Comparison of DNA Breaks at Entrance Channel and Bragg Peak Induced by Fast C^{6+} Ions

–Influence of the Addition of Platinum Atoms on DNA–

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When energetic carbon ion beam (GeV range) goes through the matter, inelastic processes such as electronic ionization, molecular and nuclear fragmentation occur. For carbontherapy (hadrontherapy) purpose, it is of interest to compare the number of DNA breaks -single SSB or double DSB- for a given dose at the entrance channel and at the Bragg peak to look for a possible differential effect in the number of DNA breaks induced at these two locations. Samples of free plasmids DNA and complexes of plasmids DNA added with molecules containing platinum have been placed at different locations of an experimental setup simulating penetration depths of the ion beam in water and irradiated by carbon ions 290 MeV/amu. The DNA breaks have been quantified by subsequent electrophoresis on agarose gels. To disentangle the respective role of the direct and indirect effect, a free radical scavenger of hydroxyl radicals HO°-dimethyl sulfoxide DMSO- has been added in some of the experiments. In the range of Linear Energy Transfert -LET 13 – 110 keV/μm-, the number of the DSB was found to be constant versus the LET for a given dose. Contrary, the number of the SSB decreases at the Bragg peak compared to the entrance channel. In the presence of platinum, the number of single and double breaks was considerably enhanced, and follows a similar behaviour than in the free-DNA experiments. Quantitative results on DNA damages do not show significant enhancement due to the nuclear or to the molecular fragmentation in the present experiments.

INTRODUCTION

When fast atomic ions like carbon ions C^{6+} at 290 MeV per amu interact with matter, an important part of the energy of the particles is released at the end of the track. The enhancement of the dose deposition at this location is named the Bragg peak (Fig. 1). This property of the dose profile finds an interesting application in medicine to treat cancer. The Bragg peak allows selective dose deposition in deeply seated solid tumours; therefore protontherapy and carbontherapy improve significantly the therapeutic index of cancer therapy.¹)

Fast atomic ions interact mainly with the electrons in the matter through inelastic collisions, resulting in the induction of secondary emitted electrons along the track. As a result of the high Linear Energy Transfer -LET- of the atomic ions, many ionisations and excitations occur in the molecules and atoms constituting the matter. Alteration of biomolecules can result in mutations or death of the cells. Cell death is often the endpoint when double strand breaks (DSB) are produced in DNA.

As a consequence of the electronic excitations or ionisations, chemical bonds of molecules in the irradiated material can be broken, thus resulting in the emission of secondary ions or molecular fragments in the matter. These fragments can induce subsequent damage in biomolecules and in particular in DNA -De Vries et al.-²) It was shown by Deng et al.³) that the interaction of the low energy ions with DNA bases is sufficient to induce fragmentation in the DNA bases. Similar effects were observed with small oligonucleotides and with lyophilized DNA -Sellami et al.,⁴) Lacombe et al.-⁵) The authors suggest that such molecular fragmentations produced in the tracks of the incident atomic ions might be an important mechanism to induce biomolecules alteration, especially at the Bragg peak location. These effects could
induce additional undesired damage beyond the Bragg peak. A quantification of the latter mechanism is of obvious interest for medical purposes and risk estimates in carbontherapy.

Another kind of fragmentation, different from the previous one, is due to the fragmentation of the incident C\textsuperscript{6+} nuclei along the track. For instance, at an initial energy of about 290 MeV/amu, carbon ions C\textsuperscript{6+} are fragmented at the Bragg peak up to 50% in secondary energetic ions like H\textsuperscript{+} and He\textsuperscript{2+}, and in smaller quantities in Li\textsuperscript{3+}, Be\textsuperscript{4+} and B\textsuperscript{5+}.\textsuperscript{6} The nuclear fragmentation of C\textsuperscript{6+} results in the production of lighter ions -H\textsuperscript{+}, He\textsuperscript{2+}, ...- in the MeV range that might play a specific role in the induction of the DNA damage around the Bragg peak. Briefly stated, the carbon beam at plateau -entrance channel- and Bragg peak is different, and this could induce different amount of DNA breaks per gray at these locations.

As pointed out by Hamada,\textsuperscript{71} the LET dependence of DNA damage remains an outstanding question. Many studies have been devoted to this problem. See for example recent review by Terato et al. and Hada et al.\textsuperscript{8,9} and references therein. It is therefore of obvious interest to investigate the number of the DNA breaks induced along the beam track, namely at the entrance channel, where the major part of the energy deposition is mediated by electronic ionisation, and at the Bragg peak where additional inelastic processes -molecular and nuclear fragmentation- exist as discussed previously. For this purpose, a quantitative analysis of the DNA breaks -single and double strand breaks SSB, DSB- produced by a C\textsuperscript{6+} ion beam at different locations along the track is made in this study.

We have shown recently that the enhancement of the SSB and the DSB in DNA irradiated by atomic ions can be observed when heavy atoms like platinum salt or nanoparticles are added to DNA.\textsuperscript{10,11} The combination of these radiosensitizers could be of interest to enhance the cell killing, and as a consequence, might improve the therapeutic index.\textsuperscript{12} One of the purpose of the present work is also to probe the effects on DNA damage of the added heavy atom, when the samples are irradiated by fast ions C\textsuperscript{6+}, at different locations of the ion track over a wide range of LET values (13.1 – 110 keV/μm).

In the experiments with the DNA and with the DNA-platinum complexes, a free radical scavenger is used to distinguish the respective contributions of the free radicals attacks -indirect effects- from those resulting from non scavengeable mechanisms -direct effects- at the entrance channel and at the Bragg peak.

**MATERIALS AND METHODS**

**Preparation of the samples**

The model used to analyze the DNA damages consists of circular double stranded DNA-plasmid pBR322, placed in aqueous solution. The DNA can be in one of the three forms:

- supercoiled when the plasmids are intact, relaxed when a single strand is broken -SSB- and linear if a double strand break -DSB- is induced. Plasmid DNA is a model system used to study the mechanisms involved at molecular level. It offers in particular the possibility to distinguish the contribution of the processes mediated by the water free radicals, and the processes due to the ionization by electrons and molecular or nuclei fragments (direct effects). For this purpose, a specific scavenger of the free hydroxyl radicals °OH, - the dimethyl sulfoxide (DMSO)- was added in some of the samples.

As a platinum containing molecule, we have chosen the platinum terpyridine chloride (PtTC), instead of other platinum salts commonly used in medicine such as cis-platin, oxaliplatin or carboplatin. The choice of PtTC stems from the fact that this molecule does not induce any artifact in the analysis of the irradiated plasmids at the concentrations used in this work. The PtTC molecule was obtained from FLUKA -Sigma Aldrich Chemie GmbH Schnelldorf Germany- and used without any further purification. Plasmid DNA pBR322 (4361 base pairs) supplied in TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid -EDTA- at pH 8.0), was obtained from TOYOBO (Osaka, Japan). The preparatory experiment showed that it contained at least 90% of supercoiled circular plasmid, less than 10% of relaxed circular conformation and no linear DNA.

Solutions of the DNA and PtTC have been prepared with ratio r, defined as the number of platinum atoms bound to DNA divided by the number of phosphorus atoms in DNA:

\[
r = \frac{\text{number of Pt atoms}}{\text{number of P atoms in DNA}}
\]

Presently the value r = 1/15 has been chosen to study the influence of the amount of platinum atoms added to DNA. The binding properties of the PtTC molecules to DNA were first studied by Jennette et al.\textsuperscript{13} Additional studies\textsuperscript{14} have shown that for ratios r smaller than 1/10 a negligible fraction of PtTC is expected to remain free in solution and PtTC is bound to DNA.

The concentrations of PtTC solutions were determined by spectrophotometry, at λ = 278 nm, with the absorption coefficient ε equal to 25100 mol\textsuperscript{-1}. cm\textsuperscript{-1}.\textsuperscript{13} When experiments are performed in the presence of the free radical scavenger DMSO, its concentration is adjusted to 25100 mol\textsuperscript{-1}. cm\textsuperscript{-1}.\textsuperscript{13} When experiments are performed in the presence of the free radical scavenger DMSO, its concentration is adjusted to 1.0 M.

The samples consist of 500 ng of DNA (1.32 μL) diluted in 12 μL of TE buffer solution. Depending on the addition of PtTC and DMSO, the total volume of the aliquots was adjusted to 18 μL with pure water. For instance when no PtTC or DMSO is present, 6 μL of pure water has been added. Finally, the concentration of DNA and salts remain constant in all the samples.

**Irradiation by fast carbon ions C\textsuperscript{6+}**

The samples are placed in Eppendorf vessels fastened to a polymethyl metacrylate support fixed perpendicularly to...
the beam. This material is known to reproduce accurately the absorption properties of water and thus of the biological tissues. One side of this support has the shape of a ladder (Fig. 1). The steps, where the samples are fixed, have different heights \( L \) that reproduce the depths of ion penetration in water. Samples have been placed at the respective positions: \( L = 0 \) (entrance channel), \( L = 50 \) mm, \( L = 100 \) mm and at the Bragg peak \( L_{\text{Bragg}} = 138 \) mm. Irradiation of the samples by \(^{6}C\) ions was performed at the Heavy Ion Medical Accelerator located in Chiba -HIMAC- (Japan). The beam was set in a small spread of the Bragg peak (SOBP 5 mm) mode in order to irradiate uniformly the samples (1.5 mm width) at the Bragg peak. The energy at the entrance channel is 276 MeV/amu (LET = 13.1 keV/\( \mu \)m). The LET at \( L = 50 \) mm, \( 100 \) mm and \( L_{\text{Bragg}} \) are respectively 14.2 keV/\( \mu \)m, 17.2 keV/\( \mu \)m and 110 keV/\( \mu \)m. The dose rate is 7 Gy/min at the entrance. The beam is able to deliver a uniform flux within 2\% in a circle with a diameter of 7 cm. The irradiation was performed under atmospheric conditions at room temperature. The analysis of the sample was performed immediately after the irradiation. Samples have not been stored frozen.

**Analysis of DNA breaks**

The samples were analyzed by electrophoresis at 850 V/m during three hours at 4°C in a 1.2% agarose gel and Tris-HCl 40 mM, sodium acetate 5 mM, EDTA 1 mM, at pH 7.8. After staining with ethidium bromide (1 \( \mu \)g/mL), the gel was placed under Ultra Violet light (302 nm) and the fluorescence image was recorded with a Charge Coupled Device (CCD) camera. The image-analysis software (National Institute of Health Image) was used to determine the integrated fluorescence of the three bands corresponding to the three distinct plasmid forms. The percentages of the three different

![Fig. 1](image1.png)  
**Fig. 1.** Full triangles represent the relative dose values against the penetration depth in mm of water (\( D/D_{0} = 1 \) at entrance channel and \( D/D_{0} = 2.9 \) at Bragg peak). Inserted in the bottom is schematic representation of the sample locations after different depth of the bolus block along the tracks of the carbon ions. Each mark (\( \times \)) stands for, before the block, at 50 mm-depth, at 100 mm-depth, and at 138 mm-depth of water equivalent.

![Fig. 2](image2.png)  
**Fig. 2.** (a) Representation of the SSB at entrance channel and Bragg peak versus dose without and with DMSO. Filled triangles stand for number of SSB versus doses at the entrance channel. Filled squares stand for the SSB at the Bragg peak. Open triangles and open squares stand respectively for the SSB at entrance and at Bragg peak in the presence of DMSO. (b) Representation of the DSB at entrance channel and Bragg peak versus dose without and with DMSO. Filled triangles stand for number of DSB versus doses at the entrance channel. Filled squares stand for the DSB at the Bragg peak. Open triangles and open squares stand respectively for the DSB at entrance and at the Bragg peak in the presence of DMSO.
conformations of DNA, the supercoiled plasmid (S), the relaxed (R) and the linear (L) forms were determined. The average fractions of the SSB, DSB per plasmid were calculated according to Spotheim-Maurizot et al.\textsuperscript{15)}

$$SSB = \ln(1 - L)/S \quad \text{and} \quad DSB = L/(1 - L).$$

**RESULTS AND DISCUSSION**

The average number of the SSB and the DSB induced per plasmid in pure DNA samples located at different locations are displayed in the Figs. 2a and 2b respectively. The laws governing the number of breaks SSB, DSB are linear in both cases.

In the Fig. 2a, the results for the SSB induction versus L are presented for the locations L = 0, L = L\text{Bragg}. The values of the slopes m\text{ssb} representing the number of single strand breaks induced per plasmid and per gray, for L = 50 mm and L = 100 mm are reported in the Table 1. The value of m\text{ssb} is found to be almost constant up to L = 100 mm, (m\text{ssb} = 35 \times 10^{-4} breaks per gray per plasmid) but decreases significantly at the Bragg peak location (m\text{ssb} = 19 \times 10^{-4} breaks per gray per plasmid).

In the Fig. 2b, the results for the DSB induction versus L are presented for the locations L = 0, L = L\text{Bragg}. The values of the slopes m\text{dsb} -Table 1- representing the number of double strand breaks induced per plasmid per gray -m\text{dsb} \sim 10^{-5}- are seen to be nearly constant at all the locations L, including the Bragg peak within the experimental error estimated to be 5%.

### Table 1. Values of the slopes m of the the SSB and the DSB -average number of breaks per plasmid per gray- (without and with DMSO respectively) obtained after irradiation of the DNA at different LET values of the ion beams. Plus or minus values are the standard error of the mean for 3 independent experiments. Each entry should be multiplied by 10^{-4} or 10^{-5} i.e. 39 should read $39 \times 10^{-4}$.

| L (in mm) | 0     | 50    | 100   | Bragg 138 |
|----------|-------|-------|-------|----------|
| LET (keV/μm) | 13.1  | 14.2  | 17.2  | 110      |
| Relative Dose | D₀    | D₀ × 1.0 | D₀ × 1.2 | D₀ × 2.9 |
| m\text{ssb} \times 10^{-4} | 39 ± 1.8 | 38 ± 1.6 | 35 ± 1.1 | 19 ± 1.0 |
| m\text{dsb} \times 10^{-4}Pt | 65 ± 1.9 | 63 ± 1.6 | 58 ± 1.9 | 37 ± 0.4 |
| m\text{dsb} \times 10^{-5} | 9.3 ± 0.09 | 11 ± 0.12 | 11 ± 0.4 | 9.4 ± 0.5 |
| m\text{dsb} \times 10^{-5}Pt | 19 ± 0.4 | 20 ± 1.1 | 21 ± 1.3 | 20 ± 0.4 |
| m\text{ssb} \times 10^{-4}DMSO | 8.3 ± 0.3 | 5.0 ± 0.3 |
| m\text{dsb} \times 10^{-4}DMSO | 3.7 ± 0.2 | 3.3 ± 0.2 |
| m\text{dsb} \times 10^{-5}DMSO Pt | 11 ± 0.5 | 6.3 ± 0.44 |
| m\text{dsb} \times 10^{-5}DMSO Pt | 6.1 ± 0.6 | 4.1 ± 0.5 |

The number of the DSB per gray is the same at the entrance channel and at the Bragg peak. This shows that the fragments from C\text{66} nuclei, which is maximum at the Bragg peak, does not show any significant enhancement of the DSB at this location. The mechanisms to induce the DSB are quite similar at the entrance channel and Bragg location.

The number of the SSB per gray decreases at the Bragg peak. This confirms that the processes inducing the SSB is not contributed by the C\text{66} nuclei fragments at the Bragg peak. Experiments made in the presence of the free HO\textsuperscript{°} radical scavenger indicate that the major part of the DNA damages are free radicals mediated as discussed below.

When DMSO is added to the samples, the slopes m\text{ssb} and m\text{dsb} of the lines are strongly reduced -Fig. 2a and 2b-. This finding illustrates that the DNA scissions are mainly mediated by HO\textsuperscript{°} free radicals attacks following the water radiolysis. Considering the values of the slopes in the Table 1, it is easy to see that the number of SSB per gray are decreased about 80% -for instance (65 – 11)/65 = 0.8- in all experiments in the presence of DMSO at both the entrance channel or at the Bragg peak. For the DSB the decrease when DMSO is added is somewhat smaller, around 70% -for instance (19 – 6.1)/19 = 0.68-. This finding is in agreement with previous results obtained with helium, carbon and iron ions\textsuperscript{10,11)} where the DSB induction were found less protected than the SSB breaks induction in the presence of DMSO.

Molecular fragmentations can be induced by fast ions in vacuum dry conditions,\textsuperscript{5,5)} but when water is present most of the effects induced by fast ions irradiation are HO\textsuperscript{°} free radicals mediated, and water is the basic constituent of the living cell.

As a first conclusion, the contribution of the molecular and nuclei fragmentation is not seen to be significant to induce additional DNA damages in the present experiments. The mechanisms proposed to explain the decrease of the amount of SSB at Bragg peak are the following.

We make the hypothesis that the DSB are in the major part due to the attack of HO\textsuperscript{°} dense clusters produced in the vicinity of the DNA. Production of clusters of HO\textsuperscript{°} close to the DNA can result from Auger effects induced by the secondary electrons in the phosphorus, carbon, nitrogen or oxygen atoms belonging to the DNA -and platinum when it is added-, and also from primary water radiolysis in the ion tracks. The SSB, contrary, are due to diffusible HO\textsuperscript{°} radicals produced relatively far from DNA. This hypothesis is in agreement with the observation that the DSB are less scavenged than the SSB in the presence of DMSO. This hypothesis is also sustained by experiments performed by Kassis et al.\textsuperscript{16,17)} with the prolific Auger emitter 125I. In this case, the induction of the SSB was found to be enhanced by 125I atoms located in the bulk of the solution, whereas the DSB were induced preferentially by 125I when the molecules containing the iodine atoms were included in a molecule intercalated in...
At the Bragg peak, the density of \(^{°}\)OH in the tracks increases with the LET and a strong recombination between the free radicals occurs.\(^{18}\) The direct consequence is a decrease of the amount of diffusible \(^{°}\)OH radicals in the solution, resulting in a decrease of the SSB yield by free radicals attacks. Similar conclusions concerning the role of the free radicals \(^{°}\)OH in SSB induction have been drawn by Jones et al. in experiments made with helium ions\(^{19}\) at different concentrations of DMSO.

**Results in the presence of platinum**

The results of the DNA breaks induction in the presence of platinum are displayed in Fig 3a for the SSB and 3b for the DSB. The Table 1 reports the numerical values of the slopes at different locations along the track. The results show a striking enhancement in the SSB and the DSB in plasmid DNA when platinum is present, in agreement with results of previous experiments made with helium, carbon and iron ions.\(^{10,11}\)

In the presence of platinum, The yield of the SSB decreases, as observed with pure DNA, when the LET increases at the Bragg peak: \(m_{\text{SSB}}(\text{Pt, entrance }) = 65 \times 10^{-4}\), \(m_{\text{SSB}}(\text{Pt, Bragg}) = 37 \times 10^{-4}\) SSB per plasmid per gray.

It is noteworthy that in the presence of platinum the values of the slopes \(m_{\text{SSB}}(\text{Pt})\) are almost constant for all LET as observed in pure DNA -Table 1-. It indicates that the contribution of the platinum atoms to the DNA breaks induction is almost constant along the track. In this case again, the nuclear fragmentation does not contribute significantly to the radiosensitization effect, for example by additional electronic excitation of the high-Z atoms by the lighter ions resulting from the fragmentation.

The amplification of the DNA breaks in the presence of PtTC has been explained in details elsewhere.\(^{10}\) Shortly, when plasmid DNA is loaded with platinum, secondary electrons emitted along the ion track can induce an Auger effect in the platinum atoms. The emitted Auger electrons resulting from the deexcitation in the high-Z atoms induces mainly water radiolysis and production of the \(^{°}\)OH hydroxyl radicals clusters. Subsequently, the free radicals induce the DSB and SSB in DNA. Briefly stated the excited platinum atom becomes a source of \(^{°}\)OH.

The decrease observed in the presence of DMSO when platinum is added -Table 1- confirms the strong implication of \(^{°}\)OH radical in the radiosensitization process. The effect of the secondary ions and secondary electrons, including Auger electrons and low energy electrons, can be considered as a minor contribution to the induction of direct -non HO\(^{°}\) mediated- damage to DNA.

**Conclusion**

Though different inelastic processes can occur along the track of a fast carbon ion beam, the present experiments show that the induction of the DSB in DNA or complexes of DNA plus platinum is constant, for a given dose, in the DNA.
range of LET studied here (13.1 – 110 keV/μm). The SSB are decreased at the Bragg peak, even though the fragmentation is maximum at this location, compared to the entrance channel. These findings suggest strongly that no significant additional damages in the DNA are due to fragmentation of molecules or carbon nuclei in the matter.

Let us finally remark that the behaviour of the amount of the DNA breaks versus the LET - DSB constant- is not able to account for the enhancement of cell death rate when the LET augment, suggesting that other pathways than induction of the DSB damages in chromosomes are involved in the cell death induction.

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