

Critical energies for SSB and DSB induction in plasmid DNA by low-energy photons: action spectra for strand-break induction in plasmid DNA irradiated in vacuum

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Abstract.

Purpose: To measure action spectra for the induction of single-strand breaks (SSB) and double-strand breaks (DSB) in plasmid DNA by low-energy photons and provide estimates for the energy dependence of strand-break formation important for track-structure simulations of DNA damage.

Materials and methods: Plasmid pMSG-CAT was irradiated as a monolayer, under vacuum, with 7–150 eV photons produced by a synchrotron source. Yields of SSB and DSB were determined by the separation of the three plasmid forms by gel electrophoresis.

Results: The yields of SSB per incident photon increased from 1.4×10^{-15} SSB per plasmid per photon/cm² at 7 eV to 7.5×10^{-14} SSB per plasmid per photon/cm² at 150 eV. Direct induction of DSB was also detected increasing from 3.4×10^{-17} DSB per plasmid per photon/cm² at 7 eV to 4.1×10^{-15} DSB per plasmid per photon/cm² at 150 eV. When the absorption cross-section of the DNA was considered, the quantum efficiency for break formation increased over the energy range studied. Over the entire energy range, the ratio of SSB to DSB remained constant.

Conclusions: These studies provide evidence for the ability of photons as low as 7 eV to induce both SSB and DSB. The common action spectrum for both lesions suggests that they derive from the same initial photoproducts under conditions where the DNA is irradiated in vacuum and a predominantly direct effect is being observed. The spectral and dose–effect behaviour indicates that DSB are induced predominantly by single-event processes in the energy range covered.

1. Introduction

For all ionizing radiations, even those that have a high initial energy, the most frequent energy loss events are around 22 eV, with the energy deposited, on average, per energy loss event, being 60 eV (Rauth and Simpson 1964). However, Monte-Carlo modelling techniques have shown that virtually all radi-

ations deposit a wide range of energies within the critical target (i.e. a small section of the DNA helix) (Goodhead 1989, Goodhead and Nikjoo 1989, Nikjoo *et al.* 1997, Ottolenghi *et al.* 1997). These will be from zero energy up to several hundred electron volts, although densely ionizing radiation will produce a greater fraction of higher energy depositions than sparsely ionizing radiation. Many modelling studies have equated this distribution of energy events with the likely yields and spectra of lesions that will be produced in DNA by radiations of differing LET (Charlton *et al.* 1989, Goodhead and Nikjoo 1989, Holley *et al.* 1990, Michalik 1993, Friedland *et al.* 1998, 1999). All these data rely on basic assumptions regarding the threshold energies for the production of lesions by ionizing radiation in DNA and the efficiency of these processes versus energy. Experimental data for this is limited at present to an early study by Martin and Haseltine (1981), which has recently been repeated (Lobachevsky and Martin 1996, Nikjoo *et al.* 1996), providing values for break induction by ¹²⁵I deoxyuridine incorporated into DNA. These studies have indicated values of 17.5 eV for the production of DNA SSB and around 35 eV for the production of DSB.

It is clear, however, that action spectra studies with both photons and electrons are required to provide fundamental information relating to the energy required to produce specific lesions in DNA and input into Monte-Carlo modelling studies that aim to predict the molecular consequences of ionizing radiation interaction with cellular DNA. The first evidence indicates that DNA strand breaks, in particular, can be induced by much lower photon energies than has been predicted in modelling studies. Early studies used predominantly gas-discharge line sources limited to studies of SSB formation. For example, Wirths and Jung (1972) found an increase, of around fivefold, in the breakage frequency in ϕ X174 DNA exposed to 21.2 eV photons produced via a low-pressure mercury vapour lamp coupled to

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a monochromator, in comparison with 4.9 eV photons, and concluded that below 10 eV only a small fraction of the ionizations in the molecule led to strand-break formation. Studies by Sontag and Dertinger (1975) using a low-pressure microwave discharge system, showed similar results for breakage formation, but it was concluded that for energies below 10 eV inactivation of ϕ X174 bacteriophage was more likely due to the production of base damage rather than SSB. Hieda and colleagues (1986), using photons from a synchrotron, showed that below 9.5 eV the cross-sections for SSB formation in ColE1 DNA decreased more rapidly with photon energy than those for base damage. In subsequent studies with a model oligonucleotide system (Saitou 1993, Saitou and Hieda 1994), they observed a decrease in the production of breakage-related products below 10 eV, in contrast to an increase in the production of photoproducts, with the yields crossing over at around 6 eV. Lücke-Huhle and Jung (1973a,b) used metastably excited gases to obtain information about excitation processes. They found an equal probability of strand breakage in ϕ X174 DNA exposed to excitation energies of 4.3 eV, 6.2 eV, 11.6 eV and 19.8 eV. For double-stranded ϕ X174 DNA, they found that the SSB induced did not lead to inactivation in a plaque-forming assay and that no significant production of base damage in the form of alkali-labile sites could be detected. Given that the first ionization potential of DNA constituents is around 9 eV (Tasaki *et al.* 1990), this suggests that excitation processes are not efficient at producing strand breakage and that these predominantly require ionization.

These studies have been augmented by work using pulsed laser sources mainly at 193 nm (6.4 eV) and 248 nm (5 eV) (Görner *et al.* 1992) carried out in hydrated DNA systems. Many of these studies have concentrated on more complex effects of intensity involving biphotonic events. At low intensities, however, they have found that with increasing energy the yields of both SSB and DSB increase. In pTZ18R DNA, Gurzadyan and colleagues (1993) found quantum efficiencies of 0.4×10^{-4} for SSB at 193 nm increasing to 15×10^{-4} for 248 nm, and 0.014×10^{-4} for DSB at 193 nm increasing to 0.6×10^{-4} for 248 nm.

Vacuum UV light from synchrotron sources has provided a useful system for studying the energy dependence of radiation damage to various cellular constituents (Dodonova 1993, Hieda 1994), in particular DNA. Vacuum UV light (5–100 eV/250–12.5 nm) studies are difficult to perform with biological systems owing to the requirements of working under rigorous vacuum conditions and because of the fluences of monochromatic photons in this energy

range currently available from synchrotrons. Studies have so far been limited to experiments with simple DNA systems, although some recent work has started to relate breakage data to mutation and inactivation in an *Escherichia coli* based transformation system (Wehner and Horneck 1995).

Of the range of lesions induced by ionizing radiations, it is clear that DSB are important in terms of biological effects. From both modelling and experimental studies, because of the spatial distributions of energy depositions within the DNA, it has been shown that clustering of damage will occur in the form of multiply damaged sites (Ward 1995, Prise 1997) and these will have a high probability of producing DSB. Recent low-energy studies have also started to address the importance of the action spectra for DSB formation. Hieda and colleagues (1994) measured both SSB and DSB in pBR322 DNA at 8.3 and 20.7 eV and found a decrease in the action cross-section for both lesions, with decreasing energy and an increase in the ratio of SSB to DSB. Preliminary data for pBR322 DNA over a limited energy range has been previously presented (Michael *et al.* 1995). In the work presented here, action spectra have been systematically produced from 7 eV up to 150 eV for both SSB and DSB in plasmid DNA irradiated under vacuum conditions. The characterization of the photon energies used and the experimental set-up have been described in detail in a previous paper (Folkard *et al.* 2000).

2. Methods

2.1. Vacuum UV irradiation

Full details of the radiation source and dosimetry have been given in the previous paper (Folkard *et al.* 2000). Briefly, irradiations were carried out at either station 3.1 or 3.3 (dipole magnets) of the 2 GeV Daresbury electron synchrotron (CLRC Daresbury Laboratory, Daresbury, UK), operating in single bunch mode with a stored current of ~ 200 mA. Photon energies in the range 7 to 25 eV (50–178 nm) were selected using a Seya-Namioka mount 1 m grating monochromator. Higher-order diffracted light was filtered out using windows of lithium fluoride (< 11.5 eV; EG Electro-optics, Friday Harbor, WA, USA), indium (12–16.5 eV; Luxel, Tarporley, Cheshire, UK) or aluminium (> 17 eV; Luxel). For energies greater than 25 eV, a Torroidal Grating Monochromator (TGM) was used with an aluminium or a polyimide window. These windows were also used to isolate the ultra-high vacuum of the synchrotron ($\sim 10^{-9}$ mbar) from the much poorer vacuum conditions at the sample position (10^{-7} to 10^{-5}

mbar). Samples were placed in a chamber at the end of a flexible bellows, which allowed raster scanning of the sample through the beam. Dosimetry was carried out using a silicon photodiode (type AXUV-100; IRD, Torrance, CA, USA) and fluorescence detection via a photomultiplier tube from a sodium salicylate window. These were used to calibrate the photo-current from a copper grid placed in the beam during irradiations (Folkard *et al.* 2000). Exposures were based on the incident photon flux per cm^2 of the sample area.

2.2. Sample preparation and assays

For these studies, pMSG-CAT (8404 bp) plasmid DNA was used. This was purified from *E. coli* strain HB101 using the alkaline lysis method (Birnboim and Doly 1979) or a commercially available kit (Quiagen, Dorking, UK); plasmids were typically 80–90% supercoiled after purification. For vacuum UV irradiation, samples were recovered into $0.068 \text{ mmol dm}^{-3}$ EDTA pH 7.0 at a concentration of $0.25 \mu\text{g}/\mu\text{l}$. DNA was prepared for irradiation using a freeze-drying technique. A $1 \mu\text{l}$ sample of DNA was spread onto a $18 \text{ mm} \times 18 \text{ mm}$ glass coverslip that had previously had $1 \mu\text{l}$ of 0.02% (v/v) Tween 80 detergent spread and dried onto it to improve spreading. For samples irradiated on station 3.1 (7–25 eV), DNA was spread over an area of $10 \times 10 \text{ mm}$ at the centre of the coverslip, whereas for station 3.3 (40–150 eV) they were spread over an area of $5 \times 5 \text{ mm}$ owing to the different beam spot sizes, although the sample volume was kept constant. The coverslip was immediately placed onto the cooled plate of the freeze-drier, which was typically at a temperature of -5 to -10°C . After a suitable number of samples were prepared (typically 15–20), the freeze-dryer chamber was evacuated to a pressure of 10^{-5} mbar and held at this pressure for 1 h. The chamber was then allowed to return to room temperature under vacuum and then dry nitrogen gas was slowly admitted into the chamber to return the samples to atmospheric pressure. Samples were held in a dessicator filled with dry nitrogen at room temperature until required and used within 24 h of preparation. The distribution of the DNA molecules over the glass coverslip was monitored using an atomic force microscope (Nanoscope III; Digital Instruments, Santa Barbara, CA, USA) to confirm that a good monolayer was obtained. Samples were then placed in the irradiation apparatus attached to the synchrotron port. In most experiments, for every sample placed in the irradiation position another sample was placed in the chamber under the same vacuum conditions but in a position that was not

exposed to synchrotron irradiation. Typically, samples were irradiated when the vacuum pressure had reduced to less than 10^{-5} mbar within the sample chamber. Irradiation times varied from 5 min to 17 h, depending on the dose and the energy of the photons. After irradiation, samples were returned to atmospheric pressure by admitting nitrogen gas into the sample chamber. DNA was rehydrated and recovered by the addition of $10 \mu\text{l}$ of TE buffer (10 mmol dm^{-3} Tris, 1 mmol dm^{-3} EDTA, pH 8.0) to the glass coverslips and removal by automatic pipette. After the addition of $2 \mu\text{l}$ of dye stop buffer (15% (w/v) Ficoll 400, 100 mmol dm^{-3} EDTA, 0.25% (w/v) bromophenol blue), samples were separated on a 1% (w/v) agarose gel, at either 16 volts for 16 h or 40 volts for 4 h at room temperature in TBE buffer (89 mmol dm^{-3} Tris, 89 mmol dm^{-3} boric acid, 2 mmol dm^{-3} EDTA, pH 8.0). No significant differences were observed between the different electrophoresis times, suggesting that heat labile sites may not be contributing significantly to the results obtained (Jones *et al.* 1994). Gels were stained for 30 min in $0.5 \mu\text{g}/\text{ml}$ of ethidium bromide in TBE followed by destaining for 30 min in fresh TBE buffer. Images of the gels were captured using a custom-built image capture system consisting of an 8-bit CCD camera (COHU, San Diego, CA, USA), image capture board (DT55-50 Hz; Data Translations, Marlboro, MA, USA: 8-bit, 786×512 pixels image capture) and an in-house developed analysis package (based on Visilog software, Datacell, Finchampstead, Berks, UK). All sample electrophoresis and analysis took place during the synchrotron beamtime.

3. Results

The use of supercoiled plasmid DNA allows the yields of SSB and DSB to be determined within the same plasmid sample by separating the three plasmid forms (relaxed, linear and supercoiled) via gel-electrophoresis. For the exposure of plasmid molecules to the photons from the synchrotron it was necessary to prepare the plasmids as a monolayer by freeze-drying to remove the water of hydration of the DNA. Under the conditions used it is assumed that most of the water is removed, leaving, at most, only three water molecules per nucleotide of DNA closely associated with the phosphate groups (Swarts *et al.* 1992). In most experiments, a control sample was also placed in the vacuum at the same time as the exposed sample and held under vacuum for the length of the irradiation time. No significant effect of the time under vacuum or of the freeze-drying process on control samples was observed (see Michael *et al.* (1994) for an example). Figure 1a show the loss

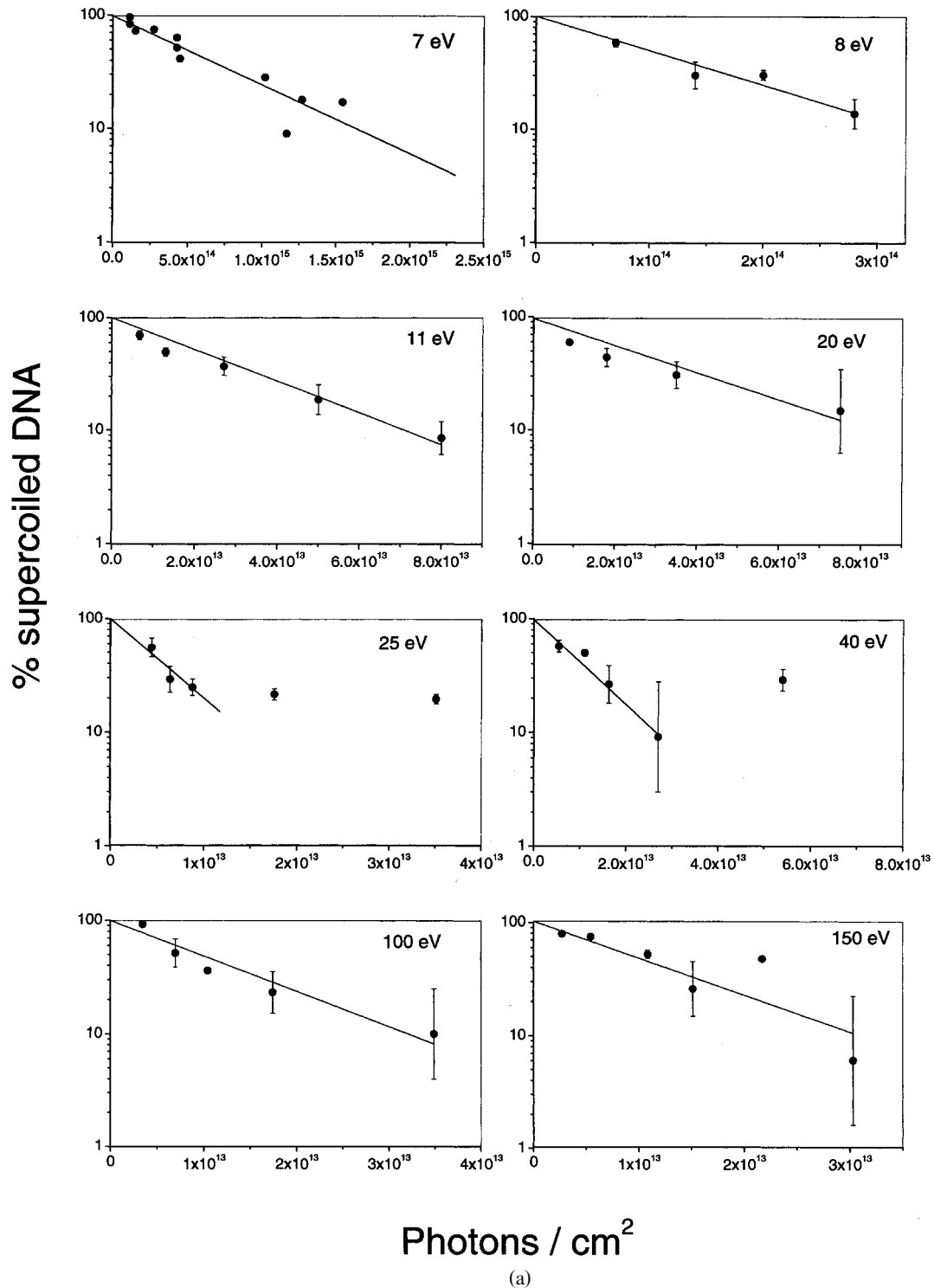
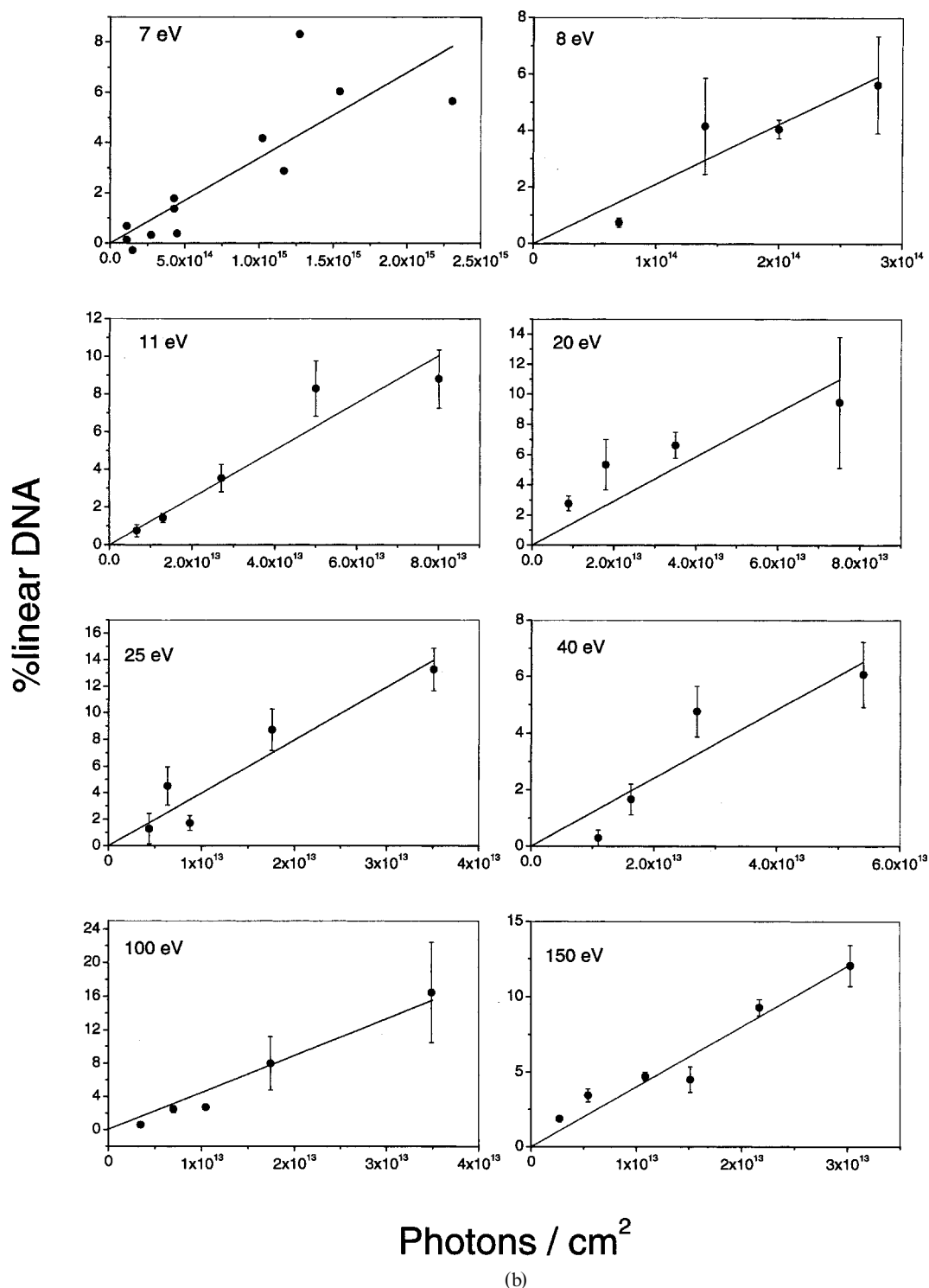


Figure 1. Dose-effect curves for the production of DNA damage in plasmid DNA irradiated with photons of specific energies. For SSB (a), the loss of supercoiled DNA versus photon fluence is plotted after irradiation with 7, 8, 11, 20, 25, 40, 100 or 150 eV photons. For DSB (b), the production of the linear form of the plasmid is plotted versus photon fluence. Data are normalized to the values at zero fluence and are the means ± 1 SEM from several experiments, with the exception of the data plotted for 7 eV, which are individual determinations.



of supercoiled form of plasmid pMSG-CAT in samples irradiated with 7, 8, 11, 20, 25, 40, 100 or 150 eV photons. The loss of supercoiled DNA is plotted versus the incident yield of photons per cm² of sample. With the exception of the data at 25 and

40 eV, the loss of supercoiled DNA appeared exponential with dose, and up to 90% conversion of the supercoiled DNA to either relaxed or linear forms. This suggests, in line with observations of the samples by atomic force microscopy, that the DNA is pre-

dominantly in a uniform monolayer in these samples. The data were fitted with an equation of the form $-\text{Log}(SC) = \alpha F$, where SC = fraction of supercoiled DNA, α is a constant and F = fluence (photons/cm²). Using this equation, the yields of SSB per plasmid per photon/cm² were 1.4×10^{-15} at 7 eV increasing by over 60-fold to 7.5×10^{-14} at 150 eV. As well as the yield of SSB in these samples, it is possible to determine the yield of DSB induced by measuring the production of the linear form of the plasmid. This is shown in figure 1b for the same energies used in figure 1a. DSB were induced by all energies studied. In general, with increasing energy there is an increase in the probability of the incident photons to induce DSB. For each energy, the yield of DSB was essentially linear with photon dose and the data were fitted with a model of the form: $LIN = \alpha F$, where LIN = fraction of linear DNA, α is a constant and F = fluence (photons/cm²). Using this relationship yields of DSB were obtained increasing from 3.4×10^{-17} at 7 eV by over 80-fold to 4.1×10^{-15} at 150 eV. The yields of SSB and DSB obtained from the dose-effect curves are summarized versus photon energy in figure 2. With increasing photon energy above 11 eV, in general there is a gradual increase in the yields of both lesions per incident photon. At energies below 11 eV the efficiency of breakage decreases more rapidly.

4. Discussion

By using photons from a synchrotron source it has been possible to measure action spectra for the

production of DNA SSB and DSB over a range of photon energies from the vacuum UV up to energies approaching those of characteristic soft X-rays (7–150 eV). In particular, it has been possible to work in the region where the most frequent energy deposition event sizes occur in DNA exposed to ionizing radiations (22 eV; Rauth and Simpson 1964) and where the threshold energies for the production of SSB and DSB have been predicted to occur (17.5–40 eV; Charlton *et al.* 1989). It also extends up to energies where the frequency of deposition is more strongly related to the quality or LET of the radiation (Goodhead and Nikjoo 1989). In these studies, DNA irradiated under vacuum conditions has been used, although in future work the role of the water of hydration will be considered. Under vacuum conditions, it can be assumed that the direct effects of photon interactions with the DNA are being measured, with virtually all energy absorption taking place on the DNA. This will consist primarily of initial ionization and secondary electron production and interaction, but also, excitation processes, which will become relatively more significant at lower energies, below the ionization potential of the DNA.

It is clear that both SSB and DSB have been detected over the entire energy range studied here with no substantial decrease in the probability of producing both lesions until the energy decreases below 8–11 eV. In general, a linear induction of DSB (figure 1b) is observed, indicative of direct production of these lesions as opposed to indirect formation via the interaction of two SSB on opposite strands of the DNA. This also rules out any significant interactions

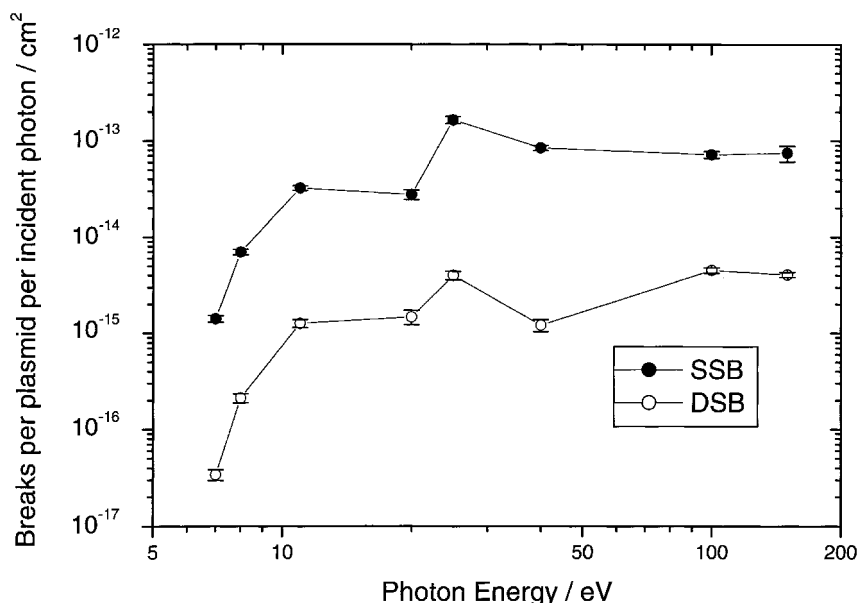


Figure 2. Action spectra for SSB and DSB plotted as breaks per plasmid per incident photon per cm² versus energy.

between other lesions, which may interact to increase the probability of DSB formation. For example, heat-labile sites converted to strand breaks during electrophoresis (Jones *et al.* 1994) or stress-induced SSB formed due to the freeze-drying and vacuum processes.

To measure the effectiveness of strand-break formation in terms of photons absorbed in the DNA (quantum efficiency), the absorption spectrum of the DNA under these conditions has been taken into account. This has been calculated from the atomic absorption data of Henke *et al.* (1993) and the DNA absorption data of Inagaki and colleagues (1974) assuming a monolayer of DNA of 2 nm thick (see Folkard *et al.* 2000). The derived quantum efficiencies for SSB and DSB induction versus energy are shown in figure 3. This shows that for SSB the probability of a break being produced when a photon is absorbed in the DNA increases from $\sim 2 \times 10^{-3}$ at 7 eV up to 0.3 at 150 eV. For DSB, the increase is from 6×10^{-5} at 7 eV to 1×10^{-2} at 150 eV. In some energy regions there is little change in quantum efficiency, for example between 8 and 20 eV. Mechanistically, this suggests that at these energies the probability of a break being produced is simply related to the probability of the photons being absorbed on the DNA. Over the whole energy range studied, however, there is a general energy dependence to break formation, with higher energies being more effective. This is likely to be related to the effectiveness of the secondary electrons produced, which are known to be more effective at energies above 100 eV (Prise *et al.*, unpublished observations 1999). Some secondary electrons may also be produced in the glass substrate and at

higher energies may have sufficient energy to interact with the DNA. No assumptions regarding their influence with the DNA have been made in this study. The authors are currently extending earlier studies with low-energy electrons (Folkard *et al.* 1993) to elucidate the likely contributions of these effects (Usami *et al.* 1999).

Overall, the ratio of SSB to DSB varies from approximately 20 to 70 with no apparent dependence on energy within the errors of the measurements made. Interestingly, these values are around those normally observed with high-energy radiations in the plasmid DNA system in solution (Krisch *et al.* 1991, Milligan *et al.* 1993). The similarity of the shapes of the action spectra, differing by a near-constant ratio throughout the energy range, suggests that both types of lesion are derived from the same initial photoproduct. Independently of energy deposited, there is a 2–5% probability of a DSB being formed instead of a SSB. Of the mechanisms that have been suggested for DSB formation by ionizing radiation, the radical transfer mechanism of Siddiqi and Bothe (1987) predicts a constant ratio of SSB to DSB. This mechanism suggests that the initial radical produced from a single OH-radical attack in the DNA leads to a SSB, then the radical is transferred to the neighbouring strand and a second break induced. A similar mechanism may be underlying studies where adjacent base damages have been observed after a single OH attack (Box *et al.* 1995, 1997). This proposed mechanism for the production of DSB in this system, however, is in contrast with what is predicted from studies in cells and plasmid DNA in solution. In those studies, the evidence points to the

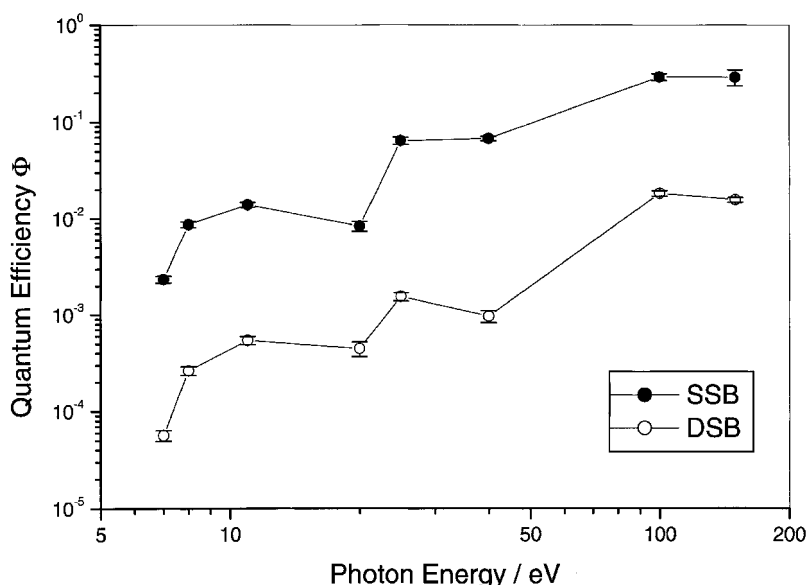


Figure 3. Quantum efficiencies (Φ) for SSB and DSB induction versus photon energy.

involvement of multiply damaged sites (Ward 1981) with two radicals being produced, one on either strand of the DNA as a radiation track crosses it (Prise *et al.* 1992, 1993, 1998, 1999, Milligan *et al.* 1995). As the system used here, of DNA irradiated under vacuum, is predominantly measuring direct effects of irradiation, it suggests that other factors such as the interaction of water with the helix and its overall structure may be important determinants of both sensitivity and damage induction mechanisms. Under vacuum conditions, it has been assumed that all but the last three water molecules (associated with the phosphate groups) have been removed. In this condition, the DNA is not in the B-form but a mixture of the A- and C-forms (Swarts *et al.* 1992). Whether this will influence the yields and/or mechanisms of break formation remains to be determined.

The data obtained are in general agreement with previously published values. For example, Hieda and colleagues (1994) measured cross-sections of $8.1 \times 10^{-19} \text{ m}^2$ and $1.6 \times 10^{-17} \text{ m}^2$ at 8.3 and 20.7 eV respectively, in comparison with $7.0 \times 10^{-19} \text{ m}^2$ and $2.76 \times 10^{-18} \text{ m}^2$ measured in this study at 8 eV and 20 eV. For DSB they measured $4.7 \times 10^{-21} \text{ m}^2$ at 8.3 eV and $2 \times 10^{-19} \text{ m}^2$ at 20.7 eV, in comparison with the present authors' measurements of $2.1 \times 10^{-20} \text{ m}^2$ at 8 eV and $1.5 \times 10^{-19} \text{ m}^2$ at 20 eV. Some of these differences may reflect the sample preparation methods used in these studies to obtain monolayers of DNA under dry conditions. For example, Hieda and colleagues (1994) used air-dried DNA in the presence of Tris buffer in comparison with the freeze-dried DNA in the presence of EDTA used in this study.

In conclusion, action spectra for the production of both SSB and DSB induced by 7–150 eV photons has been presented. A common action spectrum is observed for both lesions, suggesting that they may derive from the same initial photoproducts under conditions where DNA is exposed under vacuum conditions to low-energy photons.

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