UVB radiation-induced formation of dimeric photoproducts at bipyrimidine sites within DNA has been unambiguously associated with the lethal and mutagenic properties of sunlight. The main lesions include the cyclobutane pyrimidine dimers and the pyrimidine (6-4) pyrimidone adducts. The latter compounds have been shown in model systems to be converted into their Dewar valence isomers upon exposure to UVB light. A new direct assay, based on the use of liquid chromatography coupled to tandem mass spectrometry, is now available to simultaneously detect each of the thymine photoproducts. It was applied to the determination of the yields of formation of the thymine lesions within both isolated and cellular DNA exposed to either UVC or UVB radiation. The cis-syn cyclobutane thymine dimer was found to be the major photoproduct within cellular DNA, whereas the related (6-4) adduct was produced in an approximately 8-fold lower yield. Interestingly, the corresponding Dewar valence isomer could not be detected upon exposure of human cells to biologically relevant doses of UVB radiation.

Ultraviolet radiation represents the most deleterious part of solar light to cells and has been associated with the occurrence of skin cancer (1). UVB (290–320 nm) radiation is highly mutagenic (2, 3) and mostly induces mutations at bipyrimidine sites in cellular DNA (4–7). Interestingly, a similar mutation spectrum was observed in the p53 gene of skin tumors cells (8, 9). Altogether, these data outlined the biological role of dimeric photoproducts of pyrimidine DNA bases. In the last four decades, major efforts have been devoted to the isolation and the characterization of bipyrimidine photolesions in model compounds and isolated DNA (for reviews, see Refs. 10 and 11). These include the cis-syn and trans-syn diastereoisomers of cyclobutane dimers and the pyrimidine (6-4) pyrimidone adducts (Fig. 1). The latter lesions have been shown to undergo an efficient photocconversion into their Dewar valence isomers upon exposure to UVB light (12).

Information on the rate of formation of each specific photoproduct is still needed. Indeed, most of the assays developed for the detection of UV-induced photoproducts within DNA involve the use of indirect methods that do not allow differentiation, for a given class of photoproduct, of the lesions arising from the different possible bipyrimidinic sequences. Cyclobutane pyrimidine dimers have been extensively detected by using T4 endonuclease V, which exhibits a N-glycosylase activity at the 5’-extremity of the lesion (13). This leads to the formation of strand breaks, which can be quantified using electrophoretic techniques. A second DNA repair system, namely the Escherichia coli Uvr ABC complex, has been used in combination with a photolyase for the quantitation of cyclobutane dimers and (6-4) photoproducts (14). Another widely applied biochemical approach involves the use of either polyclonal or monoclonal antibodies able to recognize specific classes of pyrimidine photoproducts (15–22). However, most of the immunological assays suffer from a lack of calibration. Therefore, they can only provide relative values for the yields of formation. Ligation-mediated polymerase chain reaction has also been applied to monitor the formation of photoproducts at the sequence level in cellular DNA (23–25). However, the method is less sensitive than the other indirect assays.

More quantitative and specific determination of the yield of formation of a given photoproduct can be achieved upon hydrolysis of DNA into small molecules with a subsequent chromatographic separation using a method such as HPLC. The main advantage of the latter assay is that the DNA photoproducts arising from the different bipyrimidine sites can be individually measured with a high specificity depending on the detection system used. This approach has been applied to the quantitation of thymine cyclobutane dimer within isolated (26) and radiolabeled cellular (27, 28) DNA following hot formic acid treatment. Using the same hydrolysis procedure, the formation of cis-syn cyclobutane thymine dimers has been monitored within isolated DNA by GC-MS following trimethylsilylation (29). However, (6-4) photoproducts could not be analyzed in the two latter assays because of their instability under strong acidic conditions. A milder hydrolysis technique involving HF in pyridine allowed their quantitation within irradiated isolated DNA by HPLC coupled to a fluorescence detection (30). However, cyclobutane pyrimidine dimers could not be monitored in this assay because they are not naturally fluorescent. Altogether, the available chromatographic methods do not allow the simultaneous detection of the different classes of photoproducts.

Interestingly, the association of HPLC with electrospray ionization tandem mass spectrometry (HPLC-MS/MS) allows the simultaneous detection of the cis-syn and trans-syn 1 cyclobutane thymine dimers (c-s T<>T and t-s T<>T, respectively), the (6-4) adduct (6-4 TT), and its Dewar isomer (Dewar TT). The assay was first applied to the measurement of the level of...
Distribution of Thymine Photoproducts in UV-irradiated DNA

photoproducts—

TABLE I
Quantum yield of formation of the thymine photoproducts within isolated DNA exposed to UVC light

| Photoproduct | c-s T<->T<sup>a</sup> | t-s T<->T<sup>b</sup> | 6-4 TT | Dewar TT<sup>c</sup> |
|--------------|----------------------|----------------------|--------|----------------------|
|              | 10<sup>2</sup> × Φ    | <0.02                | 0.13   | <0.01                |

<sup>a</sup> Calculated from the linear part of the dose–curve of formation of the cis-syn cyclobutane dimer.

<sup>b</sup> Not detected for the lowest doses. The values reported in the table were calculated at higher doses, which were required for the detection of the photolesions.

<sup>c</sup> The four main thymine photoproducts within isolated DNA exposed to UVC and UVB radiation. The method was also found to be suitable for the quantitation of thymine dimeric photodamage within the DNA of cells exposed to biologically relevant doses of UVC and UVB lights. The major photoproduct was found to be c-s T<->T<sup>a</sup>, which was generated in a 8-fold higher yield than 6-4 TT. However, the Dewar valence isomer was not detected even at relatively high doses of UVB light.

EXPERIMENTAL PROCEDURES

Preparation of Calibrated Solution of Thymidyl-(3'-5')-thymidine (TpT) Photoproducts—The cis-syn and trans-syn 1 cyclobutane dimer together with the (6-4) photoproduct of TpT were obtained by UVC irradiation of the dinucleoside monophosphate followed by HPLC purification as previously reported (31). The (6-4) photoproduct solution was calibrated by measuring its absorbance at 320 nm. The concentration was determined by using the molecular absorption coefficient of the compound reported in the literature (32). An aliquot fraction of the latter solution was exposed to UVB light for increasing periods of time until no residual absorbance in the UVB region remained. This provided a calibrated solution of the Dewar valence isomer. The calibration of each of the solutions of c-s T<->T<sup>a</sup> and t-s T<->T<sup>b</sup> was achieved by quantitatively photoreverting an aliquot fraction of the lesions into TpT. The molecular absorption coefficient of TpT at 260 nm had been determined previously (ε = 7400 cm<sup>-1</sup>·liter·mol<sup>-1</sup>) and was used in the spectrophotometric determination of the concentration of the photoreverted solutions.

HPLC-MS/MS Detection of the Dimeric Photoproducts of TpT—Samples were injected onto a HPLC system consisting of a 7100 Hitachi-Merk pump (Merk, Darmstadt, Germany) associated to a SILL-9 automatic injector (Shimadzu, Tokyo, Japan). The column was an Upisphere ODB (150 × 2 mm internal diameter, 5 µm particle size) octadecysilyl silica gel column (Interchim, Montluçon, France). The mobile phase was a gradient of 5 m% ammonium formate that was prepared form ACS reagent grade formic acid and 99.99% ammonium hydroxide (Aldrich) and methanol (HPLC grade, Carlo Erba, Milano, Italy). The flow rate was 200 µl/min. The proportion of methanol rose from 0 to 2% within 5 min and reached 28% after 20 min. Methanol was also added at the outlet of the column prior to the inlet of the mass spectrometer at a flow rate of 0.2 ml/min. The API 3000 spectrometer (Perkin-Elmer/SCIEX, Toronto, Ontario Canada) was operated in the negative mode. Analyses were performed in the multiple reaction monitoring mode. Two transitions were monitored simultaneously: 545 → 447 and 545 → 532. The dwell time was set at 1 s for both signals. Calibration of the response of the spectrometer was obtained by injecting increasing amounts of a mixture of the authentic photoproducts prior to each series of injection. For a given series, 2 µmol of each photoreversion of the four photoproducts was injected every 10th sample to control the stability of the signal.

UV Irradiation of Isolated DNA—Calfl thymus DNA (Sigma) was exposed to UV light in aqueous solution (1 mg/ml, 5 ml) in Petri dishes (3.5 cm in diameter). The lamp (VL 215G, Bioblock Scientific, Illkirch, France), placed 10 cm above the DNA solution, was equipped with two 15-W tubes mostly emitting at either 254 or 312 nm. The emission spectrum of the UVB lamp (normalized at 312 nm) was as follows: 280 nm, 5%; 290 nm, 40%; 312 nm, 100%; 330 nm, 60%; 350 nm, 20%; 370 nm, 5%. The fluence, measured by a VLX 3W radiometer (Vilber Lourmat, Marne La Vallée, France) equipped with either a CU 254 or a CU 312 probe, was determined to be 2.4 and 2.8 kJ·m<sup>-2</sup>·min<sup>-1</sup> for the 254- and 312-nm sources, respectively. UVR-irradiated DNA was also performed at a fluence of 139 J·m<sup>-2</sup>·min<sup>-1</sup> with the lamp placed 1 m above the samples. For each irradiated solution, three 100-µl aliquot fractions were independently analyzed. The quantum yield of UVC-induced formation of thymine dimeric photoproducts within DNA was inferred from a comparison with the rate of formation of photohydrates in a 1,3-dimethyluracil (Aldrich) solution exhibiting the same absorption as the DNA solution (Table I). The slope of formation of the photoproducts in the two systems were compared and the quantum yields of formation of the DNA lesions were inferred from the value for the photohydration of 1,3-dimethyluracil, which is 0.0139 (33). The yields, expressed with respect to thymine, took into account the proportion of bipyrimidinic sites within DNA as reported by Patrick (26).

UV Irradiation of Cells—THP1 monocytes, grown as described previously (34), were irradiated as a suspension in phosphate-buffered saline (15 × 10<sup>6</sup> cells in 15 ml) in plastic Petri dishes (8.5 cm diameter) placed approximately 1 m below the lamp. Three samples were irradiated simultaneously to reduce the time during which cells were handled. The dishes were placed in a position at which the fluence was 109 and 118 J·m<sup>-2</sup>·min<sup>-1</sup> for the 254- and 312-nm irradiation, respectively. Irradiations were also performed with 15 × 10<sup>6</sup> cells suspended in 5 ml of phosphate-buffered saline, which were placed in a 3.5-cm-diameter Petri dish. The UVB source was the same than for the first series of experiments, but the UV lamp was shaded in order to provide a fluence of 9 J·m<sup>-2</sup>·min<sup>-1</sup>. DNA was extracted from cells using a NaI precipitation based chaotropic method (35). Then, purified DNA (50–60 µg) was solubilized in 100 µl of water.

DNA Analysis—Calfl spleen phosphodiesterase (0.004 units; Roche Molecular Biochemicals) and 10 units of nuclease P1 (Sigma) were added to the DNA solution (100 µl) together with 10 µl of buffer (200 mM succinic acid, 100 mM CaCl<sub>2</sub>, pH 6). The resulting solution was incubated for 2 h at 37 °C. Then, 10 µl of buffer (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.003 units of snake venom phosphodiesterase (Roche Molecular Biochemicals) and 5 units of alkaline phosphatase (Roche Molecular Biochemicals). The sample was incubated at 37 °C for 5 h. The resulting solution was centrifuged (5000 × g) and subsequently transferred into HPLC injection vials. An aliquot fraction of the sample (5 µl) was added to 45 µl of water, and the resulting solution was analyzed by HPLC with UV detection to determine the amount of DNA on the basis of the area of the peaks of normal nucleosides.

RESULTS

HPLC-MS/MS Analysis of TpT Photoproducts—Electrospray ionization (ESI) mass spectrometry is a suitable technique for the characterization and the measurement of a wide range of molecules (36). The electrospray interface allows coupling with HPLC, which is a major advantage for analytical purposes. Sensitivity is drastically increased by using tandem mass spectrometry (ESI-MS/MS) operating in the multiple reaction monitoring mode. A given molecular ion is collected by a...
first quadrupole and then fragmented in a second quadrupole that contains a low pressure of inert gas (collision cell). A third quadrupole is set to only detect the major daughter ions of the molecule of interest. This approach provides a highly specific and sensitive analytical tool. Although thymine and thymidine photoproducts can be readily analyzed by ESI-MS/MS (37), modified dinucleoside monophosphates were chosen as the analytes for the measurement of photoproducts at the DNA level. Indeed, TpT photoproducts can be analyzed as negative ions. This is a major advantage in a method involving the coupling of HPLC to mass spectrometry. Indeed, the contribution of the mobile phase to the background level of the signal is reduced in the negative analysis mode with respect to the positive one. In addition, the mass spectra of the two classes of photoproducts (c-s T<>T and t-s T<>T on one hand, and 6-4 TT and Dewar TT on the other hand) exhibit different fragmentation patterns even though their molecular weights are the same (Fig. 2). This was a major advantage in the detection of the c-s T<>T and Dewar TT, which were eluted close to each other from the HPLC column. Monitoring the two different transitions allowed to unambiguously detect them in a specific way. 6-4 TT and t-s T<>T, which were eluted more slowly from the column, were well separated. A third advantage of detecting photoproducts as dinucleoside monophosphates is that the phosphate group is easily ionized, and the response is very stable. Therefore, injection of purified photoproducts in solution either in plain water or mixed with 20 μg of hydrolyzed DNA provided the same response (Fig. 3). This observation, together with the good stability of the response during long periods of time (more than 48 h of continuous injection), avoids the use of isotopically labeled internal standards that are often necessary in chromatographic techniques associated with a mass spectrometry detection (38). The other key step in the assay is the enzymatic release of the photoproducts as modified dinucleoside monophosphates. This was achieved by the sequential use of phosphodiesterases hydrolyzing DNA first from the 5’-end to the 3’-end and then from the 3’-end to the 5’-end. Indeed, previous works have shown that the progression of phosphodiesterases is blocked by the presence of either a cyclobutane dimer or a (6-4) photoprotein (32, 39). It should be mentioned that nuclease P1 and alkaline phosphatase treatments were added to the enzymatic digestion procedure. Incubation with nuclease P1 was aimed at hydrolyzing the double-stranded DNA into shorter fragments and at eliminating the 3’-phosphate groups from oligonucleotides produced upon incubation of DNA with calf spleen phosphodiesterase. Previously published work has shown the hydrolysis of the intradimer phosphodiester bond of cis-syn cyclobutane thymine dimer by nuclease P1 (39). However, this was observed neither for the (6-4) adducts of TpdC and dCpT (32) nor for oligonucleotides containing thymine photoproducts (40). In the present work, no decrease in the signal was observed upon HPLC-MS/MS analysis of a 1 μl solution of the four TpT photoproducts incubated for increasing periods of time (up to 2 h) in the presence of 0.1 unit/μl of nuclease P1. The alkaline phosphatase treatment was aimed at releasing all the normal nucleobases as nucleosides in order to allow the determination of the amount of DNA present in the sample by a HPLC-UV measurement.

**Formation of Thymine-Thymine Photoproducts within Isolated DNA**—Two sources of UV light were used in the study. The broad band UVB lamps exhibited an emission maximum centered around 312 nm, whereas the UVC lamps emitted mainly at 254 nm. Upon exposure to UVC light, c-s T<>T was the major photoproduct (Fig. 4). However, the formation of the latter dimer was not linear and reached a plateau at a dose of 20 kJ/m². The yield of formation of c-s T<>T was similar to that measured by GC-MS within DNA irradiated under similar conditions (29). In contrast to that observed for the c-s T<>T, the yield of 6-4 TT was proportional to the dose of UVC light over the range of applied doses. t-s T<>T and Dewar TT were produced in very low yields. Interestingly, the quantum yield of formation of c-s T<>T (φ = 0.0205) is similar to a previously reported value (φ = 0.0193) (26). However, it should be added that the presently measured yield of photo-induced c-s T<>T is at least one order of magnitude higher than that determined using a 32P-postlabeling assay (41). This discrepancy may be explained by a poor quantitative aspect of the mild enzymatic release of the photoproduct as trinucleotide in this method. The HPLC-MS/MS assay allowed the determination of the formation yield of 6-4 TT, Dewar TT, and t-s T<>T, for which no reliable data were available. When DNA was exposed to UBV light within a dose range between 0 and 350 kJ/m², c-s T<>T was still the major photoproduct, and its formation was linear with respect to the dose up to 100 kJ/m² (Fig. 5A). The formation of t-s T<>T was not linear, and its efficiency increased with the overall UBV dose (Fig. 5B). In contrast to the results obtained upon UVC irradiation, the formation of 6-4 TT
rapidly reached a plateau (Fig. 6). The formation of Dewar TT was clearly quadratic, and its level increased above that of its (6-4) adduct precursor for doses higher than 50 kJ m⁻². Interestingly, Dewar TT was not detected within the DNA samples exposed to the lowest doses (below 3.5 kJ m⁻²). Therefore, irradiation of DNA was repeated with a 100-fold lower dose range (0–2.8 kJ m⁻²). Under these conditions, the formation of both c-s T<>T and 6-4 TT was linear with respect to the dose.
The respective yields of formation of the two latter lesions were 0.128 and 0.010 lesions/J m\(^{-2}\) per 10\(^6\) bases. Dewar TT was only detected at the highest dose. In the latter sample, the level of Dewar TT represented less than 2% of that of its (6-4) adduct precursor. Exposure of TpT to UVB light yielded the (6-4) photoproduct together with its Dewar valence isomer. The dose-course formation of 6-4 TT was linear, whereas that of Dewar TT was not (Fig. 7A). In contrast, the production of the Dewar valence isomer upon UVB irradiation of pure 6-4 TT was linear with respect to the dose (Fig. 7B).

Formation of Thymine-Thymine Photoproducts within Cellular DNA—The doses of UV light applied to cells were much lower than those for experiments involving isolated DNA. Doses of UVB light were kept below 600 J m\(^{-2}\) to minimize cell killing (Fig. 8A). The light was delivered with a fluence equivalent to that of natural solar light (approximately 0.2 mW cm\(^{-2}\)). Under these conditions, irradiation lasted less than 5 min. Two experiments were carried out using UVC light, at maximal doses of either 80 (Fig. 8B) or 500 J m\(^{-2}\). The yield of formation of the photoproducts were expressed in lesion/J m\(^{-2}\) per 10\(^6\) bases (Table II). As observed within isolated DNA, c-s T<>T was produced in higher yield than the related (6-4) photoproduct upon exposure to both UVC and UVB radiation. On the other hand, t-s T<>T and Dewar TT were not detected within the DNA extracted from irradiated cells. Surprisingly, the yield of photoproducts produced per J of UVC light was lower in the low dose experiment. This was explained by the fact that the size of the irradiation dishes had to be minimized in order to avoid sunburn of the cultured cells. Indeed, the total amount of DNA extracted from cells was 10–15 times lower in the low dose experiment. This was explained by the photoreversion of this class of photoproducts upon exposure to light. The observation that the formation of the cis-syn cyclobutane dimer reached a plateau upon exposure of DNA to UVC light can be explained by the photoreversion of this class of photoproducts (45). Indeed, c-s T<>T exhibits a UV absorption maximum around 230 nm but also a residual absorption at 254 nm. This leads to the photoreversion of c-s T<>T residue into thymine when their level within DNA increases. The observation that the formation of c-s T<>T is linear upon exposure of DNA to UVB can be explained by the very low absorption of the cyclobutane dimers at these wavelengths. Also interesting is the observation of the formation of t-s T<>T upon UVB and, to a lesser extent, UVC irradiation. The latter photoproduct has already been detected within photosensitized DNA (46), but this is the first observation of the formation of t-s T<>T within native DNA exposed to relatively low doses of far-UV light. The efficiency of the formation of t-s T<>T increases upon exposure to UVB. This may be accounted for by the presence of other

**FIG. 6.** Formation of photoproducts within isolated DNA exposed to UVB radiation: 6-4 TT (C) and Dewar TT (D). The results represent the average ± S.D. of three determinations for each irradiated DNA sample.

**DISCUSSION**

Even though the cis-syn thymine cyclobutane dimer was isolated 40 years ago (42), there is still a lack of basic information, such as the respective yields of formation of the different photoproducts within DNA for each possible bipyrimidinic sequence. In this respect, we report the simultaneous measurement of four thymine dimeric photoproducts by HPLC associated with electrospray ionization-MS/MS operating in the multiple reaction monitoring mode. The latter technique has already been applied to the sensitive detection of 8-oxo-7,8-dihydro-2’-deoxyguanosine, a major DNA oxidation product (43, 44). The limitation of sensitivity of the assay for UV-induced thymine lesions ranges between 10 and 50 fmol, depending on the photoproduct. This allowed the accurate quantitation of the photoproducts in isolated and cellular DNA. In this series of experiments, thymine photoproducts could be detected at doses as low as 1.5 J m\(^{-2}\) of UVC light in the DNA of cultured monocytes. Experiments were carried out with a large number of cells, but only 10–15 µg of the extracted DNA was analyzed. This shows that the HPLC-MS/MS assay is as sensitive as other methods involving the use of either antibodies or DNA repair enzymes. In addition, it allows an unambiguous assignment of the measured lesions together with accurate quantitative analysis. It can also be added that the use of an enzymatic digestion of the DNA samples allowed the release of relatively unstable photoproducts, such as the Dewar valence isomer. Another interesting feature of the reported method is its experimental work-up, which is easier than available indirect assays and allows the simultaneous analysis of a large number of samples.

Experiments involving UV-irradiation of isolated DNA confirmed previously obtained results for cyclobutane pyrimidine dimers using chromatographic analysis of radiolabeled DNA. The observation that the formation of the cis-syn cyclobutane dimer reached a plateau upon exposure of DNA to UVC light can be explained by the photoreversion of this class of photoproducts (45). Indeed, c-s T<>T exhibits a UV absorption maximum around 230 nm but also a residual absorption at 254 nm. This leads to the photoreversion of c-s T<>T residue into thymine when their level within DNA increases. The observation that the formation of c-s T<>T is linear upon exposure of DNA to UVB can be explained by the very low absorption of the cyclobutane dimers at these wavelengths. Also interesting is the observation of the formation of t-s T<>T upon UVB and, to a lesser extent, UVC irradiation. The latter photoproduct has already been detected within photosensitized DNA (46), but this is the first observation of the formation of t-s T<>T within native DNA exposed to relatively low doses of far-UV light. The efficiency of the formation of t-s T<>T increases upon exposure to UVB. This may be accounted for by the presence of other
photoproducts that destabilize the DNA structure. Indeed, the rate formation of t-s T>T significantly increases for doses higher than 40 kJ m⁻², which correspond to the induction of 52 c-s T>T, 3 6-4 TT, and 2 Dewar TT per 10⁶ bases. By taking into account the lesions produced at other bipyrimidine sites, a level of 1 photoproduct per 100 bases can be estimated. Melting temperature studies and ¹H NMR experiments have unambiguously shown that all types of photoproducts induce a destabilization of the DNA double helix (47–50). This leads to an increase in the DNA flexibility, which has been associated with an increase in the rate of formation of cyclobutane pyrimidine dimers (51). Moreover, the formation of t-s T<T>T has been observed within denatured DNA, whereas it could not be detected within irradiated native DNA (52). Altogether, these observations suggest that the formation of t-s T<T>T within DNA exposed to high UVB doses results from a drastic structural modification. To confirm this hypothesis, isolated DNA was irradiated with UVC light to induce the formation of c-s T<>T and 6-4 TT and then further exposed to UVB radiation for increasing periods of time. Under these conditions, the formation of t-s T<>T was linear and its rate was close to that observed for the highest doses in the experiment involving the sole UVB irradiation (data not shown).

The present study involving isolated DNA also provided interesting insights into the UVB photolysis of the (6-4) photoproduct. Thymine (6-4) photoproduct was produced in a lower yield than the related cis-syn cyclobutane dimer upon both UVC and UVB irradiation. The predominance of the formation
of cyclobutane dimers at the thymine-thymine sequence has already been observed using dinucleoside monophosphates as model systems (32, 53). In addition, indirect methods have shown that cyclobutane pyrimidine dimers are produced in higher yields than (6-4) photoproducts (54), even though the different bipyrimidine sequences could not be taken into consideration. The ratio between the slope of the formation curve of c-s T<>T and 6-4 TT in their linear part is 15 and 13 upon exposure to UVC and UVB light, respectively. A particular emphasis was placed on the formation of the UVC-photosomerization product of the (6-4) adducts, namely the related Dewar valence isomer. The structure of the latter compound has been unambiguously determined in dinucleoside monophosphates (12, 55, 56). However, quantitative data on the rate of formation of this class of photodamage within DNA are still lacking. In the present experiments, Dewar TT was produced in a very low yield upon exposure of isolated DNA to low doses of UVC light. However, its rate of formation increased with the dose at the expense of its (6-4) photoproduct precursor. A non-linearity of the HPLC-MS/MS detection could be ruled out on the basis of the observation of a linear formation of Dewar TT upon exposure of pure 6-4 TT to low doses of UVB light. Altogether, these observations show that the photosomerization of the thymine (6-4) photoproduct within DNA is less efficient than in model systems (12, 53, 55, 56). This can be rationalized in terms of the effect of the residual absorption of normal bases in the UVB range rather than by conformational considerations. Indeed, the dose-course formation of Dewar TT upon exposure of TpT to UVB light exhibits the same quadratic shape than within DNA.

As observed within isolated DNA, c-s T<>T is produced in a higher yield than 6-4 TT upon exposure of the cells to either UVC or UVB light. Interestingly, the ratio of the rates of formation of these two photoproducts is 8, which is lower than within isolated DNA. This may be rationalized in terms of structural differences induced by the higher compaction of DNA within chromatin fibers. The yield of formation of c-s T<>T upon exposure of cells to UVC light is similar to that reported in the literature, which ranges between 2 and 10 lesions/Jm^-2 per 10^6 bases for various cellular systems (57–62). The fact that the values determined in the present work are among the lowest reported can be explained by the relatively high cell density during the irradiation, which appeared to be a key parameter for the reproducibility of the results. The high sensitivity of the HPLC-MS/MS assay allowed the use of low doses of UV light for the irradiation of monocytes. Therefore, the secondary photochemical reactions only observed upon exposure of isolated DNA to high doses of UV light did not occur within cellular DNA under the irradiation conditions used. In particular, no formation of Dewar valence isomer was observed upon UVC irradiation. Taking into account the sensitivity of the assay for Dewar TT, this observation shows that its yield is at least 100 times lower than that of its (6-4) adduct precursor. This is a striking result, which contrasts with data based on the use of antibodies expected to be specific for Dewar valence isomers (20, 63, 64). However, it should be remembered that such an immunological approach does not differentiate among the modified bipyrimidine sequences. Therefore, the response might be due to thymine-cytosine, cytosine-thymine, or cytosine-cytosine photoproducts. Nevertheless, the present results rule out the involvement of the Dewar valence isomer at thymine-thymine sites in the mutagenic properties of solar light.

In conclusion, a sensitive and highly specific HPLC-MS/MS assay has been applied to the determination of the yield of formation of thymine photoproducts upon exposure of isolated and cellular DNA to relatively low doses of UVC and UVB radiation. This provided relevant information on the respective contribution of the cis-syn cyclobutane thymine dimer and the corresponding (6-4) photoproduct to the degradation of DNA by UVB light. In addition, a major result is the lack of detectable amount of thymine Dewar valence isomer that should not be implicated in the genotoxic properties of UV radiation. Ongoing work aimed at extending the HPLC-MS/MS assay to the detection of other photolesions will provide further information on the relative yields of the different classes of photoproducts at the various bipyrimidine sequences.
Distribution of Thymine Photoproducts in UV-irradiated DNA

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