Photosensitized Lysis of Egg Lecithin Liposomes by L-Tryptophan and N-Acetylphenylalanyl-L-Tryptophan

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Received October 12, 1985

The photosensitized lysis of egg lecithin lipid membranes (liposomes) have been performed to UV-B light (270-320nm) by L-tryptophan(L-Trp) and its peptide such as N-acetylphenylalanyl-L-tryptophan(NAPT) incorporated in the liposomes(ca. 0.1% by weight) or in the external buffer (0.1-0.3mM). Requirement of oxygenation suggests that the lysis of liposomes is caused by the photosensitized oxidation of lipids. There was significant protection against lysis photosensitized by Trp in the external buffer by low concentration of ferricyanide (0.8mM), but there was no effect on the lytic efficiency by N_3^- which is singlet oxygen($^1O_2$) quencher, indicative of an electron transfer mechanism involved in the photosensitization. The small change of the lytic efficiency with increasing pH from 4 to 9 was interpreted by large target theory and subsequently indicates that superoxide(O_2^-) may be an active intermediate for the oxidation. The efficiency of photosensitization of Trp was higher than that of NAPT under the same experimental condition. The weak lytic efficiency of liposomes photosensitized by NAPT was enhanced by incorporating NAPT in liposomes, but it was again quenched by β-carotene incorporated in the bilayer of liposomes. These results indicate that a portion of liposome lysis may be due to $^1O_2$ formation from the excited NAPT.

Introduction

It has been well known that both protein and lipid components of cell membranes are chemically changed upon absorption of ultraviolet B(UVB) radiation. Kochevar[1] suggested that the dominant chromophore causing the UVB-induced chemical changes of membrane components is tryptophan (Trp) in the membrane proteins. Many investigators have already reported that Trp photosensitizes oxidation of Trp-containing proteins by initial formation of a photodynamic sensitizer, N-formylkynurenine(NFK) from photodestruction of Trp[2] or by electron transfer both intramolecularly and intermolecularly.[3,4]

Our previous investigations[5,6] have demonstrated that Trp-peptides also photosensitize the oxidation of methyl linoleate(ML) in ethanol solution, supporting the possibility of Trp-photosensitized oxidation of membrane lipids. In this case, NFK was not involved in the photosensitization. On the contrary, the Trp-photosensitization was attributable to the intermolecular electron transfer between ML and the excited Trp in competetion with generation of singlet oxygen($^1O_2$) directly from the excited Trp by interaction with molecular oxygen.

Nonetheless, the mechanism of Trp-photosensitization at the membrane level remains unclear. Some Trp in polypeptide such as gramicidin D is located in the hydrophobic region of the membrane lipid bilayer.[7] On the other hand, glycophorin A, as a major glycoprotein of red blood cell membranes contains Trp-peptide which is located in the external to the membrane. Thus, it is worth while to examine microenvironmental effects on the Trp-photosensitization at membrane level.

This paper describes the photosensitizing activity of Trp in lysis of heterogeneous model system consisting of suspension of egg-lecithin liposomes. It is shown that Trp-photosensitization mechanism of the lysis of liposomes is influenced by location of Trp in the liposome suspension.

Experimental

L-tryptophan(L-Trp) and N-acetylphenylalanyl-L-tryptophan(NAPT) were used as received from Research Plus Lab. Egg-lecithin (L-α-phosphatidylcholine from egg yolk) and dimyristoyl phosphatidylcholine (DMPC) obtained from Sigma Chemical Co. were used without further purification, since they showed pure single spot on TLC eluted with solvent mixture (hexane/ether/acetic acid = 35/2/1 by volume). All other chemicals were reagent grade.

Liposomes were prepared by evaporating a chloroform solution of lipid to dryness in a round bottom flask under reduced pressure. The lipid was then dispersed in Tris-HCl buffer (0.1M Tris-HCl + 0.1M NaCl, pH 4-9) with or without L-Trp or NAPT by sonication at ambient temperature for 60 min with Bransonic sonifier (60Hz). The sonified dispersion was centrifuged at 154,000g in ultracentrifuge (MSE Prepsin 75 Model) to separate unilamellar liposomes from multilamellar liposomes. NAPT-incorporated liposomes were prepared by mixing a chloroform solution of egg-lecithin (4mg.ml^{-1}) with an ethanol solution of NAPT (0.40mg.ml^{-1}) and evaporating to dryness with nitrogen gas followed by sonication in Tris-buffer. Free NAPT were eliminated by dialysis in 3 l of buffer at 4°C for 24 hours. Information about binding of NAPT to liposomes were obtained by using fluorescence spectroscopic method which was performed on Jovin-Yvon JY3 spectrofluorometer.

The relative liposome concentration was measured by turbidity at 750 nm on a Beckman UV-5260 spectrophotometer. Dilution measurements showed that turbidity (measured in terms of O.D.) at 750 nm was linearly proportional to the lipid concentration of undyed liposomes up to O.D. = 1.5, which was determined by heating method of phosphorus assay.[8] Thus, the relative photolysis of liposomes were determined by decrease of turbidity (O.D.) at 750nm, where O.D. = 1.0 was a typical initial value.

Samples of 3.5 m*l* were irradiated in Pyrex test tube located in front of water-cooled Pyrex vessel. The suspension of liposomes were continuously bubbled with oxygen or nitrogen gas via syringe needle at flow rate 400 bubbles.min^{-1}. The light source was a 200W Hg lamp(Bausch & Lomb). The incident fluence rate was 1.1×10^{18} quanta.$l^{-1}.s^{-1}$ as determined by the chemical actinometry using ferrioxalate solution.[9]

Results and Discussion

Location of Trp in the liposome suspension was determined by comparing fluorescence emission maxima of Trp measured in the liposome suspension with those measured in different homogeneous solvents of various polarity. Table 1 shows the emission maxima of L—Trp and NAPT in liposome suspension as well as in homogeneous solvents, which were measured with excitation at 280nm. When liposomes were prepared by sonicating dispersion of lipid in L—Trp or NAPT-containing buffer, L—Trp and NAPT appeared to be located in aqueous phase of liposomes, because their emission maxima were the same as those in aqueous solution. On the other hand, when liposomes were prepared by sonicating aqueous dispersion of NAPT—containing lipid, the fluorescence maximum of NAPT shifted to the blue(λ_{em}^{max} = 345mn) which is close to that in ethanol, indicating that NAPT is incorporated into the ethanol—like polar site of liposome bilayer. Supporting this fact, fluorescence quenching of NAPT—incorporated liposomes by KI is less efficient than that for free NAPT, as shown in Stern—Volmer plot(Figure 1). By assuming fluorescence lifetime of NAPT in both aqueous solution and liposome bilayer 2.9ns, the bimolecular quenching constant, k_q in water was calculated to be $1.1 \times 10^9\ M^{-1}s^{-1}$ which drops to $0.8 \times 10^9\ M^{-1}s^{-1}$ for NAPT—incorporated liposomes. The small reduction of k_q again indicates that NAPT is incorporated into the polar site in the outer layer of liposomes.

Photosensitized lysis of liposomes by 0.4mM L-Trp in external buffer were monitored by decrease of turbidity at 750nm. The results in Figure 2 show that lysis of egg—lecithin liposomes are caused by 60min irradiation with oxygen bubbling. The lysis of liposomes are much more significant (40-50%) in the presence of L—Trp than those in the absence of L—Trp (less than 10%), indicating that lysis of liposomes are photosensitized by L—Trp. The weak photolysis of liposomes in the absence of L—Trp might be due to hydrodynamic effect on autooxidation of lipid as proposed by Grossweiner.[10] Actually, the lysis of liposomes appear to be caused by photosensitized oxidation of unsaturated lipid, since the lysis are completely protected by nitrogen bubbling and the photosensitized lysis of saturated DMPC are much less effective than that of the unsaturated egg—lecithin liposomes (see Figure 2). Photosensitization by Trp may occur by in-

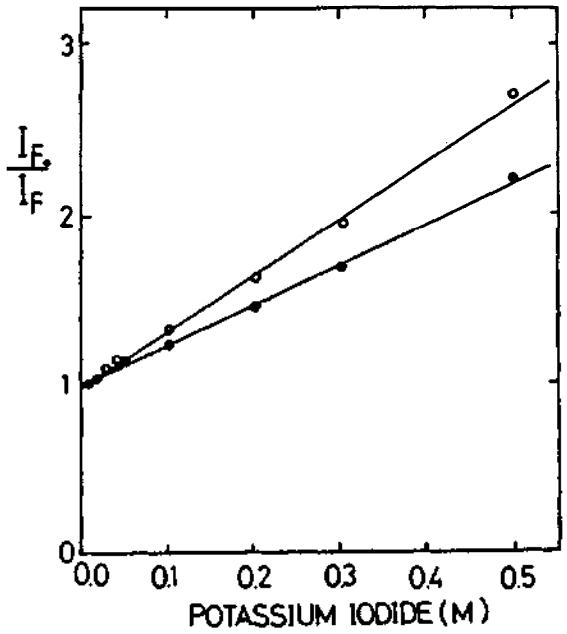

Figure 1. Stern-Volumer plot of fluorescence quenching of NAPT-incorporated liposomes (●) and free NAPT(○)by KI. NAPT concentration was 0.15 mM.

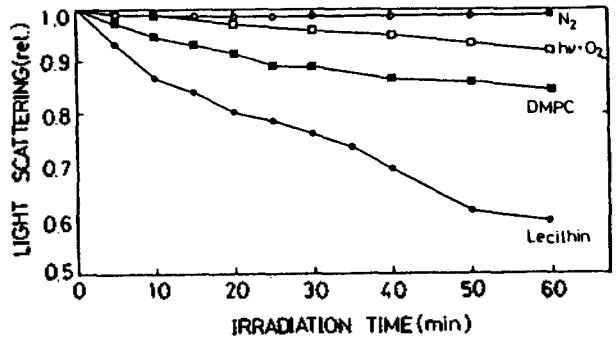

Figure 2. Photosensitized lysis of phosphatidylcholine liposomes by 0.4 mM L-Trp in the external buffer (pH 8) with O_2 bubbling (rate, 480 bubbles·min^{-1}. (●) egg lecithin + NAPT + O_2. (○) egg lecithin + NAPT + N_2. (■) DMPC + NAPT + O_2. (□) egg lecithin – NAPT + O_2.

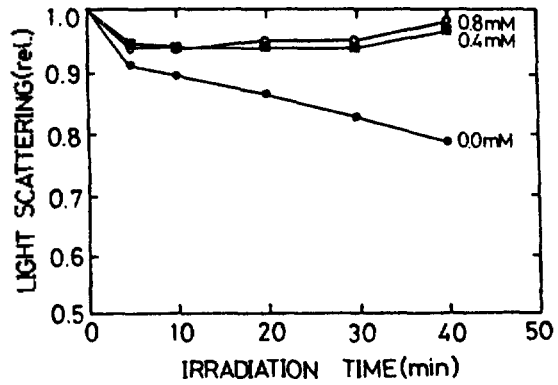

Figure 3. Ferricyanide effect on photosensitized lysis of lecithin liposomes by 0.2 mM L-Trp in the external buffer (pH 8.0) with oxygen bubbling (rate, 480 bubbles·min^{-1}).

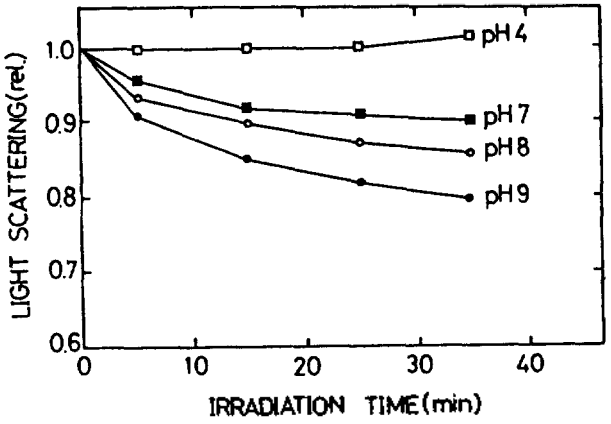

Figure 4. Effect of medium pH on photolysis of liposomes in the presence of 0.4 mM L-Trp.

itial formation of photoproduct of Trp, NFK which is known to be a photodynamic sensitizer for the photooxidation of protein.[11,12] However, the lysis of liposomes seem to be photosensitized by unchanged Trp, because the ratio of the percent Trp remaining in the preirradiated solutions (before irradiation with liposomes) to the relative photolysis of liposomes was close to unity (see Table 2).

It is well known that Trp in aqueous solution photoionizes to form hydrated electron.[13,14] Thus, the possible mechanism for Trp–photosensitization may involve the formation of hydrated electron. As shown in Figure 3, the Trp–photosensitized lysis of liposomes were completely protected by ferricyanide at low concentration (0.1–0.8mM), indicative of electron ejection from L–Trp. The hydrated electron subsequently may react with O_2, generating an active superoxide anion (O_2^-) for the oxidation of lipid components. Actually the Trp–sensitized photolysis of liposomes were also inhibited by superoxide dismutase with no observable inhibition by azide which is a singlet oxygen quencher.

If O_2^- is an active oxygen intermediate, rate of photolysis of liposomes would depend on the pH of aqueous solution

$$HO_2 \overset{k_1}{\rightleftharpoons} O_2^- + H^+ \quad (1)$$

because of the dismutation equilibrium with dismutation rate constant,[16] $k_{dis} = (8.60 \times 1^5 + 1.02 \times 10^8)/(1 + X)^2$, where $X = K_1/[H^+]$ and $pK_1 = 4.69$.[16] The superoxide concentration at steady state is given by

$$[S] = (R\,\phi_{O_2^-}/k_{dis})^{1/2} \quad (2)$$

where R is absorbed fluence rate and $\phi_{O_2^-}$ is O_2^- formation quantum yield. Assuming that $\phi_{O_2^-}$ is the same as the quantum yield of hydrated electron (0.08 in the pH range 4-8 and 0.12 at pH 9),[17] superoxide concentrations for the present absorbed fluence rate ($R = 1.1 \times 10^{16}$ quanta $l^{-1}s^{-1}$) were calculated as shown in Table 3. This calculation leads to expectation that the lysis rate increases significantly from pH 4 to pH 9 because of highly increased concentration of superoxide at higher pH. However, the results in Figure 4 show a small dependency of the lysis rate on pH. This discrepancy may be explained by Grossweiner's large target theory[18] which has been applied to the reaction of small intermediate with spherical target such as liposome. According to this theory, when the targeting space is comparable to or larger than the mean diffusion range of the intermediate, the mean number of O_2^- hits required for liposome lysis after absorbed fluence rate, R, is given by

$$\eta_{O_2^-}^{-1} = 4\pi D_{O_2^-}\tau_{O_2^-}\phi_{O_2^-}(1+r/(D_{O_2^-}\tau_{O_2^-})^{1/2})R \quad (3)$$

where r is the target radius, $D_{O_2^-}$, $\tau_{O_2^-}$ and $\phi_{O_2^-}$ are the diffusion rate constant, lifetime and formation quantum yield of O_2^-, respectively. Assuming $r = 5 \times 10^{-5}$ cm for mean radius of liposomes, $D_{O_2^-} = 3 \times 10^{-5}\ cm^2s^{-1}$,[15] $\phi_{O_2^-}$ from Table 3, $R = 1.1 \times 10^{16}$ quanta cm^{-3} and $\tau_{O_2^-} = 1/k_{dis}[S]$, $\nu_{O_2^-}^{-1}$ was calculated at each pH (see Table 3.) This calculation indicates that number of O_2^- interactions required for the lysis of liposomes increase from pH 4 to pH 9. Thus, this effect may be compensated by the increased concentration of O_2^-, resulting in small dependency of lysis on pH of aqueous medium.

Similar results were observed when NAPT was dissolved in external aqueous phase of lecithin liposomes, but the rate of the NAPT-photosensitized lysis of liposomes was significantly lower than when L–Trp was used under the same condition (see Figure 5). This indicates that Trp–

Table 1. Fluorescence Emission Maxima (λ_{max}, nm) of L-tryptophan and NAPT in Different Media

Medium / Compound	water	ethanol	isopropanol	n-hexane	liposome suspension
L-tryptophan	360	—[a]	—	—	360
NAPT	360	342	340	—	360[b]
					340[c]

[a]- indicates insolubility of the compound. [b]Measured when liposomes were prepared by sonicating dispersion of lipid in NAPT-containing buffer. [c]Measured when liposomes were prepared by sonicating aqueous dispersion of NAPT-containing lipid.

Table 2. Relative Photolysis of Liposomes Sensitized by Preirradiated L-tryptophan

% Remaining L-Trp[a] (1)	Relative photolysis[b] (2)	(2)/(1)
100	100	1.00
92	91	0.99
88	85	0.97
84	80	0.95

[a]Percent remaining tryptophan was monitored by measuring changes in the absorbance at 280 nm after a certain period of irradiation (20, 40, 60 min) with UV-B light. [b]Photolysis of liposomes were performed by 2 hours irradiation.

Table 3. Photolysis of Liposomes Sensitized by L-Trp in the External Buffer at Various pH

Parameter	pH 4	pH 7	pH 8	pH 9
$\phi_{O_2^-}$ [a]	0.08	0.08	0.08	0.12
k_{dis} [b]	1.5×10^7	5.0×10^5	5.0×10^4	5.0×10^3
S [c]	8.6×10^{-8}	4.7×10^{-7}	1.5×10^{-6}	5.8×10^{-6}
$\eta_{O_2^-}^{-1}$ [d]	1.3×10^9	6.6×10^9	2.2×10^{10}	8.5×10^{10}

[a]O_2^- quantum yield assumed to be the same as e^- quantum yield, from Bent and Hayon.[17] [b]Dismutation rate constant ($M^{-1}s^{-1}$) from Bielski.[16] [c]Steady state superoxide concentration, M. [d]Mean number of O_2^- hits for liposome lysis.

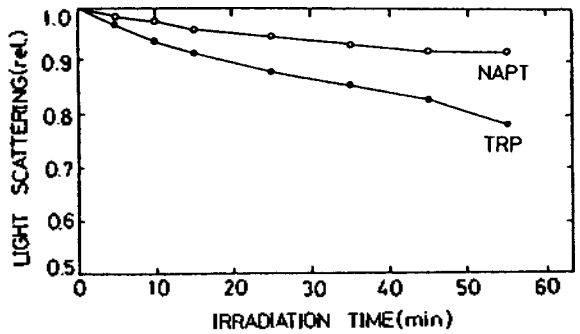

Figure 5. Photosensitized lysis of lecithin liposomes by L-Trp and NAPT in the external buffer (pH 8.00) with O_2 bubbling (rate, 480 bubbles·min^{-1}). The optical densities of both L-Trp and NAPT were adjusted to be the same at 280 nm (1.66).

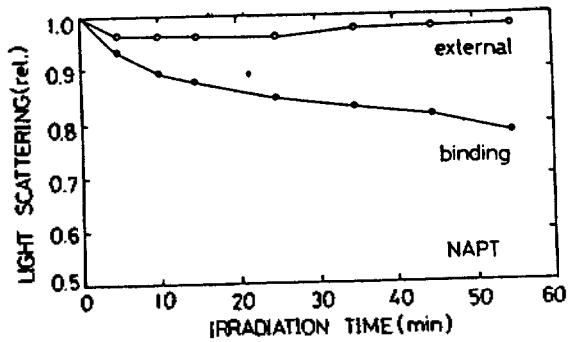

Figure 6. Comparison of photosensitized lysis of NAPT-incorporated lecithin liposomes with those of liposomes by external NAPT. O.D. of NAPT in both cases were adjusted to be the same at 280 nm (0.26).

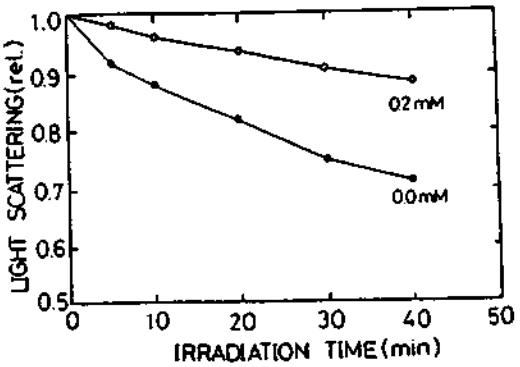

Figure 7. β-carotene effect on the photosensitized lysis of lecithin liposomes by 0.15 mM-NAPT-incorporated liposomes with O_2 bubbling (rate, 480 bubbles·min^{-1}).

photosensitization may depend on peptide constitution. NAPT as well as L-Trp in water is photodecomposed by itself through oxidation with O_2^- which is generated by photoionization of NAPT in aerobic condition.[6] Quantum yield for the photochemical destruction of NAPT in water is approximately 15 times higher than that of L-Trp.[6] Thus, O_2^- reaction with NAPT would be in competition with the reaction with liposomes more significantly than when L-Trp is used.

However, the low photosensitizing ability of NAPT was observed to be enhanced by incorporating NAPT in the liposome bilayer. As shown in Figure 6, incorporating NAPT (absorbance at 280 nm = 0.26) into liposomes resulted in 20% lysis of liposomes upon 50 min irradiation with oxygen bubbling, but dissolving the same amount of NAPT in aqueous phase (absorbance at 280 nm = 0.26) resulted in no lysis of liposomes. These results imply that mechanism of Trp-photosensitization in liposome bilayer phase may be different from that in aqueous phase. The photosensitized lysis of NAPT-incorporated liposomes was completely protected by N_2 bubbling, indicating that an active oxygen species should be involved in the photosensitization of NAPT in liposome bilayer phase, too. Our previous investigation[5] has demonstrated that NAPT, not its photoproduct, photosensitizes the oxidation of ML in ethanol partially by generating singlet oxygen, even though multiple competing pathways are not completely ruled out. Since NAPT was shown to be incorporated in the ethanol-like site of the bilayer of liposomes, one could expect the similar effect of singlet oxygen on the oxidation of lipid of liposomes. To determine whether or not singlet oxygen participates in the lysis of liposomes photosensitized by NAPT, β-carotene (0.2 mM) which is singlet oxygen quencher[19,20] was incorporated into the bilayer with NAPT before irradiation. Figure 7 shows that β-carotene quenched the photosensitized lysis of lecithin liposomes by 60%, indicating that at least a portion of liposome lysis appears to be due to singlet oxygen generated from the photoexcited NAPT, in contrast to the photosensitized lysis of liposomes by NAPT in the external aqueous phase. Unfortunately, we were not able to determine whether or not generation of singlet oxygen is the only mechanism for the lysis of liposomes photosensitized by NAPT incorporated in the lipid bilayer, because of difficulty in incorporating β-carotene at different concentration. Thus, the possibility of non-singlet oxygen mechanism remains open for further investigation.

In conclusion, lysis of unsaturated phospholipid liposomes seem to be photosensitized by Trp in aqueous phase through oxidation of lipid with O_2^- generated by photoionization of Trp in aerobic condition. However, singlet oxygen mechanism also seems to work in the photosensitization of Trp when Trp is located in the lipid bilayer phase of liposomes.

Acknowledgement. The aduthors thank the Korea Science and Engineering Foundation for financial support.

References

1. I.E. Kochevar, *J. Invest, Dermatol.* **74**, 256 (1980).
2. (a) P. Walrant and R. Santus, *Photochem. Photobiol.* **19** 411 (1974); (b) P. Walrant and R. Santus, *Photochem. Photobiol.* **20** 455 (1974).
3. L.I. Grossweiner and Y. Usui, *Photochem. Photobiol.* **13**, 195 (1971).
4. Y. Lion, M. Kawa bara and P. Riesz, *Photochem. Photobiol.* **35**, 53 (1982).
5. I.E. Kochevar and M. Yoon, *Photochem. Photobiol.* **37**, 279 (1983).
6. M. Yoon, M.Y. Song and D.W. Cho, *Bull. Korean Chem. Soc.* **6**, 291 (1985).
7. D. Marsh and A. Watts, In "Lipid-Protein Interaction (Edited by P.C. Jost and O.H. Griffin) Vol. 2, pp 53-126, Weiley, New York.
8. R.B. Grant, *J. Biol. Chem.* **234**, 466 (1958).
9. C.G. Hatchard and. C.A. Parker, *Proc. R. Soc. London, Ser. A* **235** 518 (1956).
10. L.I. Grossweiner and J.B. Grossweiner, *Photochem. Photobiol.* **35**, 583 (1982).
11. M. Pailthorpe, J. Bonjour and C. Nicho 1s, *Photochem. Photobiol.* **19** 209 (1973).
12. L.I. Grossweiner, A.L. Brendzel and A. Blum, *Chem. Phys.* **57** 147 (1981).
13. H.B. Steen, *J. Phys. Chem.* **61** 3997 (1974).
14. C. Pigault, C. Hasselmann and G. Lanstriat, *J. Phys. Chem.* **86** 1755 (1982).
15. R. Muller-Runkel, J. Blain and L.I. Grossweiner, *Photochem. Photobiol.* **33** 683 (1981).
16. B.H.J. Bielski, *Photochem. Photobiol.* **28**, 645 (1978).
17. D.V. Bent and E. Hayon, *J. Am. Chem. Soc.* **97**, 2612 (1975).
18. L.I. Grossweiner, *Photochem. Photbiol.* **28**, 645 (1978).
19. A. Farmilo and F. Wilkinson, *photochem. Photobiol.* **18**, 447 (1973).
20. A. Garner and F. Wilkinson, In "Singlet-Oxygen Reaction with Organic Compounds and Polymers" (edited by B. Ranby and J.F. Rabek) PP. 48, Wiley, New York.