OXYGEN-INDEPENDENT DIRECT DEOXYRIBONUCLEIC ACID BACKBONE BREAKAGE CAUSED BY ROSE BENGAL AND VISIBLE LIGHT†

M. J. PEAK and J. G. PEAK

Photobiology Group, Division of Biological and Medical Research, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439 (U.S.A.)

C. S. FOOTE

Chemistry Department, University of California, Los Angeles, CA 90024 (U.S.A.)

N. I. KRINSKY

Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, MA 02111 (U.S.A.)

(Received January 20, 1984; in revised form March 17, 1984)

Summary

An oxygen enhancement ratio of 10 for the induction of backbone single-strand breaks (SSBs) in purified deoxyribonucleic acid (DNA) by monochromatic 365 nm UV radiation was obtained. Similarly, a dose reduction factor of 10 was observed when the DNA was irradiated in the presence of 0.1 M diazabicyclo[2.2.2]octane (DABCO). To determine whether this breakage of DNA was due to the action of a reactive oxygen species such as singlet oxygen, we used the photosensitizing dye Rose Bengal and visible light as a system for generating singlet oxygen. Treatment of the DNA with Rose Bengal and 545 nm monochromatic light enhanced the rate of induction of SSBs six times, compared with the rate we obtained when the light was used alone. Elimination of oxygen or addition of 0.1 M DABCO during the 545 nm irradiation in the presence of Rose Bengal *did not alter* the enhancement of SSBs in the DNA caused by Rose Bengal and 545 nm radiation. The induction of SSBs in the DNA caused by irradiation of the DNA by 545 nm light in the presence of Rose Bengal was not enhanced by the use of D_2O instead of H_2O as a solvent. The results indicate that Rose Bengal plus visible light can cause biological damage without the intermediacy of reactive oxygen species, *i.e.* Rose Bengal and visible light can react directly with biological material, in reactions that appear to be type I photosensitized processes, independent of singlet oxygen as an intermediate.

---

†Paper presented at the COSMO 84 Conference on Singlet Molecular Oxygen, Clearwater Beach, FL, U.S.A., January 4 - 7, 1984.

Elsevier Sequoia/Printed in The Netherlands

1. Introduction

There has been a recent increased interest in the biological effects of solar UV radiations (at wavelengths between 290 and 410 nm) because these wavelengths are carcinogenic [1, 2], their flux might be enhanced by attenuation of the stratospheric ozone shield as a result of man's activities [3] and they are used extensively for cosmetic and medicinal purposes [4]. Our understanding is that unidentified changes in deoxyribonucleic acid (DNA) other than the formation of pyrimidine dimers (which are induced primarily by wavelengths shorter than 290 nm) are responsible for the lethal, mutagenic and carcinogenic effects of these radiations [5, 6]. One type of DNA damage that might be responsible for some of the biological effects is the induction of single-strand breaks (SSBs) in the backbone of the molecule [7, 8] by photodynamic photosensitized reactions involving reactive species of oxygen derived from ground state molecular oxygen [9, 10] and naturally occurring sensitizers such as thiolated rare nucleic acid bases [11, 12]. One of the possible reactive species of oxygen is singlet oxygen [13, 14]. Protection against biological damage caused by solar UV by diazabicyclo[2.2.2]octane (DABCO) and other reagents that quench singlet oxygen constitutes one piece of evidence of a role for this intermediate. More definitive evidence of a role for singlet oxygen is the enhanced induction of SSB in DNA by near-UV radiation when the DNA is in an environment of D_2O, compared with H_2O [14]. However, we also have evidence that reactive species other than singlet oxygen, such as the hydroxyl radical, must be involved in the damages to DNA caused by solar UV radiation [15, 16].

If our model for the effects of solar UV radiation on DNA is correct, and does indeed involve a participation of singlet oxygen in the breakage of DNA, then singlet oxygen must be capable of causing reactions that lead to breaking the covalent bonds holding the DNA backbone together. To the best of our knowledge, this has yet to be demonstrated. The experiments described here were designed to test the effect of singlet oxygen generated independently through the use of the dye Rose Bengal, which has been used extensively in the past to generate singlet oxygen (see for example refs. 17 - 21).

We have used isolated DNA as the substrate in order to eliminate diffusion problems for the reactants due to membranes and chemical barriers.

2. Methods and materials

In the experiments described here, radiolabeled DNA was first purified from *Bacillus subtilis* and then irradiated either aerobically or anaerobically *in vitro* by monochromatic 365 or 545 nm radiation in the absence or the presence of polymer-bound Rose Bengal (2 mg ml^{-1} Sensitox II, Chemical Dynamics Corporation, lot 112759, washed once with methanol and vacuum

dried before use). Irradiation, preparation of other reagents, isolation of ^{14}C-labeled DNA and measurement of DNA SSBs using alkaline sucrose gradient centrifugation were all carried out exactly as described previously [12]. The samples being irradiated were stirred vigorously during irradiation by a stream of oxygen (aerobic irradiations) or stringently prepurified nitrogen, as described [12]. The 545 nm radiation, fluence rate 420 W m^{-2}, was filtered by an LP 400 cut-off filter to eliminate scattered light. In the experiments where the H_2O was replaced by D_2O (kindly supplied by Dr. J. Katz of this laboratory), the original aqueous solvent was removed by lyophilization, and the solute was taken up in an equal volume of D_2O. This procedure did not induce SSBs in our DNA, as measured by our technique.

3. Results

Figure 1 shows that near-UV radiation at 365 nm breaks DNA [12] and that a majority (at least 90%) of these breaks require aerobic conditions. We cannot determine whether those SSBs induced in our anaerobic conditions were the result of traces of contaminating oxygen pervading our system. When DABCO was present in a concentration of 0.1 M during the irradiation, it also eliminated 90% of the SSBs. (This protection by DABCO is not due to preferential absorption of the UV radiation by the DABCO because DABCO does not absorb light at 365 nm.) This confirms previous observations [12, 13] which suggest that near-UV radiations damage DNA

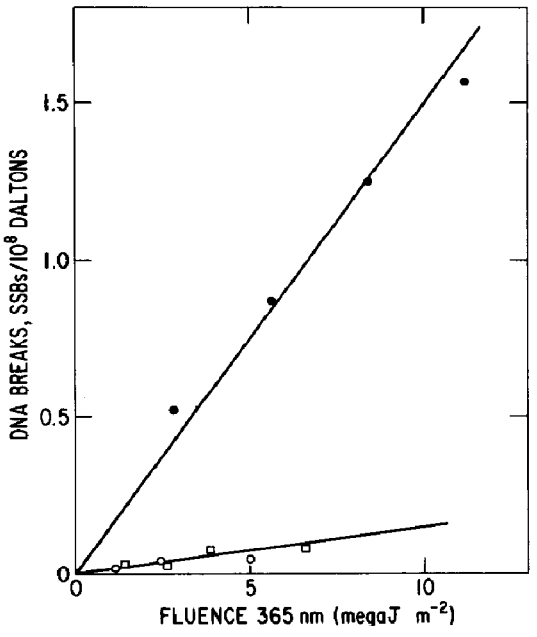

Fig. 1. Rate of induction of SSBs in isolated DNA by 365 nm radiation: ●, aerobic irradiation in the absence of DABCO; ○, anaerobic irradiation in the absence of DABCO; □, aerobic irradiation in the presence of DABCO.

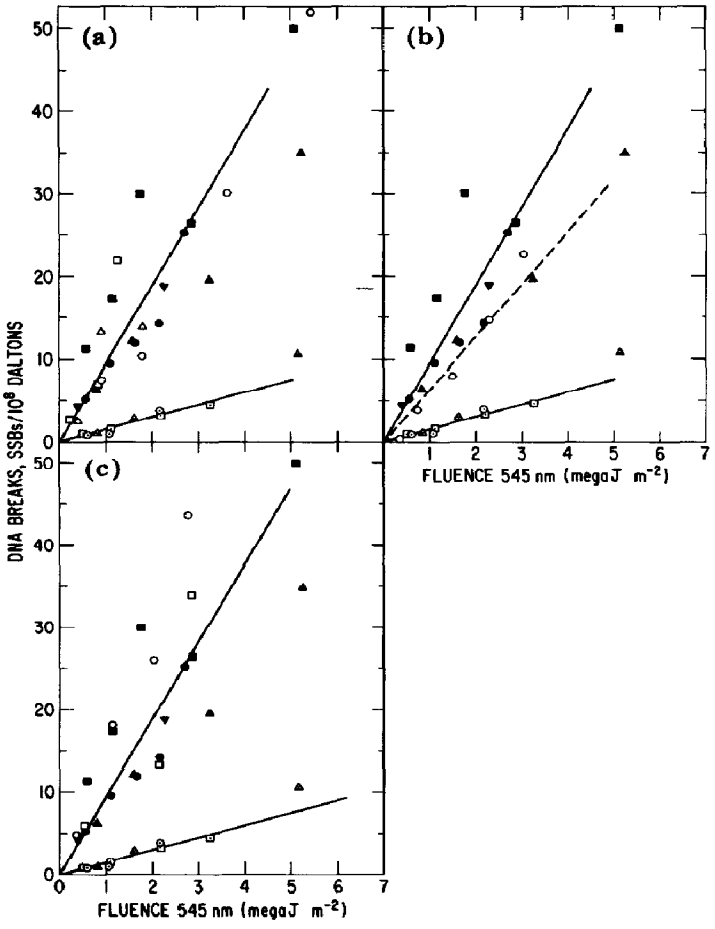

Fig. 2. Induction of SSBs in isolated DNA by 545 nm green light (⊙, △, ⊡, irradiation of the DNA in H_2O with oxygen in the absence of Rose Bengal (three replicate experiments); ■, ●, ▲, ▼, irradiation of the DNA in H_2O in the presence of oxygen and Rose Bengal (four replicate experiments)): (a) □, △, □, irradiation of the DNA in H_2O in the presence of oxygen, Rose Bengal and 0.1 M DABCO (three replicate experiments); (b) ○, irradiation of the DNA in D_2O in the presence of oxygen and Rose Bengal (one experiment); (c) ○, □, irradiation of the DNA in H_2O in the presence of Rose Bengal and in the absence of oxygen and DABCO (two replicate experiments).

by photodynamic photosensitizations involving reactive species of oxygen. At present, we do not know the identity of the photosensitizer or whether it is a contaminant of the DNA or a part of the DNA molecule itself.

Figure 2 presents results of the experiments designed to confirm previous work [13, 14] in which singlet oxygen was postulated to be the reactive oxygen species responsible for breaking the DNA. Each experiment was repeated; the different symbols on the figure signify the different repetitions. In the control experiments without Rose Bengal, the 545 nm radiation alone induced SSBs in the DNA. The nature of this SSB induction by the green light has yet to be explored. The experimental results show that the Rose Bengal plus visible light treatments caused a sixfold enhancement

TABLE 1

Induction of single-strand breaks in deoxyribonucleic acid by Rose Bengal and visible light

Conditions under which the DNA was irradiated with 545 nm light	*SSBs*[a] ($J\ m^2\ s^{-1}$)
Control: no Rose Bengal, O_2, H_2O	1.8 ± 0.8 (10)
Rose Bengal, O_2, H_2O	10.2 ± 3.8 (16)
Rose Bengal, O_2, D_2O	6.0 ± 3.5 (5)
Rose Bengal, O_2, H_2O	13.0 ± 4.2 (7)
Rose Bengal, O_2, DABCO, H_2O	11.7 ± 6.6 (10)

[a]Mean value of the slope of the induction curve together with the standard deviation; the numerals in parentheses indicate the number of determinations.

over the controls of the rate of breakage of DNA compared with the rate of induction caused by the light treatment alone. However, neither anoxia nor the presence of 0.1 M DABCO reduced the enhancement of SSBs caused by Rose Bengal and visible light. In the experiments in which D_2O replaced H_2O as solvent, the rate of induction of SSBs by Rose Bengal and visible light was not enhanced. These data are summarized in Table 1, which also demonstrates that there is no statistically significant (none of the standard deviations overlaps) effect of anoxia, DABCO or D_2O on the increase of SSBs caused by the Rose Bengal and light. In fact, there may have been a slight reduction when the D_2O was used compared with H_2O. However, the number of experiments available at present does not allow us to consider this decrease to be significant.

4. Discussion

In this paper we confirm that DNA is broken by near-UV radiation as a result of a photosensitized reaction that involves the generation of one or more species of reactive oxygen. Thus, near-UV radiation is capable of initiating type II (singlet oxygen) photosensitized oxidations. In addition, we have demonstrated that Rose Bengal plus visible light increased the rate of induction of SSBs in DNA compared with the rate in the absence of Rose Bengal. However, our attempts to demonstrate that this is a type II reaction were negative. Under anaerobic conditions, we observed the same rate of induction of SSBs as under aerobic conditions. In addition, having assumed that a type II reaction would occur, we studied the effect of the singlet oxygen lifetime modifiers, DABCO and D_2O. In both cases, the data did not support the possibility that singlet oxygen might be involved in the Rose-Bengal-sensitized reaction. It would therefore appear likely that Rose Bengal and visible light are functioning in a type I photosensitive process, involving direct electron or hydrogen atom transfer from substrate to sensitizer, in contrast with type II reactions involving singlet oxygen.

Type I mechanisms for killing cells and damaging the components in nucleic acids have been reviewed in the past [22]. Of particular interest have been the reports indicating that eosin can function as a type I photosensitizer [23] and the recent observation that methylene blue catalyzes the anaerobic reduction of oxidized cytochrome C via a type I reaction [24]. In that mechanism, the reactive species is reduced methylene blue formed by the photo-oxidation of the buffer system (either tris-HCl or sodium phosphate) by triplet state methylene blue [25].

Rose Bengal has been used extensively in the past as an agent for carrying out type II photosensitized oxidations. It has been demonstrated [26] that it can kill yeast cells in the presence of visible light, but no gene conversion has been observed, presumably because Rose Bengal did not penetrate the yeast membrane and therefore could not interact with the nuclear material. However, an additional report [17] indicates that polymer-bound Rose Bengal can kill *Escherichia coli* via a singlet oxygen mechanism thus suggesting that the reactive species generated by the polymer-bound Rose Bengal can diffuse either to the surface of the bacterium or into the cell itself.

We therefore conclude that Rose Bengal plus visible light induces SSBs in isolated DNA by a type I photosensitized reaction rather than by the expected type II (singlet oxygen) reaction. This result does not, of course, shed any light on the question of the mechanism of the near-UV-induced SSBs in DNA; the previous conclusion that this damage is at least partially due to singlet oxygen still applies. However, the type I reaction with Rose Bengal appears to be more efficient.

Acknowledgment

The work of M.J.P. and J.G.P. was supported by the U.S. Department of Energy under Contract W-31-109-ENG-38.

References

1 H. F. Blum, *Carcinogenesis by Ultraviolet Light*, Princeton University Press, Princeton, NJ, 1959.
2 J. H. Epstein, in W. Montagna (ed.), *Advances in Biology of Skin VII*, Pergamon, Elmsford, NY, 1966.
3 U.S. National Research Council, *Causes and Effects of Stratospheric Ozone Reduction: An Update*, National Academy Press, Washington, DC, 1982.
4 J. A. Parrish, R. R. Anderson, F. Urbach and D. Pitts, *UV-A: Biological Effects of Ultraviolet Radiation with Emphasis on Human Responses to Longwave Ultraviolet*, Plenum, New York, 1978, pp. 221 - 240.
5 M. J. Peak and J. G. Peak, in J. Calkins (ed.), *The Role of Solar Ultraviolet Radiation in Marine Ecosystems*, Plenum, New York, 1982, pp. 325 - 336.
6 R. B. Webb, in K. C. Smith (ed.), *Photochemical and Photobiological Reviews*, Plenum, New York, 1977, pp. 169 - 268.

7 M. J. Peak and J. G. Peak, *Photochem. Photobiol., 35* (1982) 675.
8 M. J. Peak and J. G. Peak, *Physiol. Plant., 58* (1983) 367.
9 R. B. Webb and M. S. Brown, *Photochem. Photobiol., 29* (1979) 407.
10 J. G. Peak, M. J. Peak and R. W. Tuveson, *Photochem. Photobiol., 38* (1983) 541.
11 M. J. Peak, J. G. Peak and L. Nerad, *Photochem. Photobiol., 37* (1983) 169.
12 J. G. Peak, M. J. Peak and M. MacCoss, *Photochem. Photobiol., 39* (1984) 713.
13 J. G. Peak, C. S. Foote and M. J. Peak, *Photochem. Photobiol., 34* (1981) 45.
14 M. J. Peak, J. G. Peak and C. S. Foote, submitted to *Photochem. Photobiol.*
15 J. G. Peak, M. J. Peak and C. S. Foote, *Photochem. Photobiol., 36* (1982) 413.
16 J. G. Peak and M. J. Peak, *Radiat. Res., 97* (1984) 570.
17 S. A. Bezman, P. A. Burtis, T. P. J. Izod and M. A. Thayer, *Photochem. Photobiol., 28* (1978) 325.
18 G. W. Byers, S. Cross and P. M. Henrichs, *Photochem. Photobiol., 23* (1976) 37.
19 N. M. C. Kaye and P. D. J. Weitzman, *FEBS Lett., 62* (1976) 334.
20 W. R. Midden and S. Y. Wang, *J. Am. Chem. Soc., 105* (1983) 4129.
21 A. Schaap, A. L. Thayer, E. C. Blossey and D. C. Neckers, *J. Am. Chem. Soc., 97* (1975) 3741.
22 G. Lober and L. Kittler, *Photochem. Photobiol., 25* (1977) 215.
23 A. G. Kepka and L. I. Grossweiner, *Photochem. Photobiol., 14* (1971) 621.
24 D. Harmetz and G. Blauer, *Photochem. Photobiol., 38* (1983) 385.
25 C. S. Foote, Y. C. Chang and R. W. Denny, *J. Am. Chem. Soc., 92* (1970) 5218.
26 T. Ito and K. Kobayashi, *Photochem. Photobiol., 26* (1977) 581.