The Influence of Hydrogen Donors on Breakage of Parental DNA Strands and on Biological Activity of Transforming BrU-DNA of *B. subtilis* after 302/313 nm Radiation

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Long wavelength UV irradiation (302/313 nm) of hybrid and bifilarly labeled BrU-DNA of *B. subtilis* results in a degradation of the molecular weight of BrU and of parental thymine containing DNA strands. Hydrogen donors present during UV irradiation are able to prevent these "primary" and "secondary" strand breaks. The protection factors are especially large for secondary breaks in parental normal DNA. Through the action of protective agents the biological activity could not be restored. The generation of these primary and secondary strand breaks due to BrU incorporation and the action of hydrogen donors on these breaks and on transforming activity are discussed.

Introduction

In normal DNA pyrimidine dimers, especially thymine dimers, are the main photochemical damage[1] and responsible for the inactivation of various biological functions[2-4]. Single strand breaks, however, represent the major alterations of biological importance in 5-bromouracil substituted DNA after UV irradiation[5-8]. The reactions leading to the single strand breaks in BrU-DNA even after irradiation with long wavelength UV are starting with a debromination and the formation of a vinyl type radical which abstracts the proton from the C_2' of the neighbouring nucleotide on the 5′ side[6]. The reactions of the free radical on the deoxyribose result in a decomposition of the sugar and finally in a single strand break[9-11]. These breaks will be called primary strand breaks. Besides these lesions in the BrU containing strands formed by the reactions mentioned above, it was found that 302/313 nm irradiation resulted in a significant amount of breakage in thymine containing parental DNA strands[12-14]. Furthermore double strand breaks in hybrid and bifilarly labeled BrU-DNA with a linear dose dependency have been detected[15]. These breaks are due to secondary reactions and consequently are named secondary strand breaks. Experimental evidence has been presented that these secondary breaks do not result from insertions of

BrU containing regions into parental DNA strands during replication in the presence of BrU either by resynthesis or genetic recombination[6, 12, 13]. They are rather due to reactions between the thymine containing DNA strand and the UV induced lesions on the opposite BrU substituted strand. Several possible models have been proposed to explain the secondary damage by assuming an intramolecular transfer of excitation energy[12, 16] or diffusion of UV induced radicals[3].

It is the purpose of this paper to contribute to the understanding of the nature and generation of these secondary radiation effects by investigating the influence of hydrogen donors. Furthermore the biological implications of hydrogen donors on strand breaks were tested by using transforming principal DNA. It is the essential finding of this paper that hydrogen donors do prevent the formation of primary as well as secondary strand breaks which is in accord with results obtained in other systems[13, 17, 18]. However, no protection was found for transforming principal DNA by hydrogen donors.

The obtained results are discussed and a model is proposed which might explain the experimental finding.

Materials and Methods

Materials and methods have been described earlier[12,19]. For clarity, however, the main points are summarized below.

Preparation of BrU-DNA

BrU-DNA was isolated from a thymine auxotroph mutant of *B. subtilis* (19-8 thy⁻, try⁻, met⁻) grown in a defined medium containing BU. Normal (TT), hybrid (TB), and bifilarly labeled BrU-DNA (BB) were separated by preparative CsCl density gradient centrifugation.

Irradiation with 302/314 nm radiation

For irradiation with long wavelength UV radiation the output of a high pressure mercury arc was filtered with interference reflection type filters (UV R 280; Schott & Gen., Mainz) in combination with UV absorption filters (WG 5 and WG 6; Schott & Gen., Mainz) and passed through a thin plastic film to eliminate scattered light of wavelength shorter than 300 nm. All DNA samples were at a concentration of 20 μg/ml in 1/10 SSC (pH 7.5; 20 °C). As hydrogen donor either cysteamine or mercaptoethanol was used at a final concentration of 0.01 M. The changes of the pH in the DNA solutions were corrected by adding the appropriate amount of 0.1 M NaOH. Fresh solutions of cysteamine were prepared for each experiment.

Determination of weight average molecular weights

After each UV fluence an aliquot of the sample was removed part of which was diluted for transformation assays and the remainder was taken for molecular weight determination. The weight average molecular weights (M_w) of native and alkali denatured DNA were determined in an analytical ultracentrifuge (AUZ 9100 Heraeus Christ) employing CsCl density gradient centrifugation at 44700 rpm. Since the band width of the DNA band in a CsCl density gradient is inversely proportional to the square of the molecular weights [20] changes in the molecular weight can be determined. Single and double strand breakage rates were calculated according to the theory of Charlesby [21] for random degradation of linear polymers.

Transformation assays

For testing the biological activity of the UV irradiated DNA a polyauxotroph mutant of *B. subtilis* (M 172, ade⁻, try⁻, his⁻, met⁻, leu⁻) was employed as acceptor. The transformation experiments were carried out following the method of Bott and Wilson [22].

Results

The effect of hydrogen donors on the breakage rate of BrU substituted DNA strands (primary strand breaks)

Hybrid and bifilarly BrU labeled DNA of *B. subtilis* with a thymine replacement of 50 and 100%, respectively, as measured by buoyant density determination were exposed to various fluences of 302/313 nm radiation in the presence and absence of 0.01 M cysteamine at room temperature and pH 7.5. The weight average molecular weight (M_w) of single stranded alkali denatuerd DNA was then determined as described in Materials and Methods.

In Fig. 1 the ratio M_{wo}/M_w is plotted *versus* UV fluence for the BrU containing strands of denatured TB and BB DNA. The B strand of hybrid (B from T $\longleftrightarrow$ B) and bifilarly labeled DNA (B from

Table I. Weight average molecular weights, breakage rates, and protection factor. Weight average molecular weights (M_{wo} in 10^6 dalton) for unirradiated BrU-DNA of *B. subtilis* in the native and denatured state are compiled with the corresponding breakage rates for ssb and dsb. These single strand and double strand breakage rates for primary as well as secondary damage were obtained after UV irradiation in the absence and presence of hydrogen donors (0.01 M cysteamine). The dimensions of the breakage rates per erg/mm² for ssb and dsb are (10^{-4} breaks/10^6 dalton) and (10^{-4} breaks/$2 \cdot 10^6$ dalton), respectively. The subscript cy indicates that the breakage rates were obtained from samples irradiated in the presence of hydrogen donors. The protection factors (P) are calculated as the ratio ssb/ssb$_{cy}$ or dsb/dsb$_{cy}$. The weight average molecular weight (M_{wo}) of the B-strand from TB or BB DNA is considerably smaller than expected from M_{wo} of the native DNA, thus indicating that already in the unirradiated BrU-DNA single strand breaks or alkali labile lesions are present (see text). For the determination of the single strand breakage rate of the T-strand in TB DNA a different DNA preparation was used. Here M_{wo} of native DNA was $12 \cdot 10^6$ dalton. Thus no single strand breaks or alkali labile lesions are present in the T-strand of unirradiated TB DNA.

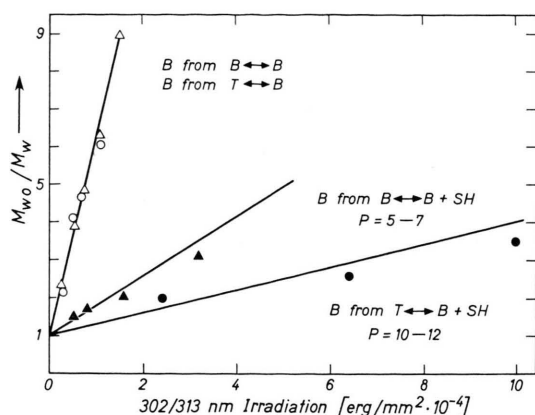|	DNA	$M_{wo} \cdot 10^{-6}$	ssb	ssb$_{cy}$	dsb	dsb$_{cy}$	P
Primary damage	B from BB	0.75	10	1.7	—	—	~5 — 7
	B from TB	0.75	10.8	0.9	—	—	10 — 12
Secondary damage	T from TB	5.5	0.5	0.02	—	—	20 — 25
	TB	3.6	—	—	0.005	<0.0008	>10
	BB	4.9	—	—	0.07	~0.01	5 — 7

Fig. 1. A plot of the reciprocal of the single strand weight average molecular weight ($M_w{}^{-1}$) of hybrid and bifilarly labeled BrU-DNA *versus* UV irradiation (302, 313 nm) normalized for zero dose. The samples were alkali denatured after irradiation, neutralized, and centrifuged in an analytical ultracentrifuge at 44700 rpm in a CsCl density gradient for 24 hours. From the band-width of the DNA bands the weight average molecular weights were determined as indicated in Materials and Methods. $\triangle$ = BrU containing strand or denatured BB DNA (B from B $\longleftrightarrow$ B); $\bigcirc$ = BrU containing strand of denatured TB DNA (B from T $\longleftrightarrow$ B). $\blacktriangle$ and $\bullet$ the same after irradiation in the presence of 0.01 M cysteamine. The protection factors (P) are also given and were obtained from the breakage rates and M_{wo} (see also legend of Table I). The protection factors are given with upper and lower limits.

B $\longleftrightarrow$ B) are degraded by 302/313 nm at roughly the same rate. The breakage rates for single strand breaks are compiled in Table I. They are derived from the data given in Fig. 1 and the single stranded molecular weight (M_{wo}) of the unirradiated DNA.

As can be seen from Fig. 1 the number of strand breaks is considerably reduced when cysteamine is present during irradiation. In a further set of experiments it was found that mercaptoethanol at a concentration of 0.01 M causes the same reduction of strand breaks as cysteamine. From the data given in Fig. 1 and M_{wo} a protection factor P defined as ratio – breakage rate without hydrogen donor/ breakage rate with hydrogen donor – can be calculated. Due to the uncertainty of the molecular weight determinations and to the scattering of the experimental points in the dose effect curves, the protection factors are given with upper and lower limit. The reduction of single strand breaks is different in hybrid and bifilarly labeled DNA. Up to 80% of the primary lesions can be suppressed in BB DNA (P 5 – 7). In hybrid DNA the protection factor is even largre (P 10 – 12). These results are not unexpected from the known reactions leading

to the primary damage and confirm the data obtained with other systems [4, 18].

The effect of hydrogen donors on the breakage rate of parental thymine containing DNA (secondary strand breaks)

Since the process by which single strand breaks are formed in unsubstituted DNA strands base paired with BrU containing strands is not yet known – though several possible mechanisms have been proposed [12, 13, 15] – the influence of hydrogen donors on these secondary breaks was investigated by irradiating hybrid DNA in the presence and absence of hydrogen donors. The molecular weight of the thymine containing strand was then determined. The results of these experiments are given in Fig. 2.
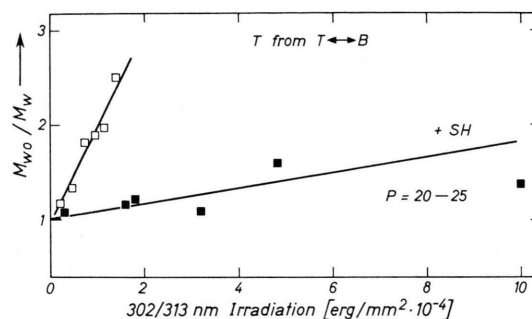

Fig. 2. The same plot as in Fig. 1 for $M_w{}^{-1}$) of the thymine containing strand of hybrid DNA, $\square$ = in the absence; $\blacksquare$ in the presence of 0.01 M cysteamine.

Here the ratio M_{wo}/M_w is plotted *versus* UV fluence for the parental thymine containing DNA strand of hybrid DNA. The generation of strand breaks in the T strand is strongly reduced by the presence of 0.01 M cysteamine during irradiation. The protection factor is about 20 to 25 as obtained from the slope of the dose effect curve and the single stranded molecular weight (M_{wo}) of the unirradiated sample. For these experiments a DNA preparation was used with a native molecular weight of $12 \cdot 10^6$ dalton. Thus a value of $5.5 \cdot 10^6$ dalton for M_{wo} for the thymine containing strand indicates that there are no single strand breaks or alkali labile lesions in the unirradiated T strand of hybrid DNA (see also legend to Table I).

The effect of hydrogen donors on the breakage rate of double strand breaks with linear dose dependency (secondary strand breaks)

In Fig. 3 the reciprocal of the weight average molecular weight normalized for zero dose is plotted
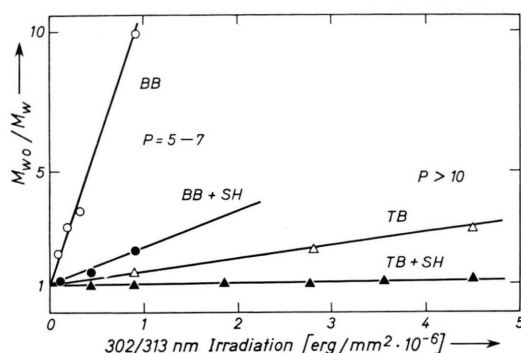
Fig. 3. A plot of the reciprocal double stranded weight average molecular weight (M_{w}^{-1}) for hybrid and bifilarly labeled BrU-DNA. ○; ● = native bifilarly labeled DNA in the absence and presence of 0.01 M cysteamine. △; ▲ = native hybrid BrU-DNA in the absence and presence of 0.01 M cysteamine. The protection factor for BB DNA is again given with upper and lower limits; for TB DNA only a lower limit can be given. Since we cannot exclude completely double strand breaks in hybrid DNA in the presence of 0.01 M cysteamine.

versus UV fluence for native hybrid and bifilarly labeled BrU-DNA. The DNA samples used in these experiments had rather small double stranded molecular weight. The same DNA samples were also used for the experiments presented in Fig. 1. The results indicate that the double strand breakage rate can be reduced substantially by 0.01 M cysteamine. The protection factor for double strand breaks in bifilarly labeled BrU-DNA is about $5 - 7$. For hybrid DNA a small decrease of M_{w} with increasing UV fluence in the presence of O.01 M cysteamine cannot be excluded from our experiments. Thus a lower limit for the protection factor is given only. In native hybrid DNA the protection against secondary breaks is certainly larger than 10.

The data obtained from Figs $1 - 3$ are compiled in Table I together with the M_{wo} values for the unirradiated DNA samples. The molecular weights of the B strands of hybrid and bifilarly labeled DNA are considerably smaller than expected from the M_{wo} values of native TB and BB DNA. This indicates that even in the unirradiated DNA single strand breaks or alkali labile lesions are present in the BrU containing strand. There is about one single strand break per 10^6 dalton formed upon denaturation in the BrU containing strand.

Influence of hydrogen donors on the transforming activity of BrU-DNA in B. subtilis

Since a reduction of primary as well as secondary strand breaks by hydrogen donors was observed it

was expected that biological activity of BrU-DNA could also be protected against the effect of 302/313 nm radiation by the presence of cysteamine. Consequently normal, hybrid, and bifilarly BrU-DNA of *B. subtilis* was UV irradiated in the absence and presence of 0.01 M cysteamine and tested for biological activity. The results of these experiments are shown in Fig. 4. Each experimental point re-
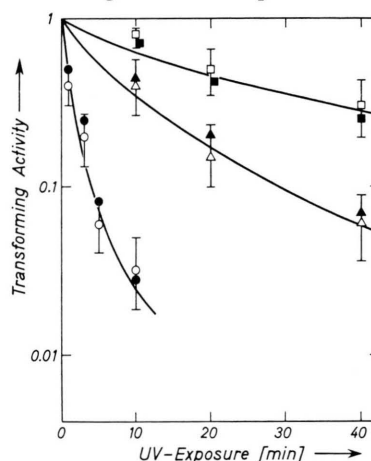

Fig. 4. Relative transforming activity of normal DNA □; ■; hybrid △; ▲ and bifilarly labeled BrU-DNA ○; ●; UV irradiated (302, 313 nm) in the absence and presence of cysteamine 0.01 M. The biological activity was tested for histidine prototrophy. The error bars indicate the mean deviation of at least 4 different irradiation experiments. For clarity they are given for the samples irradiated in the absence of cysteamine only. The mean deviations of the transforming activity of samples irradiated in the presence of cysteamine are of the same order.

presents the average of at least 4 different irradiation experiments. No protective effect was found for hybrid and bifilarly labeled DNA. Cysteamine does not affect the transforming activity of normal DNA. It was, however, observed, that the total number of transformed cells was reduced by a factor of $3 - 4$ for unirradiated samples containing cysteamine.

Discussion

Hydrogen donors like cysteamine and mercaptoethanol reduce the production of primary strand breaks in BrU substituted DNA when present during UV irradiation at a concentration of 0.01 M. It was found that the protection factor (P) is about twice as large in the B strand of hybrid DNA than in the B strand of bifilary labeled DNA. In the B strand of hybrid DNA only primary damage can occur whereas in the B strand of BB DNA energy absorbed by one strand can cause alterations in the

complementary strand. Thus the observed protection factors are not unexpected.

Hydrogen donors also reduce the generation of strand breaks in parental thymine containing DNA. Here the protection factor is about $20-25$. If this secondary damage is due to the transfer of excitation energy one would expect that hydrogen donors would not influence this reaction and a protection factor of 1 should be found. The observed values of P, however, indicate that transfer of excitation energy cannot be responsible for the generation of secondary strand breaks. Alternatively, if primary and secondary damage are due to the same molecular reactions, the same protection factor should be found. The experimental results indicate a higher protection for secondary than for primary damage. Thus either different reactions are responsible for primary and secondary strand breaks or hydrogen donors affect these reactions at different rates. This reasoning is supported by the results obtained with native DNA. It was found that double strand breaks with a linear dose dependency occur in hybrid as well as in bifilarly BrU labeled DNA after 302/313 nm irradiation, thus indicating that part of the secondary breaks due to energy or damage transfer is just a few base pairs away from the primary lesion in the complementary strand. Hydrogen donors reduce these double strand breaks in hybrid and bifilarly labeled BrU-DNA as well. The protection factors are of the same order for primary (B from B ⟷ B and B from T ⟷ B, Fig. 1) and secondary lesions (double strand breaks in BB DNA) in BrU containing DNA strands, whereas the protection for the secondary damage in parental thymine containing strands is considerably larger (ssb in T from T ⟷ B and dsb in TB, Table I). An explanation for these results can be given by assuming that a diffusible reactive species probably a brom radical can react with the complementary DNA strand. This radical has apparently a higher rate of reaction when the complementary strand contains bromouracil. Thus hydrogen donors exert a higher protection for secondary damage in the thymine containing strand. As assumed earlier [12, 16] transfer of excitation energy or exciton migration [23, 24] may not be responsible for the observed effects. These results are in general agreement with the data of Beattie [13] who reports a strong reduction of strand breaks in parental DNA of *Haemophilus influenzae* by cysteamine after 313 nm irradiation.

Under experimental conditions very similar to those used in this work, Mönkehaus [18] found an even higher reduction of secondary breaks in BrU-DNA of phage PBSH by cysteamine (protection factors of up to 100).

Bacterial transformation provides the opportunity for testing the effect of cysteamine and other hydrogen donors on the biological activity of BrU substituted DNA after UV irradiation. In a different biological system protection was reported for plaque forming ability of BrU substituted phage T_1 and T_4 [4, 25] and BrU T_3 phage [26] after irradiation in vivo with 254 nm and 265 nm radiation, respectively. In the system used in this investigation no protective effect was found or hybrid and bifilarly labeled BrU-DNA irradiated with 302/313 nm radiation in the presence of 0.01 M cysteamine (Fig. 4). The observed reduction of the total number of transformed cells in the unirradiated sample might indicate that cysteamine is interfering with the uptake of donor DNA. For this reason the transformation experiments were repeated with another hydrogen donor (mercaptoethanol 0.01 M). No such interference was here observed. There was, however, no indication for a protection either. Similar experiments were then performed with normal and bifilarly labeled DNA and 254 nm radiation. Again no protection was observed. These results are quite unexpected since it has been shown by Thorsett *et al.* [27] that single strand breaks produced either by deoxyribonuclease I or γ-rays are responsible for the loss of transforming activity. Thus restoration of the physico-chemical integrity of the DNA should be accompanied by regaining biological activity. Certainly the strand breaks produced by 302/313 nm radiation are different from those by γ-rays or enzyme treatment. Furthermore only part of the strand breaks observed after alkali denaturation may actually exist in the DNA directly after the photochemical lesion is formed (Hewitt, Hutchinson [3, 28]). Therefore it could very well be that hydrogen donors, besides delivering a hydrogen atom to the urazilyl radical, are also able to react with the alkali labile lesion by forming a DNA-cysteamine complex; thus preventing the formation of a strand break upon denaturation and yet preventing the restoration of biological activity. This model is supported by the following result: Upon mixing cysteamine and urazil in solution a new compound is found by paper chromatography (Lion, personal communication).

So far protection of biological activity against UV light by cysteamine has been observed when BrU-DNA was irradiated *in vivo* [4, 5, 25]. A protection by cysteamine has also been found with infectious BrU-DNA of phage T_1 [29] and with transforming BrU-DNA of *Haemophilus influenzae* [30] after irradiation with 255 nm radiation *in vitro*.

In those experiments the BrU substitution was about 60% and approximately 20%, respectively. In our experiments the BrU-DNA was irradiated *in vitro* in diluted solution. Certainly the DNA configuration and spatial arrangement inside a phage head or within a bacterial cell is different from DNA in solution. Furthermore the replacement of thymine by BrU is considerably higher in the experiments reported here than in *Haemophilus influenzae* [30] and infectious T_1 DNA [29]. It is quite feasible that the high BrU content renders the DNA very susceptible to alterations beyond restoration. The comparatively small protection factors found

for the production of strand breaks in the B strand of BB DNA and in native BB DNA point in this direction as well. The process of DNA uptake is also different for *E. coli* spheroplasts and competent bacterial cells. In the transformation systems discussed here the mechanism of integration of extra cellular DNA is different as well. These differences might be responsible for the different action of hydrogen donors.

Further experiments of the action of hydrogen donors on other biological functions like priming activity of BrU substituted DNA which can be investigated *in vitro* and *in vivo* are necessary to proof or disproof the proposed explanations.

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