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The variety of UV-induced pyrimidine dimeric photoproducts in DNA as shown by chromatographic quantification methods

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Induction of DNA damage is one of the major consequences of exposure to solar UV radiation in living organisms. UV-induced DNA photoproducts are mostly pyrimidine dimers, including cyclobutane pyrimidine dimers, pyrimidine (6–4) pyrimidone photoproducts and Dewar valence isomers. In the last few decades, a large number of methods have been developed for the quantification of these pyrimidine dimers. The present review emphasizes the contribution of chromatographic techniques to our better understanding of the basic DNA photochemistry and the better description of damage in cells.

1. Introduction

As illustrated by the abundant literature, induction of DNA damage by UV radiation is a major event involved in mutagenesis, carcinogenesis and cell lethality.[1–3] DNA photoproducts are also signals for other effects of solar exposure such as immunosuppression, melanogenesis or erythema. The nature and the yield of UV-induced DNA damage strongly depend on the wavelength of the incident photons.[4–6] UVC (100–280 nm) exhibits maximal efficiency because it corresponds to the maximal absorption of DNA. However, UVC is not biologically relevant since it is blocked by stratospheric ozone. Yet, UVC is widely used as an efficient DNA damaging agent in the laboratory and as a sterilizing agent in germicidal lamps. In sunlight, UVB (280–320 nm) is the wavelength range best absorbed by DNA while UVA is more abundant but less damaging for the genome.

Absorption of UVB and UVC photons by DNA leads to the production of excited states of nucleic bases.[7,8] The absorbed energy is released by triggering photochemical reactions, among other pathways. The vast majority of the resulting photoproducts results from the formation of covalent bonds between adjacent pyrimidine bases, thymine (T) and/or cytosine (C). These pyrimidine dimers are of two main types: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidine photoproducts (64PPs). Evidence is accumulating for the formation of CPDs also upon absorption of UVA photons although with a much lower efficiency than in the UVC and UVB ranges. In cells, UVA also induces a number of oxidative lesions resulting from photosensitization processes such as singlet oxygen production and release of hydroxyl radicals.[9] The most common damage resulting from this oxidative pathway is 8-oxo-7,8-dihydro-guanine (8-oxoGua), together with DNA strand breaks.[10] A special case is bacterial spores where UVC and UVB irradiation leads to the formation of a specific dimeric lesion involving two thymines, the spore photoproduct (SP).[11]

Due to the biological importance of pyrimidine dimers, their detection in isolated DNA and in cells and skin has been a constant analytical challenge since the first discovery of thymine cyclobutane dimers at the end of the 1950s.[12,13] The purpose of this review is to present chromatographic techniques, compare their advantages and limitations with other approaches and to summarize the information obtained using such assays.

2. Structure and properties of pyrimidine dimers

Outstanding research efforts have been made to identify and characterize UV-induced pyrimidine dimers. The data reported in the present chapter were mainly obtained after irradiation of free bases or nucleosides, or of small DNA model systems such as dinucleoside monophosphates. These studies showed that the simple term "pyrimidine dimers" used in photobiology hides a large variety of reaction mechanisms and chemical structures.

2.1 Cyclobutane pyrimidine dimers

CPDs arise from a [2 + 2] cycloaddition between the C5–C6 double bonds of the two pyrimidine rings. This reaction leads

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to the formation of a 4 membered cyclobutane ring linking the two bases. Reviews on these early works are available.[14–16] Triplet excited states are known to be at the origin of CPDs as shown by the specific formation of this class of pyrimidine dimers upon photosensitised triplet–triplet energy transfer.[17] Yet, recent results support additional pathways involving singlet excited states. One line of evidence is the extremely high rate of the reaction which takes place in the 15 ps range as shown in single-stranded oligonucleotides by using time-resolved infrared spectroscopy.[18] Formation of CPDs has been documented for the four possible doublets of pyrimidines: TT, CT, CT and CC as well as for the minor base 5-methylcytosine (m^5C).

Several arrangements are possible for the position of the two pyrimidine bases with respect to the cyclobutane ring[19] (Fig. 1). First, the two bases can be on the same or on opposite sides of the cyclobutane moiety. These geometrical relationships define the *cis* and *trans* stereoisomers, respectively. In addition, the two bases can be parallel with the C5 atom of one pyrimidine linked to the C5 of the other, and consequently the two C6 atoms also bound to each other. Such a configuration is known as *syn*. In contrast, an *anti* configuration is defined when C5 atoms are linked to C6, in an antiparallel orientation of the two bases. An additional level of complexity comes from the presence of chiral atoms in the photoproducts. For dithymine CPDs, two enantiomers exist for the *trans,syn* and *cis,anti* CPDs of bases while *cis,syn* and *trans, anti* diastereoisomers are *meso* forms. Consequently, 6 diastereoisomers are produced with nucleosides. An even larger variety of stereoisomers is produced for CPDs involving one thymine and one cytosine. All of these diastereoisomers have been characterized, mostly for thymine, both at the base and nucleoside level.[15,16] *Cis,syn* and *trans,syn* CPDs have also been identified in dinucleoside monophosphates containing the various combinations of thymine, cytosine and 5-methylcytosine.[19–25] A second minor diastereoisomer of *trans,syn* TT CPD[26] has also been isolated at the dinucleoside monophosphate level.

Although CPDs are stable compounds in solution, they may undergo secondary reactions. First, they can be reverted to the original bases upon exposure to UVC radiation.[27] Loss of the two double bonds of the pyrimidine bases results in a shift of the maximal absorption from around 260 nm to 230 nm. A residual absorption of photons at 254 nm is still occurring and triggers the reversion of the initial photoaddition reaction. A second property of CPDs is a dark hydrolytic process which applies to all cytosine derivatives and is known as deamination.[28–30] In this reaction, a water molecule adds to the C4 carbon bearing the exocyclic amino group of cytosine. Loss of a NH_3 molecule from this intermediate produces a uracil moiety.[31,32] This process is very slow for unmodified cytosine but is strongly accelerated when the C5–C6 double bond is saturated. Accordingly, half reaction times of a few hours have been reported for TC, CT and CC CPDs in dinucleoside monophosphates. Interestingly, deamination is slower in *trans,syn* than in *cis,syn* CPDs.[30] Deamination of m^5C photoproducts is slower than those of cytosine.[33]

2.2 Pyrimidine (6–4) pyrimidone photoproducts and Dewar valence isomers

The second major class of bipyrimidine photoproducts namely 64PPs is also produced by a [2 + 2] cycloaddition involving the C5–C6 double-bond of the 5′-end pyrimidine. The second double bond is either the C4 carbonyl group of a 3′-end thymine or the imine function of a cytosine in a tautomeric form. A 4 member ring intermediate bearing an oxetane or an azetidine depending on whether the 3′-end base is T or C is produced (Fig. 2). This cyclic intermediate rearranges into the final product where a covalent bond links the C6 of the 5′-end to the C4 of the 3′-end pyrimidine while the C4 exocyclic group of the original 3′-end base is shifted to the C5 position of the 5′-end pyrimidine. This photochemical process, known as a

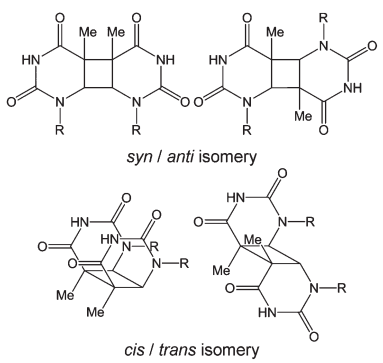

Fig. 1 The two stereoisomeries for CPDs. A *syn*/*anti* stereoisomery reflects the parallel or antiparallel orientation of the bases (upper panel) while a *cis*/*trans* stereoisomery corresponds to the relative position of the two pyrimidines with respect to the cyclobutane ring (lower panel). The figure shows thymine CPD (R = H in bases or 2-deoxyribose in nucleosides and DNA). The *cis* and *trans* isomers presented are *syn* derivatives but the equivalent *anti* isomers are also formed.

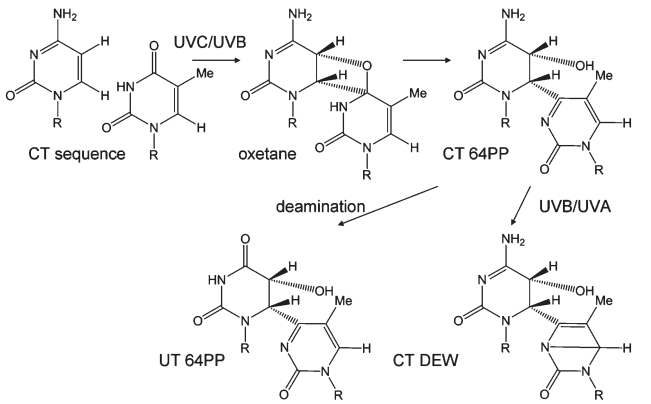

Fig. 2 Formation and fate of CT 64PP (R = H for bases or 2-deoxyribose in nucleoside and DNA). Photoinduced [2 + 2] cycloaddition leads to a transient oxetane that rearranges into 64PP. This photoproduct can then deaminate into its uracil derivative UT 64PP or undergo a secondary photoinduced intramolecular cycloaddition leading to CT DEW. The represented stereochemistry is that found in dinucleoside monophosphates and DNA.

Paterno–Bücchi reaction, is most likely triggered by singlet excited states. Evidence for the proposed mechanism has been provided by the isolation of the cyclic intermediate when a thymine-(3′–5′)-4-thiothymine dinucleoside monophosphate was irradiated.[34] Time-resolved spectroscopic measurements also showed that the final 64PPs are present after a much longer period of time than CPDs, in agreement with the formation of a transient photoproduct.[35]

64PPs were first isolated from UVC-irradiated DNA in a dehydrated form.[36,37] They were then isolated from thymine[38] and thymidine, as well as from bipyrimidine dinucleoside monophosphate.[23,39–41] The stereochemistry of 64PPs is simpler than that of CPDs since only two stereoisomers are produced upon irradiation of monomers and only one in dinucleoside monophosphates. Specific properties of 64PPs are related to the presence of a 3′-end pyrimidone ring. This moiety exhibits a maximal absorption at 325 or 315 nm in dinucleoside monophosphates depending whether or not it is substituted by a methyl at C5. Excitation at these wavelengths results in fluorescence emission at around 380 nm, with a quantum yield that is larger in constrained structures like dinucleoside monophosphates than in a base or nucleoside derivatives.[42]

An additional specific property of 64PPs, also related to the presence of the pyrimidone moiety, is the conversion into their related Dewar valence isomers upon absorption at 325/315 nm.[43,44] The resulting photoreaction is 4π electrocyclisation which leads to the creation of a new covalent bond between the N3 and C6 atoms of the pyrimidone, yielding a structure similar to that proposed for benzene by James Dewar in the XIXth century.[45] The Dewar valence isomers neither absorb UVB nor fluoresce. Yet, DEWs possess some UVC absorption which explains why they are reverted to 64PPs upon irradiation in this wavelength range.[24] Both 64PPs and DEWs deaminate when the 5′-end is a cytosine with a similar rate in dinucleoside monophosphates.[30,39] When only the 3′-end is a cytosine, deamination is not possible since the exocyclic amino group is at C5 on the 5′-end base.

2.3 Spore photoproduct

As early as 1965, analysis of DNA extracted from bacterial spores exposed to UVC revealed the presence of a photoproduct different from CPDs.[46] It was shown to be 5-(α-thyminyl)-5,6-dihydrothymine and is known as the spore photoproduct (SP) because it has never been detected in another living organism. The SP has also been isolated upon irradiation of thymidine both in frozen solution[19] and as a dry film.[47] Under the latter conditions, the two diastereoisomers resulting from the presence of the asymmetric C5 carbon could be isolated.[48] The SP is also produced in dry films and frozen solutions of DNA.[49] Yet, only one diastereoisomer also produced by photolysis in the dry state of the dinucleoside TpT is observed (Fig. 3).[50] Extensive NMR investigation showed that this compound exhibits a 5*R* configuration.[51]

Model studies have been designed to understand the specific photochemistry of DNA in spores. Dehydration was found to be a major cause of the formation of the SP since irradiation of dry films of isolated DNA leads to the formation of large amounts of this photoproduct.[52,53] Recently, it was shown that a significant fraction of these photoproducts are interstrand lesions although this was not the case in spores.[54] An additional key factor is the conformation of spore DNA in an A-like form resulting partly from dehydration but also from the presence of small acid soluble proteins which bind DNA in large amounts in spores and modify its structure.[53–55] Lastly, a small molecule present in large amounts in the spore core, calcium dipicolinate,[56,57] was found to behave as a photosensitiser.

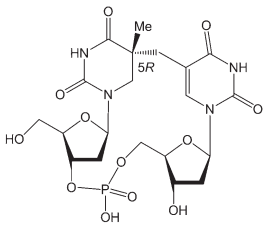

Fig. 3 Chemical structure of the spore photoproduct as a dinucleoside monophosphate. The represented diastereoisomer exhibits the 5*R* configuration observed in the SP released from irradiated DNA.

2.4 Minor UV-induced photoproducts

In addition to CPDs 64PPs and DEWs which represent the most frequent UV-induced DNA lesions, a few other photoproducts should be mentioned. Dimeric photoproducts involving adenine and thymine[58–60] or two adenine moieties[61] have been characterized in a model system exposed to UVC radiation. These lesions also arise from [2 + 2] cycloaddition. Both indirect and direct quantification in isolated DNA showed that their yield was about two orders of magnitude lower than that of the pyrimidine dimers.[61,62] Other UVC-induced photoproducts are monomeric damage: the stereoisomers of 6-hydroxy-5,6-dihydrocytosine known as cytosine hydrates.[63] They are produced upon addition of a water molecule to the C5–C6 double bond of the base. These photoproducts are unstable and either dehydrate or deaminate into uracil hydrates. Their yield is also much lower than that of CPDs and 64PPs in DNA,[64,65] although they may represent a large fraction of the photoproducts when monomeric systems are irradiated.

Another class of UV-induced DNA damage is mostly involved in the effects of UVA and results from oxidative photosensitized reactions.[9] The main process involves singlet oxygen that specifically reacts with guanine in DNA to yield only 8-oxoGua. Another possible photosensitisation pathway involves one electron oxidation that again specifically targets guanine with formation of 8-oxoGua and the ring-opened formamidopyrimidine derivative. Lastly, hydroxyl radicals ($^{\cdot}$OH) may be indirectly produced by UVA in cells. This reactive oxygen species is highly reactive and damages the 4 bases, leading to a wide variety of oxidation products. $^{\cdot}$OH can also react with the 2-deoxyribose moiety in the DNA backbone and induce

single strand breaks, but not double strand breaks as recently demonstrated.[66] It should be kept in mind that UVA is two or three orders of magnitude less efficient than UVB at damaging DNA, even in cells.[10]

3. Quantification of pyrimidine dimers in DNA

Identification of the chemical structure of pyrimidine dimers made possible the development of both biochemical and chemical tools for their quantification in DNA. A number of assays have been developed but the three main classes that are routinely applied involve immunological measurements, electrophoretic techniques and chromatographic quantification. Apart from technical flaws, no technique is better than another; the choice of approach only depends on the scientific question addressed and the type of samples available.

3.1 Main techniques available for the measurement of dimers

3.1.1 Antibody-based assays. Immunological detection of dimeric photoproducts is by far the most commonly used approach in photobiology today. This class of techniques relies on the production of polyclonal or monoclonal antibodies raised against UV-irradiated DNA or oligonucleotides.[67,68] Precise characterization works allowed several groups to produce antibodies specific against the three main classes of bipyrimidine dimers, namely CPDs, 64PPs and DEWs. These antibodies are then used in different types of assays to reveal the presence of photoproducts. These techniques, when applied to isolated or extracted DNA, include among other radioimmunoassays, enzyme-linked immunosorbent assay and immuno-dot-blot. In whole cells, flow cytometry has also been used. A unique opportunity afforded by immunological assays is the possibility to localize the photoproducts in tissues, for instance in skin, by using immunohistochemistry.[69,70] In addition, antibody-based techniques usually require rather small amounts of DNA. A limitation of immunological assays is that results are often obtained in arbitrary units rather than in absolute frequency of damage. This disadvantage can be overcome by calibrating the response of the antibodies[71] or by using a radioimmuno assay.[68] Another drawback is that, for a given type of dimeric photoproduct, no difference is made between the TT, TC, CT and CC derivatives.

3.1.2 Electrophoresis-based assays. Electrophoretic separation of biomolecules such as DNA and proteins is a powerful tool relying on large differences in molecular weight and electrical charge. Consequently, electrophoretic techniques have been extensively used for the quantification of strand breaks in DNA. Pulsed field gel electrophoresis has long been the reference technique for double-strand breaks while the Comet assay is becoming a standard for the detection of single strand breaks and alkali-labile sites.[72] Valuable data have also been obtained by alkaline gel elution.[10] Such methods cannot be directly applied to dimeric photoproducts which are stable and do not induce DNA cleavage. Yet, photoproducts can be converted into additional strand breaks by using purified repair enzymes as biochemical tools. The most commonly used is the endonuclease V of phage T4 that specifically recognizes CPDs.[73] Early works also relied on alkaline treatments to detect 64PPs which were thought to be unstable under these conditions. It appeared though that 64PPs were degraded but not cleaved and that the induction of strand breaks was more likely due to DEWs.[74] A powerful variant of the electrophoretic approach combines damage detection and sequencing. Techniques like ligation-mediated polymerase chain reaction[75,76] have thus shown the modulation of photoreactivity along specific targeted genes. A limitation of these sequencing techniques is the lack of sensitivity. Again, unless sequencing is performed, electrophoresis-based assays fail to provide information on the different bipyrimidine derivatives and are mostly applied to CPDs. Reliable information on 64PPs is much more tedious to obtain.

3.1.3 Chromatography-based assays. Analytical chemistry approaches have also been designed to quantify CPDs, 64PPs, DEWs and SP. These techniques all rely on three main steps, namely extraction of DNA, hydrolysis aimed at releasing the photoproducts and separation of the hydrolyzed DNA mixture on a chromatographic system with appropriate detection. The main advantage of this approach is that, based on the chromatographic properties of the dimers and sometimes the specific features of the detection, information is gathered for a given class of damage on the different bipyrimidine derivatives. As explained above, a very large number of diastereoisomers of all possible photoproducts involving the 4 pairs of pyrimidine bases may be produced. In principle, all these photoproducts can be separately quantified by chromatographic assays which actually were the basis of the isolation of all the diastereoisomers of dimers produced upon photolysis of bases and nucleosides. In DNA, steric hindrance and geometrical issues limit the possible configurations. Only one configuration has been observed for 64PPs and DEWs, while only *cis,syn* and to a lesser extent *trans,syn* CPDs can be produced. As a consequence, most assays focus on these latter diastereoisomers for TT, TC, CT and CC photoproducts.

A key step is the hydrolysis of DNA which should prevent degradation of the targeted lesions and be quantitative. Depending on the type of hydrolysis, photoproducts are recovered under different forms which influence the choice of chromatographic separation and detection techniques. These types of assays often require more DNA than other techniques and provide only an averaged value in a cellular population within a tissue. The main interest of the chromatographic techniques is that they provide an unequalled level of information on the formation and repair of the different bipyrimidine photoproducts. Moreover, calibration of the response of these techniques is easy and the obtained results are always quantitative, expressed in number of lesions with respect to a given number of normal bases (photoproducts per 10^6 bases for example). It should be stressed that developing chromatographic techniques rely on the availability of calibrated solutions of the

pure targeted analytes used as a reference. This part of the work is often the most tedious and time consuming step. A last important information is that cytosine-containing photoproducts are always detected as their stable deaminated derivatives. As a matter of fact, the duration of the sample treatment and the characteristics of the buffers used allow the quantitative conversion of cytosine photoproducts into uracil derivatives. A consequence is that m^5C photoproducts deaminate into T derivatives, namely TT, TC and CT photoproducts.

3.2 Radioactive detection of dimers following hydrolysis

All of the early information obtained in the 1960s and 70s on photoproducts in isolated and cellular DNA were performed on DNA labelled with radioactive atoms, mostly ^{14}C- and ^{3}H-thymine.[15,16] A few data were reported using labelled cytosine.[77] In most of these works, DNA was extracted from bacteria grown in the presence of radioactively labelled DNA bases. Hydrolysis was performed at high temperature under acidic conditions, usually formic acid[78,79] (Fig. 4). The hydrolyzed samples were then analyzed by paper, thin layer or liquid chromatography and the radioactivity of the fraction containing photoproducts was quantified.[15,78,80] A large number of very valuable data on CPDs were obtained in this way. Unfortunately, 64PPs were unstable under these rough hydrolysis conditions[81] and isolated semi-quantitatively only in a dehydrated form.[36,37] A more recent radioactivity-based technique is based on the ^{32}P approach typically used for the measurement of DNA adducts.[82] For the quantification of this class of damage, DNA is enzymatically hydrolyzed into 3′-monophosphate nucleotides which are subsequently labelled at the 5′-position with ^{32}P-phosphate by polynucleotide kinase (PNK). Unfortunately, the dimeric photoproducts released in the form of dinucleoside monophosphates are not substrates for PNK. Thus, DNA is hydrolyzed by phosphodiesterase I, a 5′-exonuclease which digests DNA fragments from the 3′- to the 5′-end. Its activity is inhibited at the photoproduct site and it consequently leaves an unmodified nucleotide. Subsequent treatment with phosphatase removes the 5′-end phosphate group and produces a good substrate for PNK (Fig. 5). A consequence of the presence of the unmodified nucleotide in the analyte is that each photoproduct has to be detected as four derivatives. Although thin layer chromatography has been used for the ^{32}P-postlabelling assay for photoproducts,[83] analyses are mostly performed on a HPLC reverse phase column using a gradient of methanol in an aqueous buffer containing ammonium formate and phosphoric acid.[84] Detection is provided by on-line radioactivity counting. ^{32}P-postlabeling was also applied to the detection of CPDs as dinucleoside monophosphate in urine.[85,86] These compounds are expected to result from DNA repair. In this case, TT CPDs are reverted to dinucleoside monophosphate bearing two normal thymines by UVC irradiation, making possible the action of PNK.

3.3 Liquid chromatography associated with mass spectrometry

Recently, high performance liquid chromatography associated with electrospray tandem mass spectrometry (HPLC-MS/MS) has received numerous applications in the field of DNA

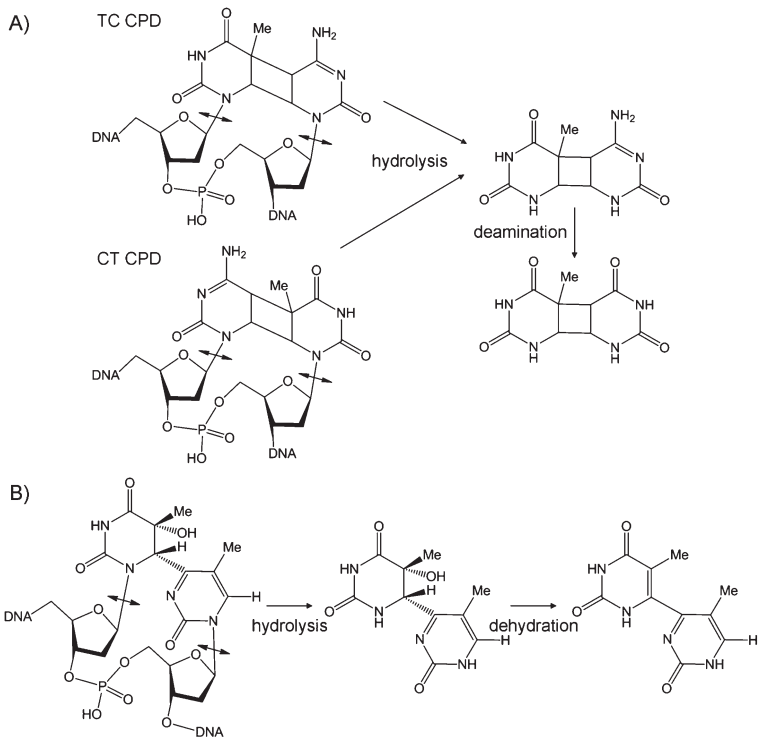

Fig. 4 Acidic hydrolysis of (A) CPDs and (B) 64PPs from DNA. The upper panel illustrates how TC and CT CPDs are released as enantiomers of the same base derivative and undergo deamination. The lower panel shows the dehydration of 64PPs under strong acidic conditions.

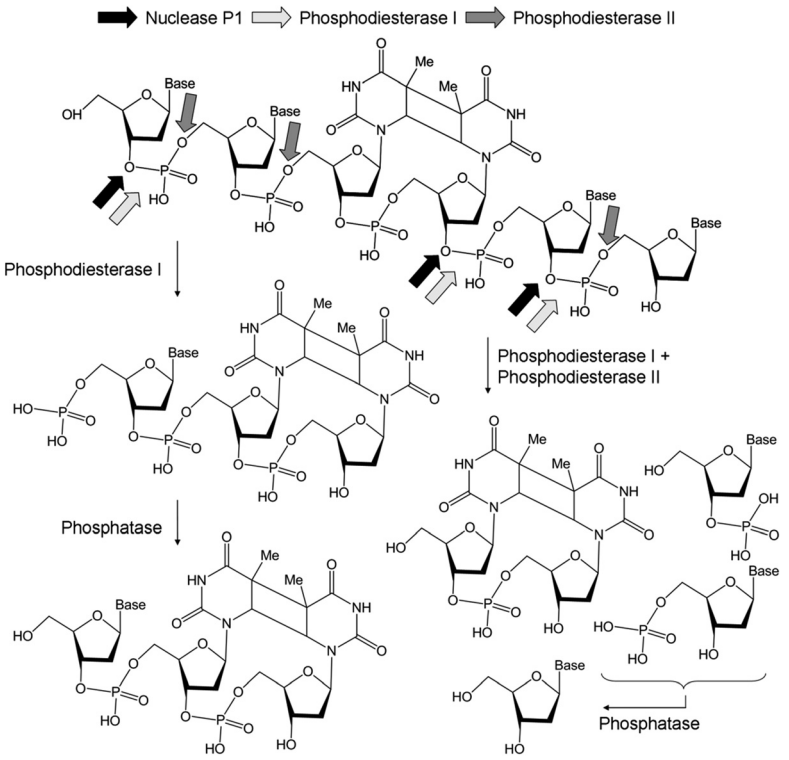

Fig. 5 Enzymatic hydrolysis of photoproduct-containing DNA with sites of cleavage of specific enzymes (arrows). Phosphodiesterase I is a 5′-exonuclease while phosphodiesterase II is a 3′-exonuclease. Nuclease P1 is also a 5′-exonuclease but is mostly used for its endonuclease activity. Acid or alkaline phosphatase is also used to facilitate the hydrolysis and produce unphosphorylated analytes.

adducts and oxidized DNA bases.[87–89] For the quantification of photoproducts, DNA is hydrolyzed by a combination of DNA endonucleases, exonucleases and phosphatases.[90,91] Phosphodiesterase II exhibits a mirror activity of that of phosphodiesterase I. It is a 3′-exonuclease hydrolyzing DNA from the 5′- to the 3′-end and leaving a normal nucleotide on the 3-end of a photoproduct. The combined action of phosphodiesterases I and II, in the presence of phosphatases which dephosphorylate DNA fragments, leads to the release of the photoproducts as dinucleoside monophosphates (Fig. 5). In this process, normal bases are released as nucleosides. The enzymatic cocktail also often contains endonucleases such as nuclease P1 or DNase II in order to cleave large fragments of genomic DNA into smaller substrates for exonucleases. Nuclease P1 is also a 5′-exonuclease. In contrast to what has been once published for nuclease P1,[92] the phosphodiester link between the two nucleotides involved in a dimeric photoproduct is not cleaved by endo- and exonucleases.[93,94] It should be stressed that this quantitative hydrolysis procedure is much milder than acidic treatments and prevents degradation of 64PPs and DEWs. Another advantage is that because part of the DNA backbone remains in dinucleoside monophosphates, separation of TC and CT derivatives is possible. This is not the case after acidic hydrolysis which releases TC and CT CPDs as undistinguishable enantiomers of the same base photoproduct.

The hydrolysed mixture is injected onto an HPLC system where separation takes place on a reverse phase column used with a gradient of methanol or acetonitrile in an aqueous buffer.[91] The latter is a solution of triethylammonium acetate which interacts with the phosphate groups of the dinucleotide monophosphates. In this ion suppression mode, the peak width of the analytes and the resolution of the separation are much improved. Normal nucleosides are quantified on-line by a regular UV detector and the eluent of the HPLC system is directed toward the inlet of a mass spectrometer with electrospray ionisation operated in the negative mode in order to take advantage of the presence of the negative charge of the phosphate groups of the dinucleoside monophosphates.[48] For sensitive and specific detection, the apparatus uses a triple quadrupolar technique for ion separation and is operated in the multiple reaction monitoring mode. In this approach, the pseudo-molecular ion of the analyte is isolated in the first quadrupole and then fragmented in the second one. The third quadrupole is set on the mass to charge ratio (m/z) corresponding to specific fragments of the targeted molecule. This detection is thus very specific, with two stages of selection of the ions, as well as very sensitive because the noise is very low. The limit of detection is in the range of 1 to 50 fmoles depending on the compound and the equipment used.[90,91] This sensitivity makes possible the detection of dimers in skin biopsies, namely 2 or 3 μg of DNA, exposed to less than 1 standard erythemal dose.[95]

Because they all exhibit different retention times or mass spectrometry features, the TT, TC, CT and CC CPDs, 64PPS

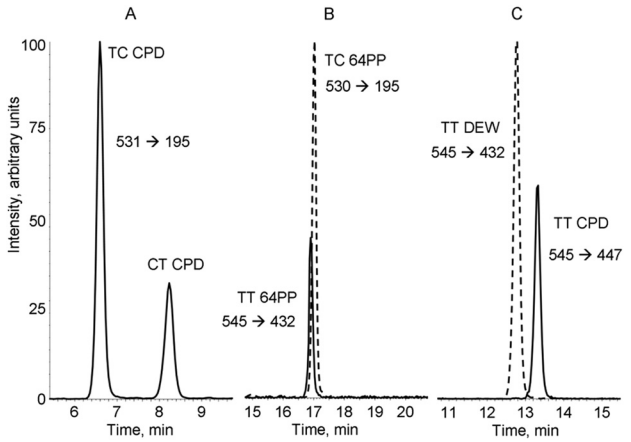

Fig. 6 HPLC-MS/MS chromatograms showing the separate detection of a series of bipyrimidine photoproducts under the form of dinucleoside monophosphates. The reported values represent the mass spectrometry fragmentation monitored for each compound.

and DEWs can all be individually detected in a single HPLC run. The interest in HPLC-MS/MS detection is illustrated by several examples (Fig. 6). The deaminated forms of TC and CT CPDs produce the same pseudo-molecular ions which generate the same fragments. Fortunately, their retention times differ by 2 min and they can be individually quantified. Conversely, TT and TC 64PPs almost co-elute. However, they do not have the same molecular weight and are easily detected on separated channels of the mass spectrometer. An even more interesting example is the detection of TT CPD and DEW. These two photoproducts are eluted in the same portion of the chromatogram. In addition, they have the same molecular weight. However, they can be separately quantified because they do not produce the same daughter ions upon fragmentation. Another advantage of HPLC-MS/MS is the large difference in retention time between *cis,syn* and *trans,syn* CPDs which can be quantified separately. This precise identification of the different photoproducts is possible only because pure standards are available. In addition, these reference solutions are necessary to calibrate the response of the mass spectrometry detector which is not equivalent for all photoproducts. A last interest of the HPLC-MS/MS analysis is a rather high throughput with approximately 100 samples analyzed per week.

3.4 Other chromatographic approaches

Other chromatographic assays although receiving limited application have been reported. An example is gas chromatography combined with mass spectrometry detection. This approach requires the release of CPDs by hot acidic treatment followed by derivation to convert CPDs into volatile derivatives.[96,97] This assay was first designed for oxidized bases[98] but was found to induce artefacts because of spurious oxidation during the derivation step. No such problem is expected for CPDs but only limited applications have been reported. Another worth mentioning technique was designed to quantify 64PPs following quantitative acidic hydrolysis. As discussed above, classical treatments such as concentrated formic acid hydrolysis lead to the degradation of 64PPs with release of a dehydrated form and several by-products. This limitation was overcome by using HF stabilized in pyridine as a mild reagent for DNA hydrolysis.[81] Quantification by HPLC with fluorescence detection provided valuable information on the respective yield of TT, TC and CT 64PPs in isolated DNA. Unfortunately, the sensitivity of this technique was not high enough to be applied at the cellular level. Other chromatography-based strategies have been designed for the quantification of CPDs such as HPLC associated with thermospray mass spectrometry[99] or capillary electrophoresis[100] but no application has been reported.

4. Photochemistry of isolated DNA

4.1 Formation in regular B-form DNA

Chromatographic techniques have provided a very precise description of the yield and relative distribution of the different photoproducts upon UVC irradiation of isolated DNA. Care should be taken not to apply too large UVC doses in order to limit reversion of CPDs. Evidence has also been provided for a UVC photolysis of 64PPs[101] but this reaction is much less efficient than reversion of CPDs. In DNA, this difference is shown by the linear increase in the level of 64PPs for UVC doses corresponding to a plateau for CPDs.[90,91] Both radioactive detection following acidic hydrolysis and later HPLC-MS/MS showed that the *cis,syn* isomer of TT CDP was the major photoproduct in B-DNA.[49,79,90,102] No *trans,syn* derivative is detected in stable duplexes. In addition, formation of *anti* derivatives is not possible between adjacent pyrimidine bases that adopt a parallel orientation because of their link to the phosphodiester backbone of DNA. TC CPD is produced in a roughly two-fold lower yield while CT and CC photoproducts are less frequent. 64PPs are accurately quantified by HPLC-MS/MS.[90] TT 64PP is produced in a 10-fold lower yield than the corresponding CPD while 64PP is only slightly less frequent than CPD at TC sites. CT 64PP can only be detected at a high UVC dose. CC 64PP is produced with a slightly higher efficiency than the CT derivative in UVC-irradiated DNA but still in a much lower amount than the TC 64PP which remains the most frequent of this class of photoproduct. These photoreactions do not exhibit a high quantum yield; it remains around 1.5×10^{-3} when all photoproducts are considered.[103] Values of 2% have been published for TT CPD[15,16,102] but these data are corrected for the frequency of TT dinucleotides. Uncorrected values are also in the range of 10^{-3}. Table 1 reports formation quantum yields in isolated calf thymus DNA exposed to 254 nm radiation.

In addition to the UVC photochemistry of DNA, studies have been performed at other wavelengths. Action spectra for the formation of CPDs obtained using radioactive detection revealed that the yield of photoproducts was reduced by at least one order of magnitude between the maximum in the UVC range and the longest UVB wavelengths.[49] Detailed

Table 1 Quantum yields[a] of formation of *cis,syn* CPDs (c,s CPD) and 64PPs in isolated calf thymus DNA[b] as determined by HPLC-MS/MS[90,103]

Photoproduct	Quantum yield (×1000)
TT c,s CPD	0.548 ± 0.031
TT 64PP	0.033 ± 0.001
TC c,s CPD	0.300 ± 0.032
TC 64PP	0.286 ± 0.008
CT c,s CPD	0.103 ± 0.010
CT 64PP	0.001 ± 0.000
CC c,s CPD	0.031 ± 0.004
CC 64PP	0.008 ± 0.005

[a] Values were uncorrected for the frequencies of the respective bipyrimidine sequences. [b] DNA was irradiated with 254 nm radiation in 0.1 M NaCl solution in ice to maintain stable double-stranded structure.

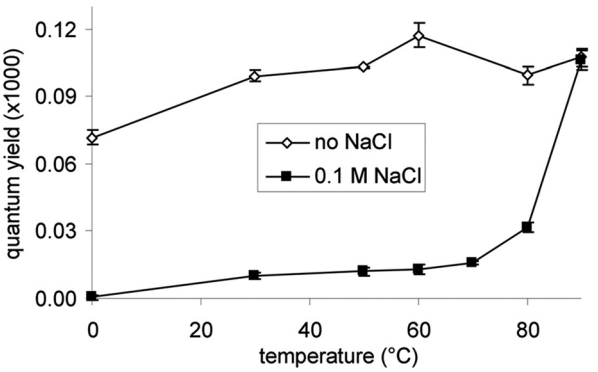

Fig. 7 Quantum yield of formation of all *trans,syn* isomers of CPDs in isolated calf thymus DNA exposed to UVC radiation (254 nm).[103] DNA was in solution either in pure water where denaturation partly took place even at low temperature or in 0.1 M NaCl where the melting point of DNA was around 85 °C.

distribution of DNA photoproducts exposed to broadband UVB was reported. Although the yield was lower than with UVC, the relative distribution of CPDs and 64PPs remained similar when low doses were applied.[90] Formation of DEWs by UVB requires first formation of 64PPs followed by absorption of a second photon for the isomerisation reaction. Consequently, the time-dependent formation of DEWs is quadratic, with minute amounts, below the detection limit, produced at low UVB dose.[90] It may be added that CPDs are produced in isolated DNA by direct UVA excitation[104–106] and by photosensitized triplet–triplet energy transfer.[107–110] In contrast to what has been reported in some work based on immunological approaches[71,111] but not all,[112] 64PPs are not detected by the more specific chromatographic assays in isolated or cellular DNA exposed to UVA or photosensitized by triplet energy transfer.[113–115] With both types of irradiation conditions, TT CPDs are the predominant damage while CT and TC CPDs are produced in much lower yields. Another type of radiation applied to DNA and for which induced photoproducts were chromatographically quantified is high intensity UVC laser.[116] It was found that as the laser intensity increased, the quantum yield of CPDs and 64PPs decreased. This was explained by the occurrence of biphotonic ionization which depleted the population of initial excited states.

4.2 Structural effects

DNA is a dynamic molecule and its conformation is not limited to the regular B-form. A number of parameters can modify its photoreactivity. The most drastic one is denaturation into two single-stranded DNA fragments. This process is easily achieved by increasing the temperature until the so-called melting point. Interestingly, only limited modulation of the distribution of photoproducts is observed below this temperature. When denaturation occurs, the overall yield of CPDs and 64PPs is significantly reduced.[78,103] In addition, *trans,syn* isomers of CPDs appear whereas they were not detected at lower temperature (Fig. 7). A more subtle effect is the decreased ratio between the amounts of 64PP and CPD, mostly at TC sites.[103] The formation of the *trans,syn* CPDs is a sign of flexibility around the *N*-glycosidic bonds of bases in denatured DNA. Accordingly, *trans,syn* CDPs are also detected in single-stranded oligonucleotides and dinucleoside monophosphates, yet always in lower amounts than the *cis,syn* isomers. Similar results can be obtained by denaturing DNA by lowering the ionic strength of the irradiated solutions.[117]

Other drastic effects can be observed when DNA keeps its double-stranded structure but changes the conformation. For instance, A-DNA, obtained when DNA is in solution in the presence of large amounts of ethanol, is characterized by a rather different photochemistry than in the canonical B-form. Under the former conditions, the photochemistry is drastically changed.[79] In particular, inter-strand photoproducts are produced which could be unambiguously identified since they are enzymatically released from DNA as dimers of nucleosides, with the 2-deoxyribose moieties but without phosphate. These inter-strand dimers could thus be specifically quantified by HPLC-MS/MS.[118] Interestingly, a large fraction of the corresponding CPDs were *anti* isomers as expected when bases with *anti* parallel orientation react with each other on opposite strands. Only little inter-strand 64PPs were produced. The formation of inter-strand photoproducts was even more efficient when freeze-dried films of double-stranded were exposed to UVC. Interstrand photoproducts may also play an important role in telomeres. In these portions of the genome, DNA exhibits a specific quadruplex structure that facilitates formation on *anti* CPDs, identified on the basis of HPLC-MS/MS analyses.[119]

4.3 Effect of DNA composition

The relative distribution of the various photoproducts does not only depend on the irradiation conditions but also on the intrinsic properties of DNA. A nice example is the composition of the nucleic acids in G : C and A : T base pairs. This topic is not really an issue for eukaryotic cells where the percentage of G : C base pairs (% GC) is always around 40–45%. In contrast a much wider variation is observed in bacteria with the %GC ranging from 25 to 75%. Obviously, differences in %GC induce differences in the respective frequency of the 4 bipyrimidine

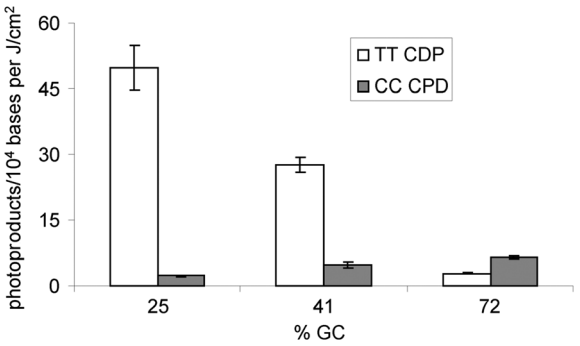

Fig. 8 Yield of formation of TT and CC CPDs in UVB-irradiated isolated DNA exhibiting increasing %GC.[120] Photoproducts were quantified by HPLC-MS/MS.

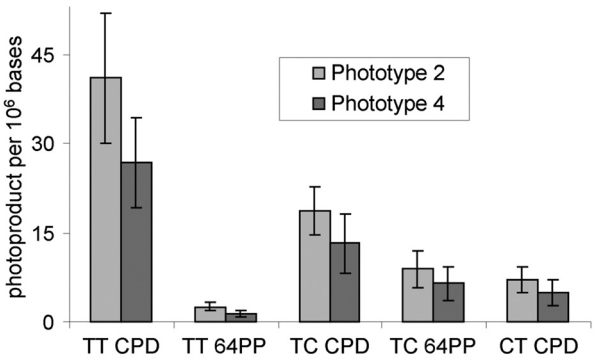

Fig. 9 Level of bipyrimidine photoproducts in human skin explants exposed to UVB radiation (200 J m^{-2}).[132] Skin was irradiated *ex-vivo*. Data were obtained from 12 phototype 2 and 12 phototype 4 donors.

dinucleotides, especially of TT and CC. This has a strong impact on the respective formation of the different CPDs and 64PPs. Data were initially obtained for thymine-containing CPDs by radioactive measurement[49] and recently extended to all photoproducts by HPLC-MS/MS.[120] At low %GC, TT CPD remains by far the major photoproduct, present in even larger amounts than in eukaryotic DNA. At high %GC, the yield of TT photoproducts decreases while that of C-containing photoproducts increases. This trend was observed for both UVC and UVB,[49,120] and recently confirmed for UVA.[115] Following exposure to UVB of genomic DNA bearing 75%GC, CC CPD is present in larger amounts than TT CPD (Fig. 8).

5. Bipyrimidine photoproducts in cells

Detailed determination of the distribution of bipyrimidine photoproducts has provided several pieces of information in living cells and organisms. The comparison with data on isolated DNA showed that the photochemistry of DNA is hardly affected by cellular components, at least in terms of relative distribution of the photoproducts. Chromatographic techniques also provided some novel data on DNA repair.

5.1 Formation of pyrimidine dimers in mammalian cells and in skin

Because UVB-induced DNA damage is a major cause of the induction of skin cancers, numerous data on pyrimidine dimers have been gathered in primary cultures of cutaneous cells and in skin. Radioactivity measurements,[121,122] ^{32}P-post-labeling[83,123] and HPLC-MS/MS[113,114,124] have been used for this purpose, in human as well as in rodent samples. A general trend is that the distribution of pyrimidine dimers is similar to that observed in isolated mammalian DNA. TT CPD is the major photoproduct followed by TC, CT and CC CPDs. 64PP at TT is low and TC 64PP is the most frequent of this class of dimeric lesions. No information has been provided by chromatographic methods on the level of CT and CC 64PPs since their amounts are below the detection limit when biologically relevant doses are applied. A similar distribution was reported for a series of mammalian cell lines, primary cultures of cutaneous cells and in human skin.[90,113,114,124–126] These observations show that the fate of excited states of bases in DNA is hardly affected by the cellular context and gives rise to the same photoproducts as in isolated DNA with a similar relative intensity. It should however be kept in mind that biochemical assays have shown local effects on formation of photoproducts such as more photoreactive sequences,[127] impact of nucleosome folding[128] and modulation by protein binding.[129–131] Differences in the absolute yield of damage have also been observed by HPLC-MS/MS between whole human skin and the cultured primary keratinocytes from the same donor upon exposure to UVB.[114] Protection by a factor of 22 was found to be provided by skin. In contrast, an almost equal yield was determined in the two types of samples after exposure to UVA. Interestingly, the yield of CPDs after both UVB and UVA has been shown to be correlated to the phototype although with a large dispersion among donors (Fig. 9),[132] as also shown by immunological approaches.[133] Another application of the accurate quantitative measurement of CPDs in skin explants by HPLC-MS/MS is the evaluation of the efficiency of sunscreen against DNA damage.[95] Such a measurement is needed to strengthen the usual sun protection factor that is defined with respect to erythema and thus does not relate to carcinogenicity.

Both HPLC-MS/MS and biochemical techniques have shown that the yields of photoproducts in cells and skin exposed to UVA are similar to that obtained with isolated DNA.[71,105,115] This observation strongly suggests that the formation of CPDs upon UVA-irradiation does not require an endogenous photosensitiser but is rather a direct photochemical process. Interestingly, the main UVA-induced oxidative lesion, 8-oxoGua, can also be quantified by chromatographic techniques, mostly HPLC associated with electrochemical detection or mass spectrometry. It is thus possible to accurately compare the yields of this lesion to that of dimeric photoproducts. In all mammalian cells and in skin, TT CPD is produced in *ca*. 5-times larger yield.[113,114,134] An exception is primary cultures of human melanocytes where the ratio between the amount of TT CPD and 8-oxoGua is only 1.4.[135]

Information on photosensitised triplet energy transfer in cells incubated with specific sensitisers has also been gathered from HPLC-MS/MS analyses.[113,136] Like in isolated DNA, TT CPD is by far the major bipyrimidine dimer and no 64PP is detected. A last piece of information regarding UVA is the major role it plays in the formation of DEWs. Indeed, the formation of this class of photoproducts results from the absorption of photons by 64PPs which exhibit a maximal absorption around 320 nm. However, HPLC-MS/MS analyses indicate that, like in isolated DNA, no DEW is observed in cells exposed to biologically relevant doses of UVB while significant formation is observed with simulated sunlight mixing UVB and UVA or with sequential exposure to UVB and UVA.[113,125] This can be explained by the initial formation of 64PPs by UVB which then absorb UVA and are converted into DEWs.

5.2 DNA repair

It is far beyond the topic of this review to summarize the amount of information gathered on repair of bipyrimidine photoproducts, in terms of regulation and effective proteins. In such studies, photoproducts are mostly quantified by antibody-based assays. Chromatographic techniques have however also been found to be a valuable source of information. Radioactive quantification provided the first evidence for repair of photoproducts in DNA.[137–139] An advantage of using this type of approach compared to antibody-based techniques is that repair kinetics of all photoproducts can be determined in the same cell sample. For instance, a strict comparison of the rate of removal of 64PPs, CPDs and DEWs was made in primary cultures of human cutaneous cells exposed to UVB and UVA.[125] This work showed that DEWs and 64PPs were repaired with the same efficiency. Numerous studies have shown that CPDs were repaired much more slowly. A specific input of chromatographic assays was the comparison of the repair kinetics of the four CPDs. They were found to exhibit different persistence times in human cells and skin with the following order of decreasing repair efficiency: CT > CC > TC > TT[69,84,140,141] (Fig. 10). Another advantage of chromatography lies in the quantitative aspect of the measurement. It was shown that the initial level of damage strongly affected the repair rate[124] and that comparison between different cell types or irradiation conditions must therefore be performed at a similar initial level of damage. With this in mind, it was shown that UVA exhibited a negative effect on the rate of repair of CPDs.[114,125]

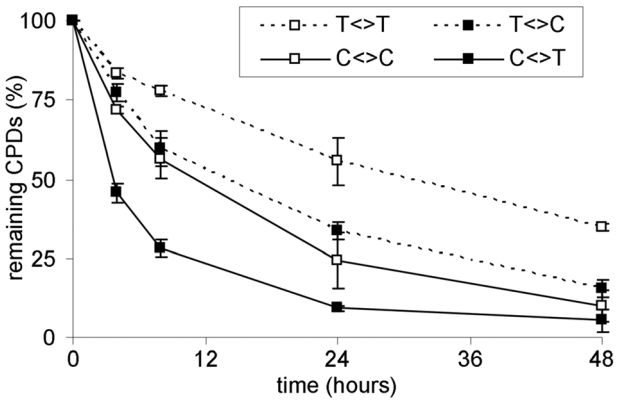

Fig. 10 Time-course repair of the four CPDs in primary cultures of human keratinocytes exposed to UVB (200 J m^{-2}).[140] Results are the average (± standard deviation) of measurements made for three donors.

5.3 Photoproducts in non-mammalian cells

DNA from bacteria and yeasts were the favoured models for a number of early studies on the formation of DNA damage mostly after UVC exposure.[14,15] Very soon after the first isolation of TT CPD from irradiated thymine, it was identified in DNA of irradiated bacteria on the basis of radioactivity measurements.[142] Dehydrated 64PPs were identified a few years later in *E. coli*.[36] Interestingly, the same authors reported the disappearance of these latter lesions after exposure to 313 nm by an unknown process that is now known to be the photoisomerisation into DEWs.[143] More recently, HPLC-MS/MS has provided additional information. More accurate data were obtained on the formation of 64PPs. Extensive description of the distribution of bipyrimidine photoproducts made possible the *in vivo* confirmation of the effect of the GC content observed on isolated DNA exposed to UVB.[120] Interestingly, in contrast to what has been sometime proposed, the %GC was not correlated to photosensitivity although more damage in the form of TT CPD was produced at low %GC.[144] Repair is likely to make a more significant contribution than only formation of photoproducts to survival after UV exposure. The distribution of DNA in archea[145] was also recently determined, with no noticeable differences with data obtained in bacteria[144,146,147] and eukaryotes (see above). Plants should not be forgotten in this survey, although most data available on the formation and repair of bipyrimidine photoproducts rely on biochemical assays. The feasibility of applying HPLC-MS/MS to this type of sample has been shown in a repair study in *Arabidopsis thaliana*.[148]

5.4 Photochemistry of DNA in bacterial spores

Spores are dormant forms of some bacterial species allowing cells to survive under unfavourable conditions. Spores are produced by asymmetric division and are characterized by a high degree of dehydration and the presence of walls and membranes around a core containing DNA and a series of specific proteins and compounds. Spores are the only cell types where SP is detected. Hydrolysis followed by radioactive detection was at the origin of the identification of SP[46] and since then chromatographic methods have been the only tools available for the study of the formation and repair of photoproducts in bacterial spores.[149–151] In spores, SP represents more than 99% of the photoproducts[53,54,152] (Fig. 11). No cytosine-containing analog of SP is detectable.[54] The main parameters involved in the specific formation of SP, also studied with isolated DNA (see section 2.3), were found to be dehydration, presence of small acid soluble proteins[55] and dipicolinic acid.[57]

Radioactive detection of SP using thin layer or liquid chromatography has been the basis of numerous experiments

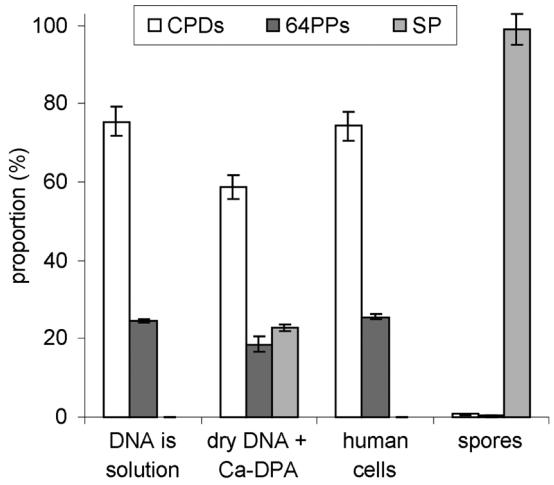

Fig. 11 Distribution of the three main classes of bipyrimidine photoproducts in various types of samples exposed to 254 nm radiation.[56,90] Human cells were THP1 monocytes and spores were from *Bacillus subtilis*. Ca-DPA is calcium dipicolinate. Quantification of photoproducts was performed by HPLC-MS/MS.

on the link between survival and exposure to UV. The effects of UVC are of major interest first because 254 nm lamps are used as germicidal tools. In addition, spores represent a highly photoresistant life form and are used as models in exobiology[153,154] to understand how living organisms could withstand the high UV fluence present on early Earth before the development of the ozone layer. Another topic of interest is the survival of life under the high UV fluences encountered in outer space. Of particular interest is also the repair machinery present in spores. As a result of the specific photochemistry of their DNA, spores have only to deal with one type of photoproduct, namely SP. DNA photoproducts accumulate SP at a high level in spores which do not exhibit metabolic activity and are thus devoid of DNA repair. Therefore, lesions have to be quickly removed upon germination, when the spore returns into a vegetative bacterial state. Nucleotide excision repair is a rather slow process involving numerous proteins and is also highly energy consuming. Removal of SP thus relies on another enzyme, the spore photoproduct lyase which reverses the lesion.[155] This repair process involves a radical reaction initiated by *S*-adenosyl-methionine. Monitoring of the repair of SP has been performed in numerous studies by chromatographic assays involving radioactivity measurements and HPLC-MS/MS. Interestingly, the latter technique allowed repaired intermediates to be trapped which provided interesting information on the enzymatic mechanism.[50]

6. Conclusions

This survey illustrates the usefulness of chromatographic techniques for the quantification of dimeric photoproducts. In the early years of DNA photobiology, these approaches were the most widely used tools for the study of formation and repair of CPDs and SP. The recent development of HPLC-MS/MS has provided an additional level of details in the understanding of DNA photoreactivity, both in solution and in cells. Chromatographic assays also provided valuable data in the field of DNA repair, both in mammalian cells and non-eukaryotic systems. The increasing number of applications of these assays shows that there is still a need for better qualitative and quantitative information of the "old class" of DNA lesions constituted of bipyrimidine photoproducts. The straightforward and highly specific chromatographic methods are very well suited for this purpose. Modern chromatographic techniques are also quite sensitive but often require more DNA than biochemical assays. Technological developments, in particular in the performances of mass spectrometers, will rapidly overcome these limitations. It can be expected that the contribution of chromatographic techniques to the field of DNA photobiology will increase in the coming years.

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