component exponential decays. However, 8MOP-DNA show heterogeneity arising from different (8MOP 3,4-, 4′,5′-Thy or Cyt bases) mono-adducts and cross-linked adducts.

TABLE I: Corrected Fluorescence Emission and Excitation Maxima of the Isolated Photoadducts in Water (pH 7) for 8MOP-DNA and in 0.3 mM Phosphate Buffer (pH 7.1) for DMC-DNA at Room Temperature.

Photoadduct (emission)	λ_{ex} (nm)	$\lambda_{F,max}$ (nm)	$\lambda_{F,sh}$ (nm)	Rel intensity[a]
8MOP-DNA	300	395	438	1.0
	320	399	434	1.4
	340	387	426	1.7
DMC-DNA	320	456		1.0
	340	449		1.4
8MOP, free	340	533		1.0
8MOP, intercal	340	485		1.0
DMC, free	340	446		0.65[b]
DMC, intercal	340	446		0.24[b]
Photoadduct (excitation)	**λ_F (nm)**	**$\lambda_{ex,max}$ (nm)**	**$\lambda_{ex,sh}$ (nm)**	
8MOP-DNA	370	291	327	
	400	285	316	
	440	270	332	
DMC-DNA	410	320		
	490	330		

[a] Relative intensity within each group. [b] Quantum yield of fluorescence.

TABLE II: Fluorescence Lifetimes of Photoadducts (Conditions Same as in Table I).

Compd	λ_{ex} (nm)	λ_F (nm)	Lifetime (ns) Phase 10 MHz	Phase 30 MHz	Demodulation 10 MHz	Demodulation 30 MHz
8MOP	350	>470[a]	1.8	1.9		
	350	520[b]	1.9	1.7		
8MOP-DNA	360	386-418[c]	1.1	0.6		
	360	>450[d]	2.0	2.0		
DMC	340	446	7.1	7.2	7.2	7.0
DMC-DNA	320	>450[d]	6.3		7.4	
	340	>450[d]	6.7	3.5	3.5	5.2

[a] 470 LP filter + $NaNO_2$. [b] 520 BP filter + $NaNO_2$. [c] 402.7 BP filter + $NaNO_2$. [d] 450 LP filter + $NaNO_2$.

The CD spectrum of 8MOP-DNA is less conserved than that of the native DNA treated under identical conditions (i.e., irradiated and chromatographed, etc., without 8MOP added), indicating some loss of helicity. Figure 5 shows the CD spectrum of DMC-DNA. The most striking feature is the negative CD band at 300-340 nm.

Interstrand Cross-linking in 8MOP-DNA. From the data presented in Tables I and II, it is clear that 8MOP-DNA is not homogeneous in its structural composition. The interstrand cross-link formed between 8MOP (through 3,4 and 4′,5′ bonds) and DNA strands represents one type of photoadduct contributing to the heterogeneity of the photochemically modified DNA. 8MOP-DNA showed only 0-2% hypochromicity upon heating-rapid cooling cycles, suggesting the occurrence of cross-linking. Hydroxylapatite column chromatography specific for double-stranded DNA confirms this result (Figure 6). Cole (1971) has earlier demonstrated cross-linking of trimethylpsoralen to DNA by the same chromatographic method. No evidence for cross-linking was found in DMC-DNA, as expected from the lack of a 4′,5′ bond in DMC.

Effects of 8MOP and DMC Binding on DNA Polymerase.

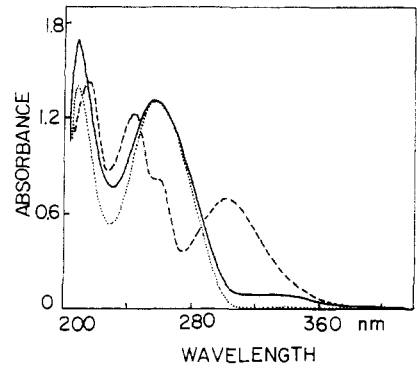

FIGURE 4: Absorption spectra of the 8MOP-DNA photoadduct (—) and control DNA (···). The photoadduct was isolated and purified as described under Methods. Irradiation time was 6 h at 365 nm. Solvent was doubly distilled water (pH 6.8). The absorption spectrum of 8MOP in doubly distilled water at room temperature (- - -).

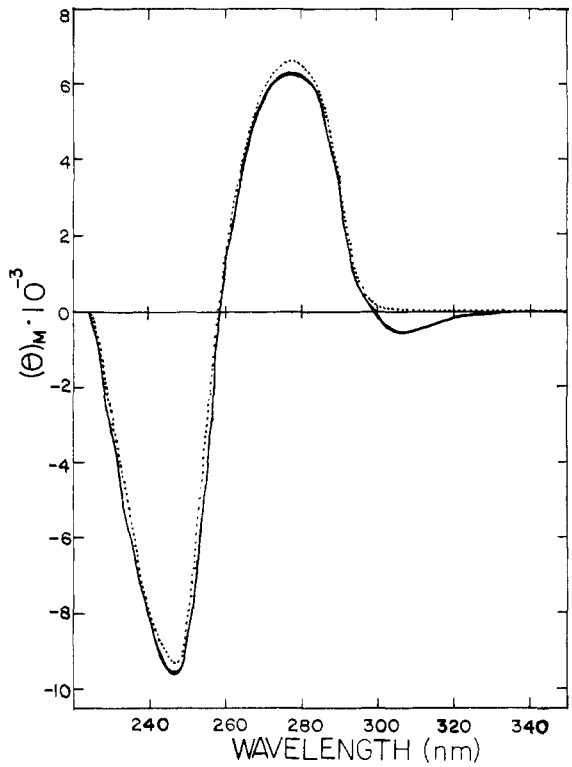

FIGURE 5: CD spectra of the DMC-DNA photoadduct (—) and control DNA (···). The DMC-DNA photoadduct was prepared as described under Methods.

In order to establish a simple assay for the photobiological activity of various skin-sensitizing and carcinogenic coumarins, we employed the mutagenicity and killing of *Bacillus subtilis* (Harter et al., 1974, 1976; Song et al., 1975b). To ascertain the role of cross-links vs. monoadducts, the template activity of photomodified DNA was tested with respect to DNA replication as catalyzed by DNA polymerase. 8MOP-DNA has only about 3% activity (Figure 7). DMC-DNA also shows a significant inhibition of the polymerase activity. The template activity of 8MOP-DNA is considerably lower than that of DMC-DNA at approximately the same sensitizer to nucleotide ratio. Figure 7 further illustrates the effectiveness of 8MOP-induced photomodification of DNA in inhibiting the polymerase activity even at the binding ratio of only about 1:1500. In these experiments, the control DNA was processed in the same way as the photoadducts (irradiation, chromatographic procedures, etc.) without light.

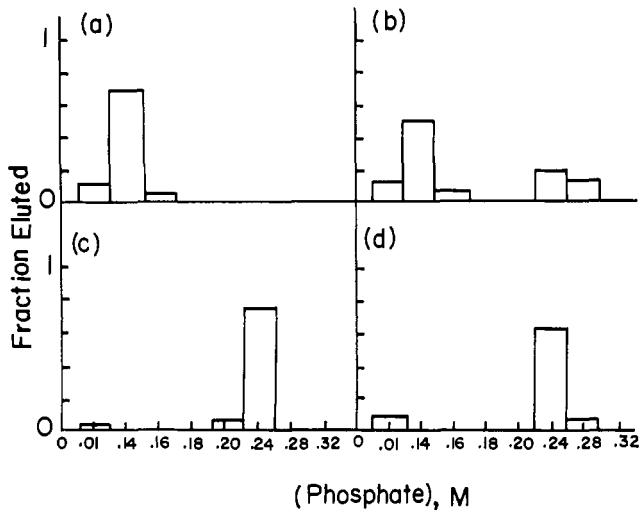

FIGURE 6: Elution profiles of hydroxylapatite column chromatography. Temperature of the column was controlled at 60 °C with a water-jacketed column and circulating water bath. (a) Heat-denatured *B. subtilis* DNA, (b) heat-denatured control calf thymus DNA, (c) native calf thymus DNA, and (d) 8MOP-DNA (calf thymus).

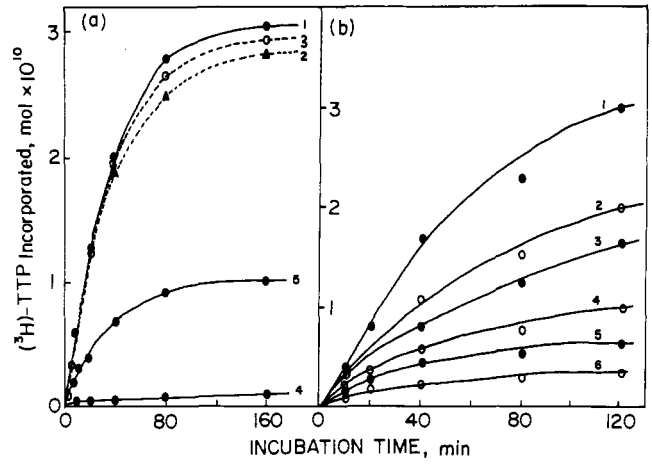

FIGURE 7: (a) The template activity of (1) native calf thymus DNA, (2) 4-h irradiated DNA, (3) ground-state complex of 8MOP and DNA with molar ratio of 8MOP equal to 1:40, (4) 8MOP-DNA, and (5) DMC-DNA. The irradiation time was 4 h for 8MOP-DNA and 8 h for DMC-DNA. All samples were in SSC buffer. (b) The template activity of 8MOP-DNA depending on the irradiation time: (1) 0 and 0.5 min (0% cross-linked DNA); (2) 1 min (photobinding ratio of 8MOP to DNA_p; 1:1500 and 10% cross-link); (3) 2 min (46% cross-link); (4) 5 min (50% cross-link); (5) 10 min (photobinding ratio 1:1500 and 88% cross-link); and (6) 30 min (photobinding ratio >1:1500 and ~100% cross-link).

Discussion

The action spectra for the photosensitizing action and photoreactions of psoralens with DNA are closely correlated (Musajo et al., 1974). The present study bears on this correlation in terms of 8MOP-DNA photobinding characteristics and effects on DNA synthesis in vitro.

Characteristics of Photobinding Kinetics and Products: 8MOP-DNA. Cole (1970, 1971, 1976) and Johnston et al. (1977) discussed various reaction schemes for the sequence of photochemical cross-link reactions of DNA strands with 4,5′,8-trimethylpsoralen and its repair. From the data shown in Figure 1, 8MOP·DNA intercalation complexes are formed in the ratio of 1 8MOP/50 nucleotides. This binding ratio is in the same range as for other psoralens under different conditions (Isaacs et al., 1977). The covalent binding ratio is 1:26, as induced by near-UV irradiation (Figure 3). The enhanced binding ratio over the intercalation complex can be accounted for either by the excited-state equilibrium for the complex in analogy to the excited state complexation equilibrium involving proflavine and GMP (Georghiou, 1977; Badea and Georghiou, 1976) or by additional intercalation of 8MOP with initial photoadducts. We propose the following reaction scheme in favor of the latter under strictly anaerobic conditions[2]

$$\text{DNA} + n\text{8MOP} \overset{K}{\rightleftharpoons} (\text{8MOP})_m\cdot\text{DNA}$$

$$(\text{8MOP})_m\cdot\text{DNA} \xrightarrow{h\nu} (\text{8MOP*})_m\cdot\text{DNA}$$

$$(\text{8MOP*})_m\cdot\text{DNA} \rightarrow (\text{8MOP})_m\text{-DNA}$$

$$(\text{8MOP})_m\text{-DNA} + j\text{8MOP} \rightarrow (\text{8MOP})_m\text{-DNA}\cdot(\text{8MOP})_j$$

$$(\text{8MOP})_m\text{-DNA}\cdot(\text{8MOP})_j \xrightarrow{h\nu} (\text{8MOP})_m\text{-DNA}\cdot(\text{8MOP*})_j$$

$$(\text{8MOP})_m\text{-DNA}\cdot(\text{8MOP*})_i \rightarrow (\text{8MOP})_{m+i}\text{-DNA}$$

where the dots and hyphens represent noncovalent and covalent bonds, respectively. "Nonproductive" reactions include photodimerization and decomposition of 8MOP itself, yielding at least seven photoproducts (two nonfluorescent and five fluorescent products on silica gel F TLC; autoradiogram solvent, cyclohexane-ethyl acetate, 1:2; J. Jung and P. S. Song, unpublished results).

To characterize the structure of the photoadduct $(8MOP)_{m+i}$-DNA, it is necessary to distinguish three possible products, notwithstanding different base adducts and stereoisomers. These are the 8MOP-3,4-, and 8MOP-4′,5′-monoadducts (with a pyrimidine base, most likely Thy) and the cross-linking adduct (Bordin et al., 1969; Dall'Acqua et al., 1978). Since dihydrocoumarin (λ_{max} 275 nm, $\lambda_{F,max}$ 313 nm; Mantulin and Song, 1973), 4′,5′-dihydrofurocoumarin (λ_{max} 335 nm, $\lambda_{F,max}$ 400 nm, with half bw 60 nm), 3,4-dihydrofurocoumarin (λ_{max} 289, $\lambda_{F,max}$ 318 nm, with half bandwidth 37 nm), and DMC-DNA (Tables I and II) are fluorescent, we suggest that all major photoadducts of 8MOP with DNA bases are fluorescent with varying quantum yields, depending on λ_{ex}. The spectral data shown in Figure 4 and Tables I and II provide indirect evidence that the 4′,5′-monoadduct does not correspond to the species with the fluorescence lifetime of 0.6–1.1 ns, as this lifetime is too short to be attributed to a 4′,5′-type adduct (Table II). The weak, short fluorescent lifetime species with $\lambda_F < 450$ may be assigned to a 3,4- or 4′,5′-monoadduct (minor isomer) or at a fluorescence quenching site. However, the former is less likely, as its fluorescence intensity would be very weak upon excitation at 360 nm which is not significantly absorbed by 3,4-dihydropsoralen. The fluorescent species predominant at $\lambda_F > 450$ nm may be assigned to the 4′,5′-adduct, on the basis of its longer lifetime (2 ns, Table II) and fluorescence excitation polarization spectrum (not shown). 4′,5′-Dihydrofurocoumarin shows λ_{max} 335 nm in coincidence with the corrected excitation maximum. Thus, the major excitation spectrum can be assigned to 4′,5′-monoadduct (see also Dall'Acqua et al., 1978). Cross-link adducts are also expected to be fluorescent, since 3,4,4′,5′-tetrahydrofurocoumarin fluoresces at $\lambda_{F,max}$ 280–300 nm. However, the cross-adduct

[2] Serious complications (e.g., chain breaks) in kinetic and product distribution patterns arise if oxygen is present to any significant extent.

fluorescence cannot be effected in the isolated 8MOP–DNA because tetrahydrofurocoumarin does not absorb at $\lambda > 310$ nm ($\lambda_{max} \sim 263$ nm which is completely masked by the DNA absorption).

It is not possible to determine relative yields of mono- vs. cross-link photoadducts from the spectral data alone. However, we estimate the cross-link adduct to be the major product under our saturating conditions leading to the 1:26 photobinding ratio. This estimate is based on the initial quantum yield of 0.015 (based on light absorbed by all 8MOP present) or 0.77 (corrected for the fraction of light absorbed by free nonintercalated 8MOP) for monoadduct formation[3] and the overall quantum yield of 0.0037 (based on light absorbed by all 8MOP initially present)[4] or 0.19 (corrected for nonintercalated 8MOP). If we assume that the decrease in quantum yield as a function of irradiation time is largely due to intramolecular photoreactions (i.e., cross-linking of monoadducts initially produced; Musajo et al., 1966), the ratio of 0.19:(0.77–0.19) then represents an upper limit (65%) for the cross-link formation when binding is saturated. This is considerably higher than the previous estimate (20–40%; Cole, 1971) under different conditions from ours and than the computer-simulated kinetic data (Dall'Acqua, 1977).[5]

Enzyme digestion of 8MOP–DNA yielded two fluorescent 8MOP–nucleotide adducts (R_f 0.83, 0.98) on one-dimensional TLC, which were resolved into four different fluorescent spots on two-dimensional TLC [relative intensities in parentheses, R_f 0.37 (strong), 0.41 (weak), 0.51 (weak), 0.80 (medium)[6]]. Further chemical identification of the base was not possible, as the photoadducts were not dissociated into 8MOP and pyrimidine nucleotides upon photoirradiation with the triplet sensitizer, sodium 2-anthraquinonesulfonate, following the method of Ben-Hur and Rosenthal (1970) and Lamola (1972).

In addition to the mono- and cross-linking photoaddition to Pyr bases of DNA, the secondary structure of DNA is significantly altered, as suggested from the CD spectrum.

DMC–DNA. There are at least two different products in the DMC–DNA photoadducts with relatively small spectral differences ($\tau_F > 6$ and <3 ns with λ_F 449 and 456, respectively) as summarized in Tables I and II. These photoadducts are clearly of the cycloaddition type, since the strong CD spectrum (Figure 5) in the region of 300–340 nm is attributable to the weak absorption by optically active cyclobutane adduct(s) of stereospecific origin (cis- or trans-syn or anti isomers). It should be noted that the intercalated DMC showed no induced CD in the near-UV region. The chemical identity of the two major products is not known, but these products may represent stereoisomers or DMC< >Thy and DMC< >Cyt monoadducts, the former being more favorable due to preferential intercalation of DMC at the d(A-T) sequence (vide supra).

Oxygen (140 μM) reduces the overall quantum yield for the photoaddition of 8MOP to DNA from 3.7×10^{-3} to 1.4×10^{-3}, suggesting that the triplet state of 8MOP is involved in the photoaddition. There also seems to be some correlation between the singlet quenching and photobinding yield (10 mM KI quenches 16% of the fluorescence of intercalated 8MOP, with $k_q = 4.2 \times 10^9$ M^{-1} s^{-1}, and 16% of the photobinding, 50 mM KI ~35% and 30% quenching, respectively), but this result cannot be unambiguously interpreted in terms of singlet 8MOP alone, as the quencher KI enhances the triplet population. Thus, we conclude that both singlet and triplet states of 8MOP are likely to participate in the photobinding of 8MOP to DNA. On the other hand, the DMC–DNA is likely to involve singlet DMC as the major reactive species (vide supra), as suggested by the fact that singlet DMC is the reactive species in the photodimerization of DMC (Shim et al., 1977; see also Harter, 1974; Leenders et al., 1973; Gervais and De Schryver, 1975).

Template Activity of 8MOP–DNA and DMC–DNA. From Figure 7, it is clear that both monoadducts and cross-links of 8MOP–DNA drastically reduce the DNA polymerase template activity. Since 10% cross-linked DNA plus 90% non-cross-linked DNA (1 8MOP/1500 nucleotides) show 33% inhibition of the enzyme activity, the monoadduct(s) is responsible for at least 23% of the inhibition, assuming that the cross-linked DNA inhibits 100%. As expected, the fully cross-linked DNA is almost completely inactive (Figure 7). DMC–DNA is also inhibitory, but its inhibitory activity is less than that of the 8MOP–DNA at the same binding ratio. This difference can be partly explained by the presence of the cross-linking in the latter. Thus, we conclude that, in addition to the cross-links, both DMC–DNA and 8MOP–DNA monoadducts are photobiologically damaging in affecting DNA synthesis. The photobinding of 8MOP and DMC also inhibits the transcriptase activity (Tapley and Song, to be published).

Concluding Remarks. The present study reveals (a) a definite binding stoichiometry and preferential sites in 8MOP– and DMC–DNA, (b) far more complexity of the photoaddition reaction in terms of spectroscopic and chromatographic characteristics of skin sensitizer–DNA photoadducts hitherto recognized, and (c) significant loss of the template activity of 8MOP– and DMC–DNA (both monoadducts and cross-link adducts). These results are useful in evaluating the molecular mechanism of skin sensitization, carcinogenicity and photomutagenicity of psoralens, as well as in interpreting photolabeling of psoralens as a probe for DNA and RNA structure in molecular biological studies. In this connection, Seki et al. (1977) showed that the psoralen monoadducts are responsible for mutation of *E. coli*, while the cross-links are lethal.

[3] The cross-link yield is negligible after a 60-s irradiation, yielding the photobinding ratio of 1:1500 (≃ monoadducts) and 10% of DNA cross-linked (see also Johnston et al., 1977).

[4] This is of the same magnitude as reported by Musajo et al. (1966).

[5] In most previous studies, O_2 was not excluded from photolysis reaction mixtures, possibly contributing to different photoreactions and yields due to participation of singlet oxygen (Poppe and Grossweiner, 1975).

[6] See Methods for details of the enzymatic digest

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