# Individual Determination of the Yield of the Main UV-Induced Dimeric Pyrimidine Photoproducts in DNA Suggests a High Mutagenicity of CC Photolesions†

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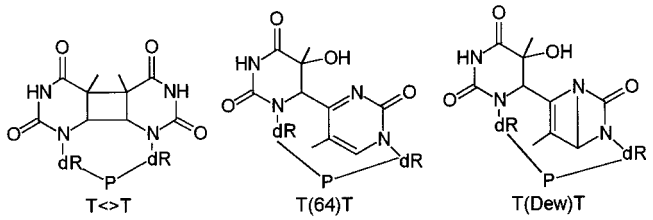
ABSTRACT: Bipyrimidine photoproducts induced in DNA by UVB radiation include cyclobutane dimers, (6-4) photoproducts, and their related Dewar valence isomers. Even though these lesions have been extensively studied, their rate of formation within DNA is still not known for each possible bipyrimidine site (TT, TC, CT, and CC). Using a method based on the coupling of liquid chromatography to mass spectrometry, we determined the distribution of the 12 possible bipyrimidine photoproducts within isolated and cellular DNA. TT and TC were found to be the most photoreactive sequences, whereas lower amounts of damage were produced at CT and CC sites. In addition to this quantitative aspect, sequence effects were observed on the relative yield of (6-4) adducts with respect to cyclobutane pyrimidine dimers. Another interesting result is the lack of formation of Dewar valence isomers in detectable amounts within the DNA of cells exposed to low doses of UVB radiation. The photoproduct distribution obtained does not fully correlate with the UV mutation spectrum. A major striking observation deals with the low yield of cytosine−cytosine photoproducts which are likely to be associated with the UV-specific CC to TT tandem mutation.

Ultraviolet B (UVB) radiation is one of the most studied genotoxic physical agents. Indeed, it is responsible for most of the nonmelanoma skin cancers induced by solar light. In the last four decades, extensive work on model compounds and isolated DNA have led to the identification of bipyrimidine sites as the main targets of incident UVB (290−320 nm) and UVC (230−290 nm) photons. Photoexcited thymine (T)[1] and/or cytosine (C) react with adjacent pyrimidine bases, leading to the formation of dimeric photoproducts (Scheme 1) (*1*, *2*). These include the cyclobutane type dimers (◇), the pyrimidine-(6-4)-pyrimidone adducts (64), and their related Dewar valence isomers (Dew). A large body of physical and chemical information on most of these photoproducts is now available, even though the photochemistry of CC is not completely elucidated. The biological features of the bipyrimidine photoproducts are widely documented in terms of mutagenic properties (*3*−*6*) and repair (*7*−*9*). However, despite this extensive accumulation of comprehensive data, it is still not possible to individually estimate the relative yield of formation of the three types of photoproducts at each of the four possible bipyrimidine sites within DNA. This is mostly due to the lack of proper analytical tools for monitoring the formation of bipyrimidine lesions within DNA.

Scheme 1: Structure of the UV-Induced Dimeric Thymine Photoproducts[a]




[a] dR, 2-deoxyribose; P, phosphate.

Several assays have been designed for the measurement of dimeric photoproducts within DNA. The most widely applied methods involve the use of poly- and monoclonal antibodies specifically raised against cyclobutane pyrimidine dimers, (6-4) photoproducts, or Dewar valence isomers. A variety of systems have been used for the quantification of the antibody binding to DNA (*10*−*17*). A limitation of the immunological approach is the lack of calibration which often prevents accurate quantitative measurements. In addition, it does not allow the differentiation between the four possible bipyrimidine lesions for a given class of dimeric photoproducts. Another biochemical approach involves the conversion of the photoproducts into strand breaks. The level of photoproduct is then inferred from the quantification of enzymatically or chemically generated strand breaks (*18*, *19*). Endonuclease V of phage T4, which exhibits *N*-glycosylase

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[1] Abbreviations: ◇, cyclobutane pyrimidine dimer; (64), pyrimidine-(6-4)-pyrimidone photoproduct; (Dew), Dewar valence isomer; C, cytosine; HPLC−MS/MS, high-performance liquid chromatography coupled to tandem mass spectrometry; IT-ES-MS, electrospray mass spectrometry with ion trap detection; U, uracil; dUpdC, 2′-deoxyuridylyl-(3′-5′)-2′-deoxycytidine; dUpdU, 2′-deoxyuridylyl-(3′-5′)-2′-deoxyuridine; dUpT, 2′-deoxyuridylyl-(3′-5′)-thymidine; T, thymine; TpdC, thymidylyl-(3′-5′)-2′-deoxycytidine; TpdU, thymidylyl-(3′-5′)-2′-deoxyuridine; TpT, thymidylyl-(3′-5′)-thymidine.

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activity at the 5′-end of cyclobutane dimers, has been widely applied to the detection of the latter lesions (*20*). Attempts have been made to study the formation of (6-4) adducts in heavily irradiated DNA followed by piperidine treatment (*21, 22*). However, the revealed alkali-labile sites were shown to be Dewar valence isomers (*23, 24*). This indirect approach suffers from the same drawbacks as the immunological detection since it only provides a global measurement of the level of each class of photoproducts. Sequence analysis has also been used to determine the location of the damage within targeted DNA sequences (*25*−*28*). Such analyses allowed the visualization of a higher frequency of cyclobutane dimers at TT sequences with respect to TC sites in various genes or plasmids (*26, 28*−*32*). In addition, evidence has been provided for an increase in the yield of dimeric damage at 5-methycytosine-containing bipyrimidine sites (*33*), in agreement with results obtained on model systems (*34*). However, the gel sequencing analysis provides results that are strongly gene- and sequence-dependent. The partial information obtained in this way on the yield of photoproducts cannot be readily extrapolated to the overall DNA. It can be added that these assays often require exposure of small amounts of DNA to high UV doses which may induce secondary photoreactions of the initial lesions. In summary, even though biochemical methods are powerful tools for investigations of key biological processes, they did not provide a complete distribution of the photoproducts within cellular or even isolated DNA exposed to UV radiation.

This important piece of information may be obtained from specific chromatographic analyses following DNA hydrolysis which allow individual quantification of defined lesions. Several attempts have been reported but have received only limited applications. The yield of cyclobutadithymine has been measured within isolated and cellular radiolabeled DNA using paper or liquid chromatography (*35*−*38*). HPLC has also been used for the fluorescence detection of (6-4) adducts within isolated DNA hydrolyzed under mild acidic conditions (*39*). It must be emphasized that none of these assays allowed the simultaneous detection of all types of photoproducts because of either drastic hydrolysis conditions or limitations in the detection technique used. To overcome these difficulties, we recently optimized a HPLC−tandem mass spectrometry method (HPLC−MS/MS) aimed at measuring the four main dimeric thymine photoproducts within both isolated and cellular DNA (*40*). In the present work, we report the extension of the method to the measurement of the photoproducts of the three other bipyrimidine sequences. In the course of this work, we obtained the first unambiguous evidence for the formation of the CC (6-4) adduct and its related Dewar valence isomer within DNA. The yields of formation of the *cis,syn*-cyclobutane dimers, the (6-4) adducts, and the Dewar valence isomers of TT, CT, TC, and CC were then determined within isolated and cellular DNA exposed to UVB and UVC radiations.

## EXPERIMENTAL PROCEDURES

*Preparation of the Photoproduct Standards.* The *cis,syn*-cyclobutane dimers of thymidylyl-(3′-5′)-thymidine (TpT), thymidylyl-(3′-5′)-2′-deoxyuridine (TpdU), 2′-deoxyuridylyl-(3′-5′)-thymidine (dUpT), and 2′-deoxyuridylyl-(3′-5′)-2′-deoxyuridine (dUpdU) were obtained by acetophenone-mediated sensitization to UVB radiation and characterized by $^1$H NMR and ion trap/electrospray mass spectrometry (IT-ES-MS) on a LCQ spectrometer (Thermoquest, San Jose, CA). The (6-4) adducts of TpT, thymidylyl-(3′-5′)-2′-deoxycytidine (TpdC), dUpT, and 2′-deoxyuridylyl-(3′-5′)-2′-deoxycytidine (dUpdC) were prepared upon exposure of the corresponding dinucleoside monophosphates to UVC light. They were further identified by IT-ES-MS and UV spectroscopy. Further support for the structure of the isolated (6-4) adducts was obtained from UVB photoisomerization experiments. A solution of the (6-4) adduct (ca. 1 optical density at 320 nm) was exposed to increasing doses of UVB light. A decrease in the 320-nm absorption was observed, and irradiation was resumed until the complete disappearance of the residual absorption. The Dewar valence isomers thus obtained were characterized by IT-ES-MS.

*Irradiations and DNA Hydrolysis.* Solutions of isolated calf thymus DNA (1 mg·mL$^{-1}$, 2 mL) were exposed to increasing doses of far-UV light in a 4-cm glass Petri dish. The fluence was measured by a VLX 3-W radiometer (Vilber Lourmat, Marne Le Vallée, France) equipped with either a CX 254 or a CX 312 probe. The UVC lamp was placed either 8 cm or 1 m above the samples. The fluence of the light was 2.64 and 0.16 kJ·m$^{-2}$·min$^{-1}$, respectively. These values were confirmed by actinometry involving photohydration of 1,3-dimethyluracil. The same irradiation conditions were used with the UVB lamp (spectrum of the UVB lamp normalized at 312 nm: 280 nm, 5%; 290 nm, 40%; 312 nm, 100%; 330 nm, 60%; 350 nm, 20%; 370 nm, 5%). The fluence was 2.75 and 0.17 kJ·m$^{-2}$·min$^{-1}$, respectively. Three aliquot fractions (100 $\mu$L) of each sample were collected. THP1 monocytes ($10 \times 10^6$ cells in 15 mL phosphate-buffered saline) were exposed to UVB light (0.17 kJ·m$^{-2}$·min$^{-1}$) at room temperature in 8.5-cm plastic Petri dishes. Immediately after irradiation, cells were recovered by centrifugation (5 min, 254*g*) and DNA was extracted using a NaI-based chaotropic method. DNA was subsequently solubilized in 100 $\mu$L of water. Irradiation and extraction were performed in triplicates. All DNA samples were hydrolyzed by sequential incubation with a 3′-exonuclease, nuclease P1, a 5′-exonuclease, and an alkaline phosphatase as previously reported (*40*). Following neutralization of the hydrolysis mixture, samples were left in the dark at 37 °C for 5 days.

*HPLC−MS/MS Analyses.* Samples were injected (20 $\mu$L) by a SIL-9A autosampler (Shimadzu, Kyoto, Japan) onto the chromatographic system consisting of a 150- × 2-mm i.d. Uptisphere HDO column (Interchim, Monluçon, France) connected to a L 6200 Merck-Hitachi pump (Darmstadt, Germany). A gradient of acetonitrile in 2 mM TEAA solution was used. The proportion of the organic phase was increased linearly from 0 to 2% within the first 8 min. It was then gradually increased to 20% over a 20-min period. The latter composition was maintained for 5 min. Pure methanol was added at the outlet of the column at a flow rate of 200 $\mu$L·min$^{-1}$. The eluent was then directed to a Waters 484 UV spectrophotometer (Mildford, MA) and then to the inlet of an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer/SCIEX, Thornhill, Canada). The UV signal was collected on a D-2500 Merck-Hitachi integrator. HPLC−MS/MS chromatograms were recorded in the multiple reaction monitoring (MRM) mode. Negative ions were monitored. The tuning parameters of the spectrometer were determined by using TpT as a reference compound. The

transitions used and the retention times observed for the different bipyrimidine photoproducts were the following: 545→447, T◇T (16.2 min); 545→432, T(64)T (19.3 min) and T(Dew)T (15.7 min); 531→433 and 531→195, T◇U (7.4 min) and U◇T (9.6 min); 531→432, U(64)T (14.2 min) and U(Dew)T (8.8 min); 530→195, T(64)C (19.3 min) and T(Dew)C (17.9 min); 517→419, U◇U (4.4 min); 516→195, U(64)C (14.1 min) and U(Dew)C (10.2 min). Some analyses of the dUpdC photoproducts were performed in the product ion scan mode which provides the fragmentation mass spectrum of a targeted molecular mass (mass range: 150−550).

## RESULTS

*Observation of the Cytosine (6-4) Adduct and Its Dewar Valence Isomer within UV-Irradiated DNA.* The principle of the HPLC−MS/MS assay has been reported in detail in a previous work dealing with the measurement of dimeric thymine photoproducts (*40*). The major difference in the present protocol is the incubation of the hydrolyzed samples at 37 °C in order to allow cytosine photoproducts to quantitatively deaminate (*41*, *42*). In addition, triethylammonium acetate was used as the ion suppression buffer instead of ammonium formate in order to increase the retention time of the fast eluting photoproducts. Another key step in the setup of the assay was the preparation of the photoproduct standards. The cyclobutane dimer, the (6-4) photoproduct, and the Dewar valence isomers of TT, TC, and CT (or their deaminated derivatives) were readily prepared from the corresponding dinucleoside monophosphates. The CC photoproducts could not be obtained by photolysis of dCpdC which yields very low amount of dimeric photoproducts with respect to thymine-containing analogues (*34*). The deaminated *cis*,*syn*-cyclobutane dimer was thus obtained by acetophenone-mediated photosensitization of dUpdU, while the (6-4) adduct was prepared in its deaminated form by UVC photolysis of dUpdC. Indeed, UV irradiation of the latter dinucleoside monophosphate yielded higher amount of a fluorescent photoproduct, tentatively identified as the (6-4) photoproduct, than exposure of dCpdC to UVC light (*43*). To further identify the 315-nm-absorbing compound isolated upon irradiation of dUpdC, its fragmentation mass spectrum was recorded and compared with that of T(64)C whose structure differs only by the presence of a methyl group at the C5 position of the 5′-end pyrimidine base (Figure 1). Both spectra exhibited high similarities including the presence of phosphorylated 2-deoxyribose (*m*/*z* = 195) as the major ion. In addition, an ion corresponding to the deprotonated base moiety was observed together with its M − $NH_3$ fragment corresponding to the loss of the amino group at the C5 position of the 5′-base. The latter feature provides strong evidence that the studied compound actually exhibits a (6-4) adduct structure. Further support for the U(64)C assignment was gained from its conversion into its Dewar valence isomer upon exposure to UVB light. The mass spectrum of the photoisomerization product was similar to that of U(64)C, in good agreement with the expected Dewar isomer structure. Definitive evidence for the formation of U(64)C and U(Dew)C within DNA was obtained by monitoring the chromatogram of a DNA hydrolysate with the three main fragments of U(64)C and U(Dew)C (Figure 2). The relative intensities of the signals and the retention

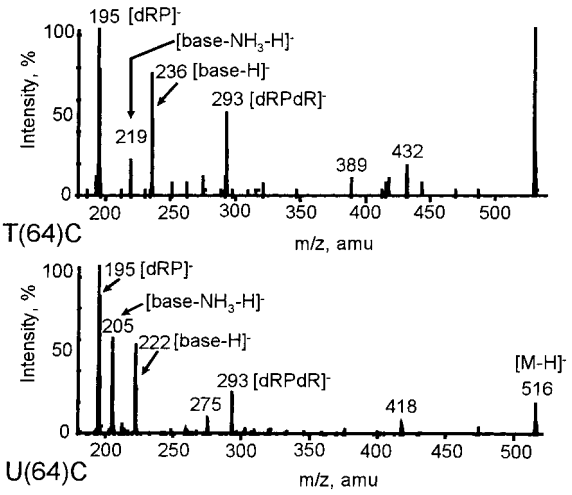


FIGURE 1: Fragmentation mass spectra of T(64)C and U(64)C (dR, 2-deoxyribose; P, phosphate). Pure standards prepared from the corresponding dinucleoside monophosphates were injected on the HPLC−MS/MS system. The parent ion was isolated in the first quadrupole. The third quadrupole was used in the scanning mode in order to record the fragmentation spectra of the compound (parent ions: $m/z = 530$ for T(64)C, $m/z = 516$ for U(64)C).

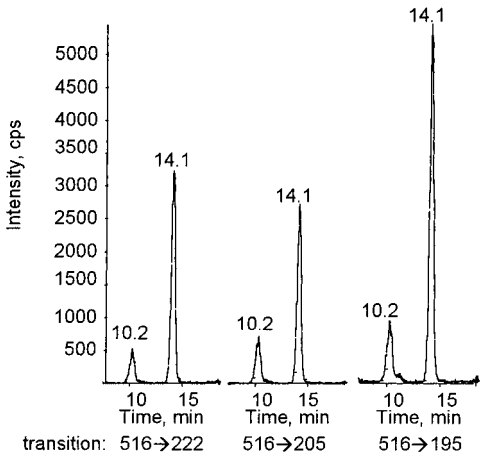


FIGURE 2: HPLC−MS/MS chromatogram of a hydrolysate of UVC-irradiated DNA. Three transitions corresponding to the main ions observed in the fragmentation spectra of U(64)C and U(Dew)C were simultaneously recorded. The respective retention times of U(64)C and U(Dew)C were 14.1 and 10.2 min.

times were similar to those obtained for the pure standards. Additional proofs of structure for U(64)C and U(Dew)C were inferred from the exposure of a digested UVC-irradiated DNA sample to increasing doses of UVB. As expected, the peak corresponding to U(Dew)C increased at the expense of U(64)C.

*Formation of the Dimeric Pyrimidine Photoproducts within Isolated DNA.* A first series of experiments was carried out in order to monitor the formation of the 12 available dimeric photoproducts within samples of isolated DNA exposed to high doses of UV light. When DNA was exposed to UVC radiation, the (6-4) photoproducts and the *cis*,*syn*-cyclobutadipyrimidines were obtained in high yields. However, the formation of the latter class of damage at TC, CT, and CC sequences rapidly reached a plateau (Figure 3), as already observed for T◇T (*40*). A striking observation was the formation in a relatively high yield of T(Dew)C upon exposure of DNA to the highest doses of 254-nm light. Indeed the yield of the latter lesion represented 15% of that

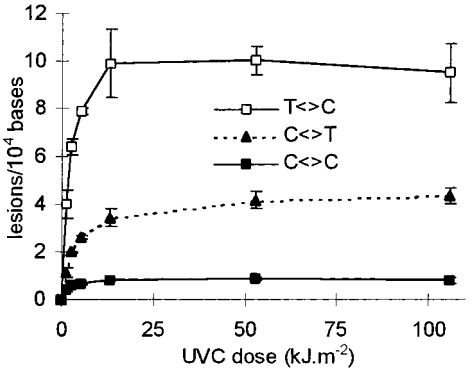


FIGURE 3: Formation of TC, CT, and CC cyclobutane dimers within isolated DNA exposed to high doses of UVC radiation. Each point represents the mean ± standard deviation of three determinations.

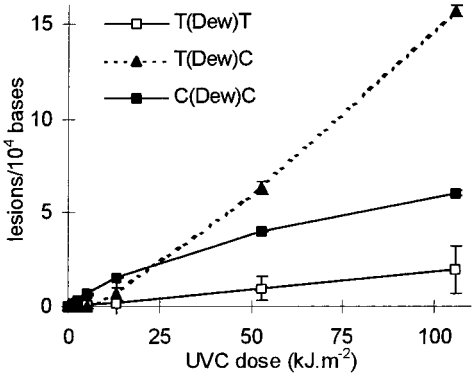


FIGURE 4: Formation of TT, TC, and CC Dewar valence isomers within isolated DNA exposed to high doses of UVC radiation. Each point represents the mean ± standard deviation of three determinations.

of T(64)C after exposure of DNA to 100 kJ·m$^{-2}$ UVC light (Figure 4). This contrasts with the induction of T(Dew)T which was detected in much lower amounts in the same samples. Interestingly, C(64)C was also formed, together with its Dewar valence isomer. Exposure of DNA to high doses of UVB light yielded the *cis*,*syn*-cyclobutane dimers as the major lesions. (6-4) Adducts were also generated in high amounts, mainly at TC sites. The photoconversion of the TT, CT, and TC (6-4) adducts into their Dewar valence isomers during the irradiation was shown by the quadratic shape of the dose−course plot of the latter class of photoproducts and the plateau reached in the level of their (6-4) adduct precursors. In contrast, the production of C(Dew)C was linear with respect to the dose of UVB radiation. This first series of experiments showed that the occurrence of secondary photoreactions may greatly influence the relative yield of the lesions. Therefore, the distribution of DNA photoproducts was determined within samples exposed to much lower doses of UV light. Under these conditions, a linear dose−course relationship was obtained for all compounds with the exception of the *cis*,*syn*-cyclobutane dimers upon UVC photolysis for doses higher than 0.8 kJ·m$^{-2}$, likely because of a partial photoreversion. Because of the lack of monochromaticity of UVB lamps, precise quantum yields could not be calculated. Therefore, results are reported as yields expressed in level of lesion per kJ·m$^{-2}$, inferred from the linear regression of the dose−course plot for all photolesions. These values are not directly comparable with those obtained from experiments carried out under different conditions. For instance, 2 mg of calf thymus DNA was used,

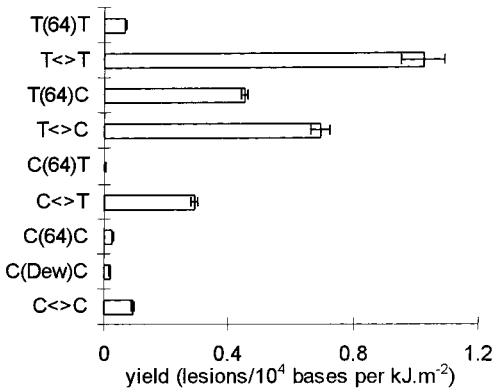


FIGURE 5: Distribution of pyrimidine photoproducts within isolated DNA exposed to low doses of UVB light. Results are expressed in lesions/10$^4$ bases/kJ·m$^{-2}$. The error bars represent the standard error of the slope.

Table 1: Yield of Formation of the Photoproducts within Isolated and Cellular DNA Exposed to UV Radiations[a]

| | isolated DNA + UVC | isolated DNA + UVB | cellular DNA + UVB |
|---|---|---|---|
| dose range | 0−6.4 kJ·m$^{-2}$ [b] | 0−3.4 kJ·m$^{-2}$ | 0−2.6 kJ·m$^{-2}$ |
| 64 TT | 0.238 ± 0.003 | 0.069 ± 0.002 | 0.245 ± 0.007 |
| Dewar TT | <0.002[c] | <0.003[c] | <0.01[c] |
| ◇ TT | 2.970 ± 0.131 | 1.023 ± 0.070 | 3.147 ± 0.095 |
| 64 TC | 1.503 ± 0.027 | 0.451 ± 0.009 | 1.400 ± 0.034 |
| Dewar TC | <0.002[c] | <0.003[c] | <0.01[c] |
| ◇ TC | 1.823 ± 0.111 | 0.694 ± 0.030 | 1.286 ± 0.047 |
| 64 CT | 0.012 ± 0.001 | 0.0024 ± 0.0003 | <0.01[c] |
| Dewar CT | <0.002[c] | <0.003[c] | <0.01[c] |
| ◇ CT | 0.573 ± 0.024 | 0.289 ± 0.012 | 0.577 ± 0.051 |
| 64 CC | 0.114 ± 0.007 | 0.028 ± 0.003 | 0.062 ± 0.028 |
| Dewar CC | 0.049 ± 0.002 | 0.019 ± 0.004 | <0.03[c] |
| ◇ CC | 0.069 ± 0.005 | 0.094 ± 0.006 | 0.279 ± 0.067 |

[a] Values were inferred from the linear regression (slope ± standard error of the slope). Yields are reported in lesions/10$^4$ bases/kJ·m$^{-2}$. [b] The yields of formation of the cyclobutane dimers were calculated from the linear part of the curve, namely between 0 and 0.8 kJ·m$^{-2}$. [c] Estimated from the detection limit of the method which is around 50 fmol for T(Dew)T, T(Dew)C, and C(Dew)T. The latter value is 150 fmol for C(Dew)C.

whereas studies involving plasmids are usually performed with a few micrograms of material. As a consequence, the same dose would lead to a higher level of damage in the latter than the former case. However, the linearity of the response established in our low-dose experiments allows to compare the relative yield of the various photoproducts. Similar distributions were obtained with either UVB (Figure 5) or UVC (Table 1) radiations. In both cases, T◇T was the main photoproduct. T(64)C and T◇C were produced in similar yields. The level of dimeric photoproducts was much lower at CT and CC sites. Interestingly, C(Dew)C was produced in a linear way with respect to the UV dose, whereas it is expected to be a secondary product of C(64)C. In contrast, no Dewar valence isomer was detected at TT, TC, and CT sites, even upon exposure of DNA to UVB radiation, at least within the range of applied doses.

*Distribution of the Photoproducts within Cellular DNA.* The HPLC−MS/MS assay was then applied to the quantification of the photoproducts within the DNA of cultured human monocytes exposed to relatively low doses of UVB light. The formation of the photoproducts was linear with respect to the dose (Figure 6). The distribution of the lesions

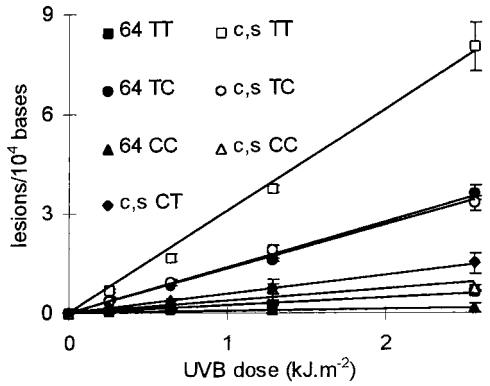


FIGURE 6: Formation of dimeric pyrimidine photoproducts within the DNA of THP1 cells exposed to UVB light. Each value represents the mean ± standard deviation of three independent experiments.

was similar to that obtained within isolated DNA (Table 1). Indeed, T◇T was the major photoproduct, while its TC analogue was produced in a 2-fold lower yield. In contrast, C◇T and C◇C were detected in a significantly lower yield. T(64)C was produced in an amount similar to that of T◇C and in a 5-fold higher yield than its TT analogue. In contrast, C(64)C was produced in a very low amount, in agreement with results obtained with isolated DNA. The amount of the corresponding CT lesion was below the detection limit. It can be added that none of the Dewar valence isomers was detected even at the highest doses of UVB light (2.6 kJ·m$^{-2}$).

## DISCUSSION

The structural assignment of the *cis,syn*-cyclobutane thymine dimer was reported more than 40 years ago (*44*). In the last four decades, many efforts have been devoted to the characterization of other classes of far-UV-induced dimeric pyrimidine photoproducts within DNA models (*1*, *2*). In addition, a large amount of information has been obtained on the biological consequences of the formation of the pyrimidine photoproducts within cells. However, basic data, such as the distribution of the three classes of photoproducts at the four different bipyrimidine sites, remain to be obtained. Indeed, available techniques based on the use of biochemical tools such as antibodies and repair enzymes failed to individually determine the yield of formation of the various bipyrimidic photoproducts. Until recently, chromatographic assays received only a few applications for the quantification of UV-induced photoproducts, with the exception of the measurement of thymine cyclobutane dimers in radiolabeled isolated and cellular DNA (*36*−*38*) and the fluorescence detection of (6-4) adducts (*39*). Recently, mass spectrometry with electrospray ionization has been successfully applied to the characterization of dimeric thymine photoproducts (*45*, *46*). We have also shown that tandem MS used in the multiple reaction monitoring mode and coupled to HPLC was a very sensitive and specific technique for the detection of dimeric thymine photoproducts (*40*). The method was extended in the present work to the measurement of photodamage at the three other bipyrimidine sites: namely CT, TC, and CC. The detection of the cytosine derivatives is made more complicated by the deamination reaction which converts cytosine into uracil in the cyclobutane dimers and at the 5′-position of the (6-4) adducts. Therefore, all photoproducts were analyzed in their deaminated form. All the authentic samples of *cis,syn*-cyclobutane dimers, (6-4) adducts, and related Dewar valence isomers were prepared from the related dinucleoside monophosphates. It can be added that the setup of the assay led to the first unambiguous observation of the formation of the CC (6-4) adduct and its Dewar isomer within DNA.

The formation of the 12 main bipyrimidic photoproducts was measured within samples of isolated DNA exposed to UVC and UVB radiations. When the irradiation was carried out at a low fluence, a similar distribution of photoproducts was obtained with both UV sources. These results, together with the observation of linear plots in the dose−course formation, indicate that no secondary reactions occurred under the irradiation conditions used. The major lesion was T◇T which was produced in a 10-fold higher yield than the related (6-4) adduct. T(64)C and T◇C were generated in similar yields. The overall amount of the two latter TC photoproducts was similar to that of the TT lesions. The rate of formation of the cyclobutane dimer is thus higher at TT than TC sites in agreement with previous gel sequencing measurements (*26*, *28*−*32*). The yield of photoproducts at CT and CC sites was 5 and 10 times lower than those of the TT and TC lesions, respectively. Beside the lower reactivity of the 5′-cytosine-containing bipyrimidine sites, comparison of the yields of the photoproducts revealed a drastic effect of the sequence on the relative formation of *cis,syn*-cyclobutane pyrimidine dimers and (6-4) adducts. Indeed, in 3′-thymine-containing sequences (TT and CT), the ratio between the yield of Pyr◇Pyr and Pyr(64)Pyr is 1 order of magnitude higher than in TC and CC. This trend has already been observed in dinucleoside monophosphates (*43*, *47*). The overwhelming formation of T(64)C with respect to the corresponding TT and CT adducts has also been already observed within irradiated DNA on the basis of HPLC analyses involving fluorescence detection (*39*). It may be also emphasized that no Dewar valence isomer was observed for TT, TC, and CT at these low doses. Interestingly, C(Dew)C was produced in a significant yield in a linear relationship with respect to either UVC or UVB doses. This may suggest that the photoisomerization of C(64)C is efficient in both wavelength ranges. An alternative mechanism would be that C(Dew)C is a primary product of irradiation of DNA. However, both pathways remain to be further documented. Irradiation of isolated DNA at higher fluences of UV light confirmed observations made on model systems. All *cis,syn*-cyclobutane dimers studied were found to undergo photoreversion by UVC light as shown by the plateau reached in their level within DNA. As already observed for TT (*40*), the Dewar valence isomers of TC and CT were produced as secondary products upon exposure of DNA to high doses of UVB light. Interestingly, a similar result was obtained for T(Dew)C within isolated DNA irradiated with UVC light. This unexpected result may account for the presence of alkali-labile sites in UVC-irradiated DNA which were tentatively identified as (6-4) adducts (*22*, *48*). It can be added that the ratio between the overall yield of cyclobutane dimers on the one hand and (6-4) photoproducts on the other hand was found to be around 3. This is close to values ranging between 2 and 8 previously proposed on the basis of immunological measurements (*13*, *49*) but much lower than the ratio of 40 determined following T4 endoV and alkaline treatment of DNA (*48*).

The photoproduct distribution within the DNA of cells exposed to UVB light was very similar to that obtained within isolated DNA exposed to low doses of UV radiation. With the exception of that of T◇T, the yield of the photoproducts presently reported had never been accurately determined before within cellular DNA. In addition, doses were carefully chosen to provide linear dose−course relationships for the formation of the photoproducts. This has not always been checked in other works that involved the use of relatively high doses of UV light (*48*). T◇T, T(64)C, and T◇C were found to be the three main lesions, produced in a 2:1:1 ratio. The yield of T◇T was in agreement with available data involving various cellular systems exposed to UVB light (*49*, *50*). T(64)T was obtained in a 10-fold lower yield than the corresponding *cis*,*syn*-cyclobutane dimer, as previously reported (*40*). The yield of the CT and CC photoproducts was much lower. Indeed, the lesions induced at these sites represented 8% and 5% of the overall detected dimeric pyrimidine photoproducts. Interestingly, the ratio between the yields of cyclobutane pyrimidine dimers and (6-4) adducts was 3 as determined within UVC- and UVB-irradiated isolated DNA. It can be also emphasized that none of the Dewar valence isomers was produced in detectable amounts. This result contrasts with the few available data based on the use of an antibody recognizing this class of damage (*15*, *49*). It would be interesting to calibrate the response of the latter immunoassay by using the HPLC−MS/MS method in order to understand this discrepancy. Indeed, HPLC−MS/MS allows the direct measurement of Dewar isomers, whereas the previous characterization of the antibody recognizing Dewar isomers mainly involved indirect approaches based on the UVB-induced conversion of the (6-4) adducts.

It appears interesting to compare the photoproduct distribution accurately determined by HPLC−MS/MS to the already known mutation spectrum of UV light. Some lack of correlation may be found between the two pieces of information. Indeed, the major photoproduct is T◇T, whereas TT sites are not mutational hotspots following UV irradiation (*5*, *6*, *31*, *51*). In contrast, CC sequences are sites of relatively frequent CC to TT tandem mutations, while the corresponding photoproducts are produced with the lowest yields (*4*, *6*). These observations emphasize the differences in mutagenic properties of the different pyrimidine dimeric photoproducts. For instance, T◇T has been reported to be only weakly mutagenic when incorporated into oligonucleotides used as templates for in vitro DNA synthesis (*52*). This may account for the relative low rate of mutation observed at TT sites (*5*, *6*, *31*, *51*). In contrast, T(64)C induces a much higher rate of mutation (*53*). This explains why, despite a photoreactivity similar to that of TT, TC sites are mutational hotspots. It can be added that deamination of a cyclobutane dimer can further explain the high mutation frequency at TC sites (*54*). The influence of the mutational properties of the photoproducts is even more drastic at CC sequences where only 5% of the photoproducts are produced but where mutations are induced in a high frequency. In addition, the CC to TT tandem mutation is a major event induced by UV light as shown by its high occurrence in either irradiated cells or the p53 gene of skin tumor cells (*55*, *56*). These data suggest that the CC photoproducts, or their deamination derivatives, are highly mutagenic. In agreement with this proposal, the uracil *cis*,*syn*-cyclobutane dimer has been shown to lead to the incorporation of two adenines with an accuracy similar to that of its thymine analogue (*57*). This indicates that the presence of a deaminated CC dimer is likely to induce a mutation with high probability. Data are still missing for the CC (6-4) photoproduct which is produced in low yield but may significantly contribute to mutagenesis. An alternative explanation for the apparent lack of correlation between the photodamage distribution and the mutation spectrum may be differences in the ability of the lesions to be repaired. Indeed, the rate of excision of a dimeric photoproduct may vary from one bipyrimidinic site to another or from one class of damage to the other. Evidence has already been obtained to support the latter aspect since the repair of (6-4) adducts is known to be more efficient than that of cyclobutane dimers (*58*). The availability of the HPLC−MS/MS assay should now allow to investigate the effect of the bipyrimidine sequence for a given class of photoproducts on the rate of excision.

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