Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, S. H. (1977) *Nucleic Acids Res. 4*, 2811–2820.
Jack, A., Ladner, J. E., & Klug, A. (1976) *J. Mol. Biol. 108*, 619–649.
Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–328.
Johnston, P. D., & Redfield, A. G. (1978) *Nucleic Acids Res. 5*, 3913–3927.
Johnston, P. D., & Redfield, A. G. (1981) *Biochemistry 20*, 1147–1156.
Kearns, D. R., & Shulman, R. G. (1974) *Acc. Chem. Res. 7*, 33–39.
Leontis, N. B., & Moore, P. B. (1986) *Biochemistry 25*, 3916–3925.
Noggle, J. H., & Shirmer, R. E. (1971) *The Nuclear Overhauser Effect*, Academic Press, New York.
Quigley, G. L., Wang, A. H. J., Seeman, N. C., Suddath, F. L., Rich, A., Sussman, J. L., & Kim, S. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4866–4870.
Quigley, G., Teeter, M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68.
Redfield, A. G. (1986) *NATO ASI Ser. 107*, 1.
Reid, B. R. (1981) *Annu. Rev. Biochem. 50*, 969–996.
Rich, A., & RajBhandary, U. L. (1976) *Annu. Rev. Biochem. 45*, 805–860.
Roy, S., & Redfield, A. G. (1983) *Biochemistry 22*, 1386–1390.
Roy, S., Papastravros, M. Z., & Redfield, A. G. (1982) *Nucleic Acids Res. 10*, 8341–8349.
Roy, S., Papastravros, M. Z., Sanchez, V., & Redfield, A. G. (1984) *Biochemistry 23*, 4395–4400.
Sampson, J. R., & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033–1037.
Sanchez, V., Redfield, A. G., Johnson, P. D., & Tropp, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5959–5662.
Sprinzl, M., & Gauss, D. (1984) *Nucleic Acids Res. 12*, r1–r131.
Sundaralingam, M. (1978) *Acta Crystallogr. B34*, 1529.
Sussman, J. L., Holbrook, S. R., Wade-Warrant, R., Church, G. M., & Kim, S. H. (1978) *J. Mol. Biol. 123*, 607–630.
Tropp, J., & Redfield, A. G. (1981) *Biochemistry 20*, 2133–2140.
Wrede, P., Wurst, R., Vournakis, J., & Rich, A. (1979) *J. Biol. Chem. 254*, 9608–9616.

Secondary Structure of DNA Modified by Monofunctional Psoralen Derivatives†

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ABSTRACT: Monofunctional psoralens such as 3-carbethoxypsoralen (3-CPs), 7-methylpyrido[3,4-*c*]psoralen (MePyPs), or 7-methylpyrido[4,3-*c*]psoralen (2N-MePyPs) are generally less genotoxic than the bifunctional ones presently used in the photochemotherapy associated with UV-A light (PUVA therapy). In spite of the structural similarities of their respective monoadducts, these compounds widely differ in their photobiological effects. In this paper we compare the local structure of the alterations that are respectively induced within DNA by 3-CPs, MePyPs, and 2N-MePyPs in a first attempt to explain these photobiological differences from a conformational point of view. The internal location of the monoadducts with respect to the helix does not depend on the chemical structure of the derivatives and is consistent with a cis stereochemistry. In contrast, sterical effects related to the chemical structure of the bound residues seem to play a major role in the DNA secondary structure in the vicinity of the alterations. The single-strand-specific S_1 endonuclease, which is used as a probe of the local denaturation around each monoadduct in DNA, does not hydrolyze the MePyPs-induced alterations. In contrast, the photobinding of one 3-CPs or one 2N-MePyPs molecule locally induces the destruction of about 7 base pairs as detected by the sensitivity of the respective modified DNA toward the S_1 endonuclease. Such disruption suggests a possible classification of the monofunctional psoralen derivatives related to the DNA conformation around their monoadducts.

The photochemotherapy of some skin diseases (psoriasis, micosis fungoid, vitiligo) using psoralens such as 8-methoxypsoralen (8-MOP)[1] or 4,5′,8-trimethylpsoralen (TMP) associated with UV-A light (PUVA) has been widely stimulated for the past two decades [for a review, see Pathak et al. (1981)]. A large number of photochemical studies have been developed to understand, at the molecular level, the mechanism of action of these photosensitizing derivatives [for a review, see Song and Tapley (1979) and Averbeck (1984)]. The photobiological properties of psoralens have been partly related

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[1] Abbreviations: 3-CPs, 3-carbethoxypsoralen; MePyPs, 7-methylpyrido[3,4-*c*]psoralen; 2N-MePyPs, 7-methylpyrido[4,3-*c*]psoralen; 8-MOP, 8-methoxypsoralen; TMP, 4,5′,8-trimethylpsoralen; 5-MOP, 5-methoxypsoralen; 4′,5′-DHCPs, 4′,5′-dihydro-3-carbethoxypsoralen; Thy, thymine; dThd, thymidine; dThd<$^{5'4'}_{5'3'}$>3-CPs, C_4 cycloadduct of 3-CPs to dThd involving the 4′,5′-furan ethylenic bond and the 5,6 pyrimidine bond of Thy; UV-A, ultraviolet light of class A (320–400 nm); bp, base pairs; CL, interstrand DNA cross-links; RFI, relative fluorescence intensity.

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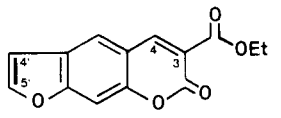
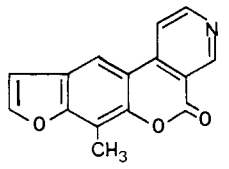
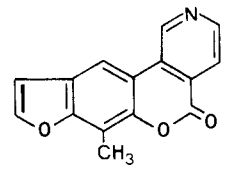


3-carbethoxypsoralen (3-CPs)



7 - methyl, pyrido [3,4-c] psoralen (MePyPs)



7 - methyl, pyrido [4,3-c] psoralen (2N-MePyPs)

FIGURE 1: Chemical structures of 3-CPs, MePyPs, and 2N-MePyPs.

to their ability to photoreact with the nucleic bases upon UV-A exposure (Musajo et al., 1965; Musajo & Rodighiero, 1970, 1972). This photoreaction involves a C_4-cycloaddition reaction with the 5,6 double bond of the Thy residues. Due to their two photoreactive site (3,4 and 4′,5′ double bonds, Figure 1) two classes of photoaddition products can be formed: monoadducts on each of these two double bonds and biadducts involving both sites of the molecule and giving rise to interstrand DNA cross-links (CL) [for recent reviews on chemical structure of DNA photoadducts, see Hearst et al. (1984) and Vigny et al. (1985)]. It is generally admitted that CL are more cytotoxic than monoadducts. This justifies the wide use of bifunctional derivatives as antimitotic agents for PUVA therapy. Moreover, CL constitute premutagenic alterations, which could explain the carcinogenicity in mouse of bifunctional compounds such as 8-MOP and 5-MOP (Zajdela & Bisagni, 1981; Urbach et al., 1982).

The search for purely monofunctional psoralen derivatives has constituted an interesting approach that has pointed out the cytotoxicity of monoadducts and their potential interest in photomedicine. 3-Carbethoxypsoralen (3-CPs, Figure 1) appears as a precursor in the synthesis of the first generation of monofunctional psoralens (Queval & Bisagni, 1974). 7-Methylpyrido[3,4-*c*]psoralen (MePyPs, Figure 1), which corresponds to the second generation (Moron et al., 1983), exhibits an activity for clearing psoriatic lesions similar to that observed with 8-MOP as shown from preliminary clinical observations (Averbeck et al., 1983). More recently, a structural isomer of MePyPs, namely, 7-methylpyrido[4,3-*c*]psoralen (2N-MePyPs, Figure 1) was also studied to determine the photochemical and photobiological effects of the nitrogen heteroatom position in the pyridinic ring (Blais et al., 1987).

In a previous work, the main DNA–3-CPs photoadducts have been identified as the two cis–syn diastereoisomers dThd$<^{54'}_{65'}>$3-CPs C_4 cycloadducts involving the intercalation of the psoralen residue within the DNA helix (Moysan et al., 1986; Gaboriau et al., 1987). Structural data concerning the furan-side monoadducts of MePyPs with DNA suggest that this compound photoreacts with the same target (Thy residues) and leads to the same cis–syn stereochemistry (Moysan, 1987, and unpublished results).

The aim of this conformational study is the comparison of the alterations respectively induced in the DNA helix by 3-CPs, MePyPs, and 2N-MePyPs. Location and mobilities of their various monoadducts with respect to the helix have been respectively investigated by fluorescence quenching experiments and anisotropy measurements. The sensitivities of modified DNA toward the single-strand-specific S_1 endonuclease (from *Aspergillus orizae*) was used as a probe of the DNA secondary structure in the vicinity of their alterations.

Materials and Methods

Chemicals. Preparation procedures of 3-carbethoxypsoralen and 4′,5′-dihydro-3-carbethoxypsoralen (Queval & Bisagni, 1974), 7-methylpyrido[3,4-*c*]psoralen (Moron et al., 1983), and 7-methylpyrido[4,3-*c*]psoralen (Blais et al., 1987) were previously described. The adduct used as a reference for fluorescence quenching experiments was obtained from the photoreaction between 3-CPs and dThd according to a method previously reported (Cadet et al., 1983). Salmon sperm DNA (type III) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The single-strand-specific S_1 endonuclease from *A. orizae* was purchased from Biochemical.

Preparation of the Modified DNAs. The photoadducts between DNA and 3-CPs, MePyPs, or 2N-MdPyPs were prepared as previously described (Gaboriau et al., 1981; Blais et al., 1984). Extensive dialysis against a Tris–NaCl buffer (5×10^{-3} M, pH 7.5) was performed to remove the noncovalently bound products. DNA modified samples, which were finally kept in a Tris–NaCl buffer (10^{-3} M, pH 7.5), exhibited a hypochromicity higher than 35%. All these purely monofunctional psoralen derivatives form only monoadducts in DNA involving their 4′,5′ double bonds (Blais et al., 1984, 1987; Moysan et al., 1986; Gaboriau et al., 1987).

3-CPs photobinding rates were measured by counting aliquots of 100 μL of modified DNA (0.5 mg·mL^{-1}) deposited on a Whatman fiber disk (GF/A). The fluorescence of the scintillator (NE-220 from Nuclear Enterprise Ltd.) was detected on a Packard liquid scintillation spectrometer (Model Tri-carb). MePyPs and 2N-MePyPs photobinding rates were estimated by spectrophotometric measurements, taking the molar extinction coefficients previously reported as reference (Blais et al., 1984, 1987). All experiments performed in this study were made at similar photobinding rates for each type of furan-side monoadduct. The extent of binding of 3-CPs, MePyPs, and 2N-MePyPs to DNA, obtained for the same dose of UV-A light absorbed by complexed molecules (3 KJ·m^{-2}), was respectively equal to 2, 10, and 2 per 1000 base pairs.

Fluorescence Anisotropy Measurements. Steady-state fluorescence anisotropy measurements were performed with a spectrofluorometer built in the laboratory (Vigny & Duquesne, 1974) using a monochromatic excitation light (λ_{exc} = 334 nm) vertically polarized. Fluorescence emission intensities were detected through an analyzer oriented parallel ($I_\parallel$) or perpendicular ($I_\perp$) to the direction of polarization of the excitation light. Corrections from polarization caused by instrumental factors were calculated by measuring the apparent polarization of the fluorescence from a solution of diphenylhexatriene (DPH) in hexane, which presents a complete depolarization at room temperature (Shinitzky & Barenholz, 1974). The ratio between parallel ($I_\parallel'$) and perpendicular ($I_\perp'$) components of its fluorescence excited with a horizontally polarized excitation beam gave the relative transmission value of the analysis monochromator (T). The corrected polarization

and the fluorescence anisotropy were then respectively calculated according to

$$P = (1 - T)(I_\perp/I_\parallel)/(1 + T)(I_\perp/I_\parallel)$$

$$r = 2P/(3 - P)$$

Fluorescence Quenching Experiments. Various potassium iodide concentrations were obtained by successively adding 20-μL aliquots of a 2 M potassium iodide solution to 1 mL of the DNA–3-CPs solution contained in a quartz cuvette (0.5 mg·mL^{-1} Tris–NaCl buffer 5×10^{-3} M, pH 7.5). After each addition, the solution was gently stirred and the characteristic fluorescence spectrum of the 4′,5′ monoadduct was recorded (λ_{exc} = 334 nm). The blank was subtracted, and the dilution factor was taken into account. The relative fluorescence intensity (RFI) was further obtained by integrating the signal under the spectrum in the range 390–520 nm. Comparison of the fluorescence intensity, F, observed in the presence of a quencher concentration [Q] to that obtained without quencher, F_0, gave a measure of the quenching efficiency by the iodide ions. According to the Stern–Volmer equation, F_0/F (and τ_0/τ) = $1 + K_{SV}[Q]$, a linear relationship between F_0/F and [Q] can be understood in terms of a dynamic quenching process in which $K_{SV} = k_Q\tau_0$, where k_Q is the bimolecular reaction rate constant and τ_0 is the fluorescence lifetime of the chromophore when no quencher is added. From the measure of the slope K_{SV} and of τ_0 (in an independent experiment), k_Q can be deduced and gives information on the accessibility of the chromophore within the DNA helix.

Contribution of the K^+ cation in the quenching process was checked with potassium chloride (KCl) in a similar way.

Kinetics Analysis of the S1 Nuclease Hydrolysis. (*a*) *Principle of the Enzymatic Assay.* The single-strand-specific S_1 endonuclease from *A. orizae* (Ando, 1966) is able to recognize structural distortions induced in UV-B-irradiated DNA (Shishido & Ando, 1974) and DNA chemically modified by *N*-acetyl-2-aminofluorene (Yamasaki et al., 1977; Fuchs, 1975). This enzyme has been shown to specifically hydrolyze the C_8 adduct from AAAF while the N_2 adduct remains unsensitive to S_1. Both monoadducts from the photoreaction of 5-methylisopsoralen with DNA- and TMP-induced cross-links were resistant to S_1 nuclease (Cleaver et al., 1985). The S_1 nuclease exhibits a low but constant hydrolytic activity with duplex DNA (0.05 μg·h^{-1}·unit^{-1}).

A calibration curve was obtained in a first step from the action of S_1 on the unmodified denatured DNA. It showed a linear relationship between the initial rate of the S_1 hydrolysis (V_i) and the denatured bp concentration. It was further used to estimate, from the V_i values obtained with 3-CPs-modified DNAs, the concentration of denatured bp induced by the 3-CPs monoadducts.

(*b*) *Preparation of Denatured DNA Samples.* Heat-denatured DNA samples with various concentrations of denatured bp were prepared as follows. Unmodified DNA was dissolved in a Tris–NaCl buffer (5×10^{-3} M, pH 7.5) at a nucleotidic concentration of 3×10^{-3} M and thermically denatured by heating 10 min at 95 °C. After cooling, its percentage of hypochromicity was 37%. Various dilutions were operated in the S_1 nuclease buffer (0.03 M sodium acetate, 0.05 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6). An intrastrand folding occurs at this ionic strength (Studier, 1969), leading to a relative decrease of the optical density at 258 nm. Assuming that a completely denatured DNA exhibits 40% hypochromicity, the percentages of hypochromicity measured in these conditions (30%) involved a folding of 35% of the denatured DNA samples.

(*c*) *Hydrolysis and Kinetics Analysis.* A spectrophotometric method was used to monitor the S_1-induced hydrolysis of the denatured DNA samples. The enzymatic reaction was initiated by adding 100 units of S_1 nuclease to 1 mL of the denatured DNA samples (Θ = 45 °C). During the hydrolysis process, the separation of the intact DNA from the hydrolysis products was achieved by exclusion chromatography (Sephadex G-25). Such an analysis has clearly demonstrated that the molar extinction coefficient of the base at 258 nm is higher in the hydrolyzed DNA (ϵ_{di}) than that in the native (ϵ_n) or in the denatured DNA (ϵ_d). This relative hyperchromicity of the bases in the hydrolyzed DNA, which has been previously reported (Szyfter & Kedzierski, 1976), explains the increase of the optical density at 258 nm observed during the hydrolysis process in the whole DNA hydrolysates. Such an increase is proportional to the concentration of the hydrolyzed DNA and provides a convenient method to monitor the hydrolysis kinetics. The absorption of the hydrolysate contained in a closed quartz cuvette was averaged on a 30-s time period (two measurements per second) at a controlled temperature (Θ = 45 °C). The approximative value of ϵ_{di} ($\epsilon_{di} \approx 1.7\epsilon_n$) obtained from the literature (Voet et al., 1963) has been verified by using native and partially denatured DNA sample ($\epsilon_{di} = 1.85\epsilon_n$).

(*d*) *Calibration Curve.* A calibration curve was obtained by plotting the initial rate values V_i as a function of the denatured bp concentration. The V_i values themselves were determined from the slope of the kinetics at the beginning of the enzymatic reaction. For denatured DNA concentrations lower than 10^{-5} M, V_i is proportional to the single-strand DNA concentration. The enzymatic reaction is thus first order with respect to the substrate, and the S_1 nuclease does not exhibit any cooperativity (calibration curve). As expected from Michaelian enzymatic kinetics, V_i reaches a maximal value (V_m) reflecting the saturation of the enzyme for higher substrate concentrations.

(*e*) *Measurement of the Single-Strand DNA Concentration of the 3-CPs-Modified DNA.* The calibration curve was used in this part to estimate (from the V_i measurements) the number of denatured bp induced by the photobinding of each 3-CPs molecule. Such estimation was performed at various binding rates. These were obtained by irradiating the [^{3}H]-3-CPs–DNA complexes with increasing doses of UV-A light (365 nm; incident dose, 15 J·m^{-2}·s^{-1}). 3-CPs-modified DNAs were purified by two precipitation steps using ethanol (Gaboriau et al., 1987). The resulting 3-CPs–DNA pellets were then solubilized in the S_1 nuclease buffer, and 100 units of nuclease was added to 1 mL of each DNA sample contained in a closed quartz cuvette. For each irradiation time the initial rate of the S_1-induced hydrolysis reactions was measured by averaging three to four kinetics measurements (Θ = 45 °C). The number of denatured bp was deduced from the V_i values by using the calibration curve. This number was then corrected from the photobinding rate to estimate the denaturation extent per each photobound molecule (number of denatured bp per alteration). Such an estimation is supported, assuming that the kinetics constants of the S_1-induced hydrolysis reaction are not modified by the 3-CPs monoadducts.

Results

Location of the 3-CPs, MePyPs, and 2N-MePyPs Monoadducts with the Helix. (*a*) *KCl Effect.* Due to their high fluorescence quantum yield, the fluorescence emissions of these three furan-side monoadducts (Table I) appear as convenient markers to determine their locations with respect to the helix. The effects of the K^+ concentration on the RFI values, ob-

Table I: Spectroscopic Properties of the Furan-Side DNA Monoadducts from the Photoreaction with 3-CPs, MePyPs, and 2N-MePyPs

DNA–monoadduct	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	ϕ_f	$^1E(o\text{–}o')$ (μm^{-1})
DNA–3-CPs[a]	360	420	0.06	2.58
DNA–MePyPs[b]	340	420	0.02	2.65
DNA–2N-MePyPs[c]	370	462	0.02	2.37

[a] Gaboriau et al. (1981). [b] Blais et al. (1984). [c] Blais et al. (1987).

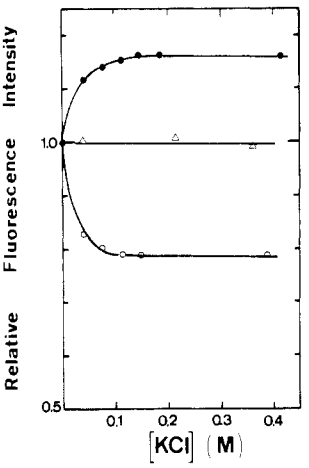


FIGURE 2: Effects of the KCl concentration on the relative fluorescence intensity (RFI) of the monoadducts from the photoreaction of DNA with 3-CPs (○), MePyPs (△), and 2N-MePyPs (●). RFI was measured by integrating the fluorescence emission signal (λ_{exc} = 334 nm) in the spectral range 390–550 nm. The value obtained without KCl(Tris–NaCl buffer 10^{-3} M, pH 7.5) was taken as a reference.

tained by successive additions of KCl (no quenching effect due to Cl^-), are reported in Figure 2.

The stabilizing effect of the cation K^+ on the DNA secondary structure of a 3-CPs-modified DNA has been previously described (Gaboriau et al., 1987). Interaction of the K^+ ion with phosphate residues results in a lowering of the intrastrand electrostatic repulsion and thus in a stabilization of the double-strand form of DNA (Manning, 1978). Such a stabilizing effect of K^+, which is expressed in Figure 2 (○) by a decrease of the fluorescence intensity of the 3CPs-Thy furan-side monoadduct, reaches a maximum for K^+ higher than 0.1 M. It suggests that the structure of the helix is locally disrupted around the 3-CPs alteration.

In the case of MePyPs (Figure 2, △) the RFI values of its furan-side monoadduct remain constant whatever the K^+ concentration. However, it has to be noted that this fluorescence intensity depends on the DNA secondary structure since a 100% increase is observed after heat denaturation of the MePyPs-modified DNA. Moreover, the fluorescence of a 4′,5′ saturated [3,4*c*]pyridopsoralen, considered a model compound for MePyPs 4′,5′ monoadduct, has been shown to increase when the solvent polarity decreases (unpublished results). These last observations indicate that the RFI values of the furan-side monoadduct of MePyPs depend on its microenvironment in DNA. Consequently, the absence of ionic strength effect observed on the fluorescence intensity reflects, in the case of MePyPs, that the DNA secondary structure remains unmodified in the vicinity of its furan-side monoadduct.

In contrast, a marked increase of the RFI value of 2N-MePyPs monoadduct is observed (Figure 2, ●) by increasing the K^+ concentration. The fluorescence emission (λ_{em} = 458 nm) and absorption maxima (λ_{abs} = 363 nm) remain unchanged as does the molar extinction coefficient of the bound

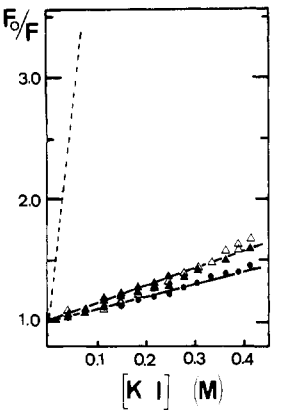


FIGURE 3: Stern–Volmer plots of the fluorescence quenching induced by KI on DNA modified by 3-CPs (▲), MePyPs (●), 2N-MePyPs (△) and on the dThd<$^{54'}_{65'}$>3-CPs model photoadduct [(---) product **2**; Cadet et al., 1983] corresponding to the fully exposed chromophore. Data were corrected for the effect of the cation K^+ previously described (Figure 2).

residue, indicating an increase of the fluorescence quantum yield. This shows, as observed with 3-CPs, that the fluorescence intensity of the 2N-MePyPs-bound residue is connected to the stabilizing effect of K^+ on the DNA secondary structure.

During the heat denaturation of this 2N-MePyPs-modified DNA a weak fluorescence quantum yield increase (≈10%) was observed. The most significant effect of the DNA strand separation involved a shift of the absorption (λ_{abs} = 357 nm) and of the emission (λ_{em} = 469 nm) maxima (unpublished results). Such spectral modifications related to the DNA secondary structure do not correspond to those observed at high ionic strength.

(*b*) *KI Effect.* Iodide ions are able to interact with residues located outside a polyelectrolyte such as DNA. Their collisional interaction with an external fluorescent adduct may give rise to a quenching of its fluorescence emission according to a Stern–Volmer process (see Materials and Methods). Figure 3 shows the F_0/F plots of the KI-induced quenching of the fluorescence of DNAs modified by 3-CPs (▲), MePyPs (●), and 2N-MePyPs (△). After correction of the K^+ effects discussed above, linear variations are observed whose slopes provide the dynamic quenching constant (K_{SV}). The Stern–Volmer plot of the KI-induced fluorescence quenching of the furan-side C_4 cycloadduct dThd<$^{54'}_{65'}$>3-CPs (product **2**; Cadet et al., 1983) is also reported as a reference (---). The dynamic quenching constant measured from this purified model adduct gives the upper value for the fully exposed chromophore (K_{SV} = 28 M^{-1}). K_{SV} values obtained with 3-CPs and 2N-MePyPs adducts are similar and respectively equal to 1.40 and 1.45 M^{-1}. These values are higher than that observed for MePyPs adducts (K_{SV} = 1.0 M^{-1}). Such values indicate that MePyPs and 2N-MePyPs residues like 3-CPs (Gaboriau et al., 1987) exhibit a very reduced accessibility to the quencher due to their internal location within the helix.

Mobility of the Monoadducts. The shortest correlation time of the various DNA motions involving the phosphate–sugar backbone (50 ns) (Early & Kearns, 1979) remains long compared to the movements of the free isolated monoadducts. Furthermore, it may be assumed that these motions of the helix cannot occur during the short fluorescence lifetime of the bound chromophore (2–5 ns). Therefore, conformational constraints induced by DNA lead to typical increases of the fluorescence anisotropy values. Such values are characteristic of the mobility of each bound residue with respect to the helix.

Results from the steady-state anisotropy measurements with 3-CPs, MePyPs, and 2N-MePyPs are summarized in Table

Table II: Steady-State Fluorescence Anisotropy Values of DNAs Modified by 3-CPs, MePyPs, and 2N-MePyPs[a]

	native	hydrolyzed	denatured
DNA–3-CPs	0.18	0.04	0.18
DNA–MePyPs	0.28		0.20
DNA–2N-MePyPs	0.40		0.20
4′,5′-DHCPs	0.37*	0.03**	

[a] Modified DNAs were analyzed in native conditions, after heat denaturation, or hydrolyzed by a mild acidic treatment (0.4 M HCl, 4 h, 75 °C). Values obtained in glycerol at 0 °C (*) or in water at 20 °C (**) with the model molecule 4′,5′-DHCPs are reported as a comparison. Fluorescence measurements ($I_\perp$ and $I_\parallel$) were performed at 420 nm by using a monochromatic excitation light (λ_{exc} = 334 nm) vertically polarized. The polarization (P) and the fluorescence anisotropy (r) were then respectively calculated according to

$$P = (1 - T)(I_\perp/I_\parallel)/(1 + T)(I_\perp/I_\parallel)$$

$$r = 2P/(3 - P)$$

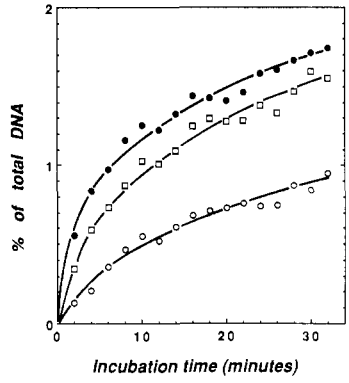


FIGURE 4: Kinetics of the nuclease S_1 induced hydrolysis of the DNAs modified by 3-CPs (□), MePyPs (○), and 2N-MePyPs (●) (Θ = 45 °C, 100 units·mL^{-1}). The percentage of total DNA that has been degraded was estimated from the variations of the OD at 258 nm (see Materials and Methods).

II. Values obtained from the 4′,5′-DHCPs in various solvent conditions are reported as a reference. In aqueous solution (20 °C) the fluorescence emission of this latter model compound is completely unpolarized (r = 0.03). In glycerol (0 °C) the anisotropy value is found to be high (r = 0.37) due to the absence of molecular movement during its radiative lifetime.

The values of anisotropy measured from the modified DNAs indicate that DNA constrains movements of the bound residues partially with 3-CPs (r = 0.18), strongly with MePyPs (r = 0.28), and completely in the case of 2N-MePyPs (r = 0.4). As previously reported, thermal denaturation does not modify the mobility of the 3-CPs-bound residue (Gaboriau et al., 1987). In contrast, the partial release of the steric constraint that results from the strand separation leads to a significant decrease of the anisotropy values of MePyPs- and 2N-MePyPs-modified DNAs. In all cases, mobilities appear to be similar after heat denaturation ($r \approx 0.2$) and characteristic of denatured sites of DNA. The total release of such sterical constraints (r = 0.04) is observed with 3-CPs-modified DNA by cleavage of the N-glycosidic bonds induced by a mild acidic hydrolysis treatment (0.4 M HCl, 75 °C).

Enzymatic Analysis of the DNA Secondary Structure in the Vicinity of the Alterations Photoinduced by 3-CPs, MePyPs, and 2N-MePyPs. To analyze the DNA secondary structure around the alterations induced by the three compounds, sensitivities of the modified DNAs toward the single-strand-specific endonuclease S_1 have been tested. Hydrolysis kinetics obtained from optical density measurements at 258 nm are reported in Figure 4 (100 units·mL^{-1}, 45 °C). They are corrected from the residual double-strand activity of S_1 measured with a native DNA (0.05 μg·h^{-1}·unit^{-1}).

Table III: Initial Rates (V_i) of the S_1 Hydrolysis of DNAs Modified by 3-CPs, MePyPs, and 2N-MePyPs[a]

compound photobound to DNA	V_i (μg·h^{-1}·unit^{-1})	photobinding rate (per 1000 bp)	relative corrected V_i
MePyPs	6	10	1
2N-MePyPs	30	2	25
3-CPs	26	2	22

[a] Initial rates (V_i) correspond to the mean values obtained from three kinetic measurements. Extent of binding was measured by using radioactive 3-CPs or by spectrophotometric measurements in the case of MePyPs and 2N-MePyPs (Blais et al., 1984, 1987). To compare the denaturation efficiency of each bound derivative, these initial rates were corrected from the photobinding rates, taking MePyPs as a reference.

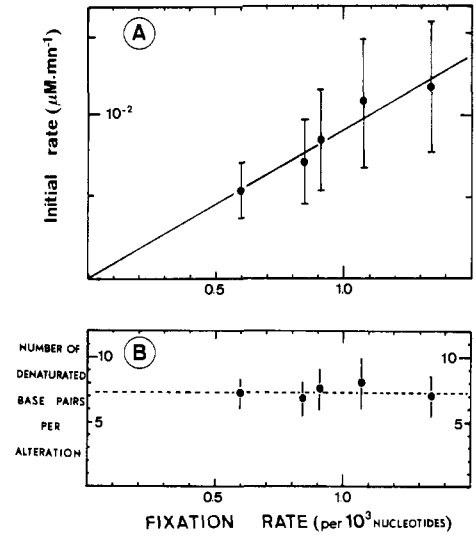


FIGURE 5: Effect of the 3-CPs photobinding on the DNA secondary structure. (A) Relationship between its fixation rate with DNA and the initial rate of the S_1 hydrolysis (Θ = 45 °C, 100 units·mL^{-1}). (B) Estimation of the average number of denatured base pairs induced by the photobinding on DNA of one 3-CPs molecule.

Relative sensitivities of the modified DNAs toward S_1 are similar in the case of 3-CPs and 2N-MePyPs and higher than that observed with MePyPs. Initial rates (V_i) obtained from the slopes of the hydrolysis kinetics at the beginning of the enzymatic reaction (t < 10 min) correspond to the mean values of three kinetic measurements (Table III). They are proportional to the single-strand DNA concentration. To compare the relative denaturing capacity of each alteration, V_i values have been corrected from the photobinding rate of each derivative. Such normalized values reported in Table III clearly show that the photobinding of one 3-CPs or one 2N-MePyPs molecule on DNA gives rise to the formation of single-strand regions involving approximately the same number of bases. This quantitative aspect will be examined later in the case of 3-CPs. In contrast, the low value of the initial rate observed with MePyPs-modified DNA reflects a very weak disruption of the DNA secondary structure around this type of alteration.

To connect the photoreaction of 3-CPs with DNA bases with the formation of single-strand DNA regions and to estimate the size of such disruptions of the helix, the sensitivity toward S_1 of modified DNAs has been estimated at various photobinding rates by 3-CPs. The rates obtained for increasing irradiation times of the DNA–3-CPs complexes have been measured by using $^{14}C_3$-labeled 3-CPs. The experiments have been triplicated on each sample. The contribution of the

nonirradiated DNA–3-CPs complex to the initial rate has been deduced (Figure 5A).

As shown in Figure 5A, a linear relationship between the initial rate of the S_1-induced hydrolysis of the 3-CPs-modified DNA and the number of bound residues is found. Assuming that the single-strand DNA concentration is directly related to the number of 3-CPs-induced alterations, our results show that the enzyme exhibits, in this range of concentration, no cooperativity toward the 3-CPs-modified DNA. For each photobinding rates, the average number of denatured bp per alteration reported in Figure 5B was deduced from the comparison of the initial rate measured on 3-CPs-modified DNAs with the previously obtained calibration curve (see Materials and Methods). As expected, these calculated numbers are constant and reflect that photoreaction of one 3-CPs molecule with DNA gives rise to the denaturation of 7 ± 2 base pairs.

Discussion

The flow linear dichroism study of furan-side psoralen–DNA monoadducts had previously shown that the adducts exhibit the same orientation relative to that of DNA bases (Vigny et al., 1987). In the case of MePyPs and 3-CPs monoadducts, the mean angles between the psoralen residue and the normal-to-the-plane of the DNA bases are respectively equal to 74 ± 2° and 72 ± 4°. These values are consistent with a pseudointercalative geometry. The chemical structure of the furan-side monoadduct isolated from 3-CPs-modified DNA was previously elucidated (Moysan et al., 1986; Gaboriau et al., 1987). The cis–syn stereochemistry of the two dThd$<^{5'4'}_{6'5'}>$3-CPs diastereoisomers is consistent with their internal location deduced from fluorescence quenching experiments (Gaboriau et al., 1987) and from the linear dichroism data discussed above. The structural assignment of the main furan-side monoadduct from the photoreaction of MePyPs with DNA is now under study (Moysan, 1987, and unpublished results). One can assume in a first attempt that the pseudointercalative geometry also observed in this case reflects the same cis–syn stereochemistry.

Our present results from fluorescence quenching experiments by iodide ions confirm the internal location of the 3-CPs and MePyPs monoadducts and demonstrate that of 2N-MePyPs-bound residues. Furthermore, they indicate that the conformation of the alterations respectively induced by 3-CPs and 2N-MePyPs in DNA are similar. The disruption of the helix around the 3-CPs and 2N-MePyPs monoadducts has been detected by the stabilizing effect of the cation K^+ on the secondary structure of the modified DNAs. Further evidence of the denaturing sterical effect locally induced in the helix by the photobinding of 3-CPs and 2N-MePyPs was obtained from their sensitivity toward the single-strand-specific endonuclease S_1. The extent of denatured base pairs per alteration is similar in both cases and approximatively equal to 7 bp. In the case of 3-CPs, this local disruption of the DNA secondary structure is consistent with the unwinding of the modified DNA helix reported in a previous study (Isaacs et al., 1984). The unwinding angle observed with 3-CPs (26 ± 3°) is similar to that measured from bifunctional derivatives such as 8-MOP (28 ± 3°) or 5-MOP (25 ± 3°) but is relatively higher than induced by the monofunctional 5-methylisopsoralen (18 ± 3°). The S_1 endonuclease has been previously shown to cleave DNA reacted with AAAF (Fuchs, 1975) and to a lesser extent UV-irradiated DNA (Shishido & Ando, 1974; Cleaver et al., 1985). Such alterations induce local denaturation or distortion in the DNA helix and constitute recognition sites for this enzyme. The binding of the AAAF molecules induces large loops in DNA (12–13 bp per bound residue) as shown from kinetics of unwinding induced by the formaldehyde (Fuchs, 1975). The size of these loops appears to be smaller in the case of 3-CPs and 2N-MePyPs. This local change of the DNA secondary structure could provide characteristic recognition sites for repair enzymes. Assuming that the same repair process leads to the same repair efficiency, these similar recognition sites could explain similarities in their genotoxic and cytotoxic effects.

These photobiological properties strongly differ from those of MePyPs, which exhibits higher lethality and mutagenic efficiency relative to the 3-CPs and 2N-MePyPs. Moreover, the peculiar step repair of the MePyPs-induced alterations has been shown by the persistance of the single-strand breaks produced in DNA after their excision. The present study suggests that these photobiological differences relative to the 3-CPs and 2N-MePyPs derivatives could be related to the secondary structure around each alteration.

Therefore, the conformation of alterations induced by MePyPs appears to be different from those induced by 3-CPs and 2N-MePyPs. A very weak disruption of the DNA secondary structure can be detected in this case. This has been deduced from the very low sensitivity of the MePyPs-modified DNA toward the endonuclease S_1 and from the absence of effect of the cation K^+ on the relative fluorescence intensity of this bound residue. This secondary structure of DNA around the MePyPs-induced alterations appears to be similar to that induced by 5-methylisopsoralen, which is resistant to S_1 nuclease (Cleaver et al., 1985) and involves a weak unwinding of the DNA helix (Isaacs et al., 1984). These data suggest a typical repair process in the case of MePyPs due to the absence of local disruption of the DNA helix, which likely constitutes the most usual triggering signal for the repair processes.

The present study indicates that similarities in the chemical structures of the furan-side adducts of 3-CPs and MePyPs lead to the same internal location of these photoadducts within the helix. In a general way such predominant location, which is consistent with their cis–syn stereochemistry, appears to be predetermined by the geometry of the noncovalent intercalation complexes formed prior to any irradiation. However, due to the sterical hindrance related to the molecular structure of the bound residue, the same internal location of each monoadduct does not imply the same conformation for their resulting alterations in DNA. Between the chemical structure of the adducts and the conformation of the DNA helix around them there is another unknown level involving the interactions of the bound residue with its environment. Our fluorescence anisotropy data provide some information about this environmental factor. Fluorescence anisotropy values measured for the 3-CPs-bound residue are identical before and after denaturation ($r = 0.18$). This indicates a mobility characteristic of a single-strand DNA region. In the case of 2N-MePyPs the high anisotropy value observed in native modified DNA ($r = 0.4$), characteristic of the absence of motion, cannot be directly connected to its local single-strand environment as reported for the 3-CPs monoadduct. One can assume in this case that unexplained interactions of the 2N-MePyPs-bound residue with locally denatured DNA bases could block its movement. In such a hypothesis the apparent increase of its fluorescence intensity observed at high ionic strength could rather reflect a quenching of its fluorescence emission at low ionic strength. This quenching could be due to nonradiative deactivation processes such as charge-transfer complex.

Conclusions

Several photobiological properties of various psoralen de-

rivatives have been related to their ability to produce interstrand DNA cross-links. The role of the monoadducts relative to the biadducts in cytotoxic and genotoxic effects has been investigated by using purely monofunctional psoralen derivatives. Some of these synthetic compounds, such as 3-CPs and MePyPs which exhibit promising therapeutic activities, strongly differ from one another in their photobiological properties. Such differences are not due only to the chemical structure of their respective furan-side monoadduct formed in DNA, since these structures seem to be similar in the case of 3-CPs and MePyPs. The internal location of these adducts relative to the helix does not depend on the molecular structure of the bound residues and is consistent with their cis stereochemistry.

In contrast, the structure of the bound compounds appears to play a major role in the stability of the helix around the alterations. Monofunctional psoralen derivatives could be classified on the basis of the DNA conformation in the vicinity of their monoadducts. Such classification as denaturing (3-CPs and 2N-MePyPs) or nondenaturing (MePyPs) involves differences in their subsequent steps of repair.

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Registry No. 3-CPs, 20073-24-9; MePyPs, 85878-63-3; 2N-MePyPs, 108736-00-1; KCl, 7447-40-7; KI, 7681-11-0.

References

Ando, T. (1966) *Biochim. Biophys. Acta 114*, 158.

Averbeck, D. (1984) *Proc. Jpn. Soc. Invest. Dermatol. 8*, 52.

Averbeck, D., Dubertret, L., Bisagni, E., Moron, J., Papadopoulo, D., Nocentini, S., Blais, J., & Zajdela, F. (1983) presented at the Joint Meeting of the Society for Investigation in Dermatology Research, April 27–May 1, Washington, DC.

Blais, J., Vigny, P., Moron, J., & Bisagni, E. (1984) *Photochem. Photobiol. 39*, 795.

Blais, J., Averbeck, D., Moron, J., Bisagni, E., & Vigny, P. (1987) *Photochem. Photobiol. 45*, 465.

Cadet, J., Voituriez, L., Gaboriau, F., Vigny, P., & Della Negra, S. (1983) *Photochem. Photobiol. 37*, 363.

Cleaver, J. E., Killpack, S., & Gruenert, D. C. (1985) *EHP, Environ. Health Perspect. 62*, 127.

Dall'Acqua, F. (1977) in *Research in photobiology* (Castellani, A., Ed.) p 243, Plenum Press, New York.

Dall'Acqua, F., Marciani, S., Ciavatta, L., & Rodighiero, G. (1971) *Z. Naturforsch. 26b*, 561.

Early, T. E., & Kearns, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4165.

Fuchs, R. P. P. (1975) *Nature 257*, 151.

Gaboriau, F., Vigny, P., Averbeck, D., & Bisagni, E. (1981) *Biochimie 63*, 899.

Gaboriau, F., Vigny, P., Cadet, J., Voituriez, L., & Bisagni, E. (1987) *Photochem. Photobiol. 45*, 199.

Hearst, J. E., Isaacs, S. T., Kanne, D., Rapoport, H., & Straub, K. (1984) *Q. Rev. Biophys. 17*, 1.

Isaacs, S. T., Wiesehahn, G., & Hallick, L. M. (1984) *Natl. Cancer Inst. Monogr. 66*, 21.

Magana-Schwencke, N., & Moustacchi, E. (1985) *Photochem. Photobiol. 42*, 43.

Manning, G. (1978) *Q. Rev. Biophys. 11*, 179.

Moron, J., N'Guyen, C. H., & Bisagni, E. (1983) *J. Chem. Soc., Perkins Trans. 1*, 225.

Moustacchi, E., Cassier, C., Chanet, R., Magana-Schwencke, N., Saeki, T., & Henriques, J. A. P. (1983) in *Cellular responses to DNA Damage*, p 87, Liss, New York.

Moysan, A. (1987) Thèse de doctorat de l'Université Paris 6.

Moysan, A., Gaboriau, F., Vigny, P., Voituriez, L., & Cadet, J. (1986) *Biochimie 68*, 786.

Musajo, L., Rodighiero, G., Colombo, G., Torlone, V., & Dall'Acqua, F. (1965) *Experientia 21*, 22.

Musajo, L., & Rodighiero, G. (1970) *Photochem. Photobiol. 11*, 27.

Musajo, L., & Rodighiero, G. (1972) *Photophysiology 7*, 115.

Nocentini, S. (1986) *Mutat. Res. 161*, 181.

Papadopoulo, D., Averbeck, D., & Moustacchi, E. (1986) *Photochem. Photobiol. 44*, 31.

Pathak, M. A., Parrish, J. A., & Fitzpatrick, T. B. (1981) *Farmaco 36*, 479.

Queval, P., & Bisagni, E. (1974) *Eur. J. Med. Chem. 9*, 335.

Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652.

Shishido, K., & Ando, T. (1974) *Biochem. Biophys. Res. Commun. 59*, 1380.

Song, P. S., & Tapley, K. J. (1979) *Photochem. Photobiol. 29*, 1177.

Studier, F. W. (1969) *J. Mol. Biol. 41*, 188.

Szyfter, K., & Kedzierski, W. (1976) *Stud. Biophys. 60*, 163.

Urbach, F., Forbes, P. D., & Davies, R. E. (1982) *JNCI J. Natl. Cancer Inst. 69*, 229.

Vigny, P., & Duquesne, M. (1974) *Photochem. Photobiol. 20*, 15.

Vigny, P., Gaboriau, F., Voituriez, L., & Cadet, J. (1985) *Biochimie 67*, 317.

Vigny, P., Blais, J., Ibanez, U., & Geacintov, N. E. (1987) *Photochem. Photobiol. 45*, 601.

Voet, D., Gratzer, W. B., Cox, R. A., & Doty, O. (1963) *Biopolymers 1*, 193.

Yamasaki, H., Pulbrabek, P., Grunberger, D., & Weinstein, I. B. (1977) *Cancer Res. 37*, 3756.

Zadjela, F., & Bisagni, E. (1981) *Carcinogenesis 2*, 121.