ELSEVIER

Biophysical Chemistry 88 (2000) 111–117

Biophysical Chemistry

www.elsevier.nl/locate/bpc

# Pulse radiolytic oxidation of β-carotene with halogenated alkylperoxyl radicals in a quaternary microemulsion: formation of retinol

S. Adhikari[a,*], S. Kapoor[a], S. Chattopadhyay[b], T. Mukherjee[a,1]

[a] *Radiation Chemistry & Chemical Dynamics Division, Bhabha Atomic Research Centre, Mumbai 400 085, India*
[b] *Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai 400 085, India*

Received 26 June 2000; received in revised form 20 August 2000; accepted 23 August 2000

**Abstract**

Pulse radiolytically generated halogenated alkylperoxyl radicals, namely $CCl_3OO^{\bullet}$, $CBr_3OO^{\bullet}$, $CHCl_2OO^{\bullet}$, $CHBr_2OO^{\bullet}$, etc. have been employed for a study of the oxidation of β-carotene in a quaternary microemulsion. All these halogenated alkylperoxyl radicals produce a radical cation (absorption maximum at 840 nm), either via the initial formation of an adduct (absorption maximum at 740 nm), or by direct reaction. There was a considerable blue shift of both absorption peaks in the present system as compared to those earlier reported in non-polar as well as in micellar media. An additional intense absorption peak at approximately 345 nm was noted at a longer time scale and was stable up to 5 ms. On irradiation with a large number of electron pulses, a stable product with an absorption maximum at 315 nm appeared. On excitation at 315 nm, this product gave a fluorescence spectrum with an emission maximum at 525 nm. The absorption and fluorescence spectra of the stable product compared with those of retinol; this was further confirmed by HPLC analysis. A suitable mechanism has been proposed. 

*Keywords:* β-Carotene; Pulse radiolysis; Retinol

* Corresponding author.
*E-mail address:* asoumya@apsara.barc.ernet.in (S. Adhikari).
[1] Co-corresponding author.

0301-4622/00/$ - see front matter 
PII: S0301-4622(00)00202-7

## 1. Introduction

There is growing evidence that retinoids, and perhaps carotenoids, can be anti-carcinogenic. As is to be expected, considerable attention has been given vis-à-vis the intake and metabolism of preformed vitamin A (retinal, retinol and its esters) and carotenoids possessing pro-vitamin A activity. Between these two classes of compounds, the retinoids have drawn extensive attention for their cancer prevention activities. Available data suggest [1] that retinoids can protect the advanced stage of neoplastic progression [2] and, depending on their concentration in blood level, can reduce cancer risk. It has been rationalised that retinoids can: (i) suppress the malignant behaviour of cells transformed with viruses, chemicals and ionising radiation; (ii) delay or prevent the onset of cancer for animals treated with DNA-binding carcinogens; and (iii) suppress some of the biochemical reactions causing tumour promotion [3].

In contrast to the retinols, however, the exact role of the lipid-soluble natural carotenoids, viz. β-carotene (β-C), against cancer risk is still speculative and controversial [4], although these are well-known as ubiquitous free radical quenchers [5–7]. Indeed, the efficacy of β-C (and other carotenoids) as anticarcinogens and for the prevention of cardiovascular diseases have been attributed to their antioxidant properties in combination with immuno-modulatory activity [8,9]. However, the antioxidant effect of β-C has yet to be proven in humans.

It would be of relevance to address: (i) the direct effect of β-C on cell differentiation; (ii) other types of protection of tissues, e.g. against singlet oxygen, superoxide, etc.; or (iii) the conversion of β-C to retinol-type molecules. Amongst these, the first two factors have been studied to some extent, and a correlation between β-C intake and lowering risk of both cancer and heart diseases have been established by epidemiological studies [10,11]. The β-C and several other carotenoids appear to upregulate the gap junctional intercellular communications and chemically suppress induced neoplastic transformation. [12,13]. Whether these biological effects are caused by β-C itself, or by some of its metabolites, like the retinoids, is still not clear. This factor might be very crucial, considering the established efficacy of retinoids in cancer prevention.

β-Carotene is known as 'provitamin A' due to its conversion into retinal by the enzyme dioxygenase via its central [14,15] or excentric [16] cleavage. This has even been demonstrated [17] by in vivo tests with vitamin A-deficient animals. Surprisingly, although the enzyme is present chiefly in the intestine and possibly in the liver, accumulation of vitamin A has been noticed in many tissues in mice [18]. This leads to the speculation as to whether it is possible that vitamin A can also be derived from β-C by some purely chemical protocol. This question can be addressed by focusing investigation on the interaction of β-C with various reactive oxygen species (ROS).

Earlier, the interaction of ROS with β-C has been investigated in aqueous–organic solvents, micellar membranes using flash photolysis [19], or pulse radiolysis [20,21]. All these studies revealed easy oxidation of the polyolefinic system of β-C by halogenated alkylperoxyl radicals, generating a radical adduct and cation $[\beta\text{-C}]^{\bullet+}$. In an attempt to mimic the in vivo system, the present pulse radiolytic study was carried out in a quaternary microemulsion. The water pool in the microemulsion mimics the water pockets often found in various bio-aggregates, such as proteins, membranes and mitochondria [22]. There is strong evidence about the structural similarity between protein embedded in phospholipids and surfactant aggregates in a microemulsion. The enzymes incorporated in the aqueous core of the reverse micelles are protected against denaturation [23–25]. Moreover, interfacial reactions of the lipid-soluble biomolecules solubilised in the organic phase can be easily studied in these systems. To the best of our knowledge, this is the first report on the peroxyl radical-mediated oxidation of β-C in a microemulsion system. The microemulsion used for the study was composed of sodium lauryl sulphate (3.23% w/v), cyclohexane (75% v/v), water (6.45% v/v) and 1-pentanol (15.32% v/v).

## 2. Experimental

### 2.1. Materials

β-Carotene (β-C) and retinol were purchased from Sigma and used as such. Spectroscopic grade 1-pentanol was obtained from Fluka. All other chemicals used were of analytical reagent grade. The solvent used was freshly prepared deionised 'nanopure' water (conductivity 0.06 μS $cm^{-1}$).

### 2.2. Methods

The pulse radiolysis system using single pulses of 7-MeV electrons has been described earlier [26]. The width of the pulse was 50 ns. The dosimetry was carried out using air-saturated $5 \times 10^{-2}$ mol $dm^{-3}$ KSCN ($G_{\varepsilon} = 21\,522$ $dm^3$ $mol^{-1}$ $cm^{-1}$ per 100 eV at 500 nm) [27]. The kinetic spectrophotometric detection systems covered the wavelength range from 250 to 1100 nm. The optical path length of the cell was 1.0 cm. The dose/pulse was 15 Gy. The bimolecular rate constants given were obtained from the slope of the plot of the pseudo first-order rate constants obtained at different β-C concentrations, using a minimum of five points. Microemulsions were prepared just prior to each experiment.

### 2.3. HPLC analysis

The HPLC analyses were carried out with a Bruker instrument. Both retinol and β-carotene were analysed on a reverse phase RP-18 column (LichroCART 250-4 Lichrosphere, 5 μm, $4 \times 250$ mm, E. Merck, Germany) with acetonitrile/water as the mobile phase. The best resolution of the peaks for retinol and β-carotene was achieved using acetonitrile/water (9:1 v/v) with a flow rate of 1.0 ml $min^{-1}$. The peaks were detected at 305 nm using a UV-visible absorption detector and identified by comparison with those obtained from standard retinol and β-carotene samples. For quantification of the retinol formed, the peak was detected at 325 nm and the yield of retinol assayed by external calibration.

## 3. Results and discussion

In the present study, $CCl_3OO^{\cdot}$ was chosen as the ROS, as $CCl_4$ is one of the best-studied [28] endogenous toxicants in biological systems. $CCl_4$ is easily activated by the omnipresent NADPH-cytochrome P-450 system, generating $CCl_3^{\cdot}$ and $CCl_3OO^{\cdot}$, the latter being implicated for acute cell death. Although the reduction potential of the $CCl_3OO^{\cdot}$ radical is 1.5 V [29], which is on the high side compared to the physiologically relevant peroxides, and the *tert*-butylperoxyl radical might have been a better choice as a model peroxyl radical, we have still used the $CCl_3OO^{\cdot}$ radical as a representative one for its inherent simplicity in performing the experiments. Moreover, this radical had been used earlier to study a very important biophysical phenomenon, namely free radical interaction between vitamin E and vitamin C [30].

In an earlier report, we studied the generation and kinetics of hydrated electrons in a quaternary microemulsion [31,32]. In this medium, the hydrated electrons are produced via the following two-step reactions:

$$C_6H_{12} \rightarrow C_6H_{12}^{+} + e^{-} \xrightarrow{\text{captured by water pool}} e_{aq}^{-}$$

$$H_2O\,(\text{water pools}) \rightarrow e_{aq}^{-} + OH^{\cdot}$$

The $OH^{\cdot}$ radicals produced in situ are scavenged by pentanol present in the system. The hydrated electrons subsequently react with $CCl_4$ in the presence of oxygen to furnish the halogenated alkylperoxyl radicals as shown below:

$$e_{aq}^{-} + CCl_4 \rightarrow CCl_3^{\cdot}$$

$$CCl_3^{\cdot} + O_2 \rightarrow CCl_3OO^{\cdot}$$

The other possible source of $CCl_3OO^{\cdot}$ radicals can be direct scavenging of dry electrons from the oil phase by $CCl_4$ following the addition of oxygen.

The formation of the halogenated alkylperoxyl radicals in the microemulsion was confirmed from the typical radical cation peaks of bilirubin, DNA bases [33], etc., generated by their oxidation in

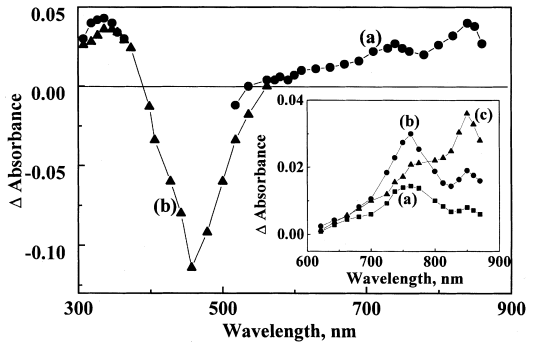


Fig. 1. Transient absorption spectra obtained from an air-saturated microemulsion solution ($w_0 = 32$) at two different β-carotene concentrations, containing $5 \times 10^{-2}$ mol dm$^{-3}$ $CCl_4$ at 50 μs after the electron pulse. Dose, 15 Gy/pulse. (a) $1 \times 10^{-4}$ mol dm$^{-3}$ and (b) $1 \times 10^{-5}$ mol dm$^{-3}$ β-carotene. Inset: time-resolved absorption spectra within a narrow wavelength region for the transients obtained at: (a) 5; (b) 15; and (c) 80 μs after the electron pulse.

the presence of $CCl_4$. Since the above system ensures the exclusive formation of the above radicals, the oxidation of the biomolecules might be caused exclusively by the halogenated alkylperoxyl radicals.

The transient absorption spectra recorded at 50 μs following the reaction of the $CCl_3OO^{\bullet}$ radical with all-*trans* β-C are shown in Fig. 1. Earlier, in similar studies [19–21] with β-C, the transient absorption spectra were shown in the wavelength range above 550 nm only. In contrast, to gain more insight into the oxidative process and to search for the evolution of the radical cation, we have measured the full range spectrum from 300 to 900 nm. Interestingly, in addition to the bleaching region, three distinct absorption peaks were noted. The difference in kinetics for the formation and decay of these peaks suggest that they belong to three independent species. The first absorption peak formed at 740 nm was ascribed to the addition radical $[CCl_3OO\text{–}\beta\text{-}C]^{\bullet}$, while the slowly forming absorption peak at 840 nm could be due to the radical cation $[\beta\text{-}C]^{\bullet+}$. This is supported by the mechanism suggested by Hill et al. [34], and is shown prominently in the inset in Fig. 1. Some selective oscilloscope traces for the transients are shown in Fig. 2. However, the formation of the $[\beta\text{-}C]^{\bullet+}$ species (absorption peak at 840 nm) seemed to involve a two-step process, which is not clear in Fig. 2B(b). To probe this aspect, the radiolysis experiment was carried out over a smaller wavelength range in a shorter time (inset Fig. 1). It was found that at an early stage (5 μs), both the above absorption peaks were present. Thus, the radical adduct and the radical cation were formed due to direct interaction of the $CCl_3OO^{\bullet}$ radical and β-C. Even at a later time (15 μs), growth of the transients continued. At a comparatively later stage (80 μs), when there was no $CCl_3OO^{\bullet}$ radical present in solution, the peak due to the radical adduct started decreasing. However, the growth of $[\beta\text{-}C]^{\bullet+}$ continued, suggesting that this is formed both by a direct route as explained above and also through the intermediate radical adduct.

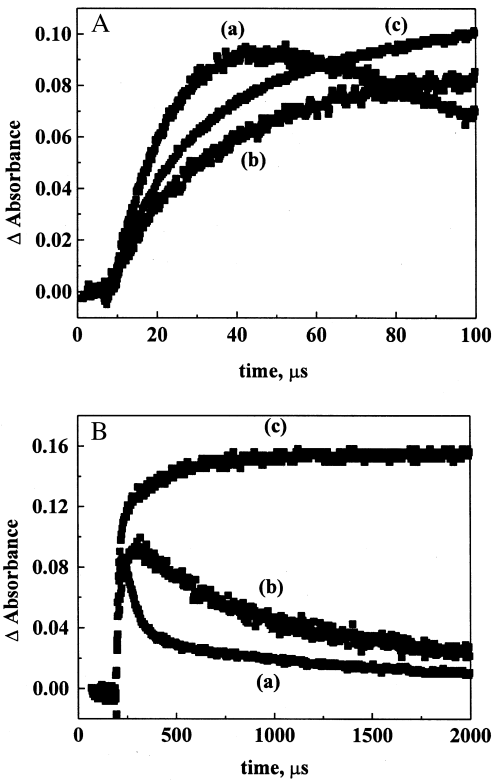


Fig. 2. Absorption vs. time plot for the transients obtained from an air-saturated microemulsion solution ($w_0 = 32$) containing $1 \times 10^{-4}$ mol dm$^{-3}$ β-carotene and $5 \times 10^{-2}$ mol dm$^{-3}$ $CCl_4$. (A), formation: (a) 740; (b) 840; and (c) 345 nm. (B), decay: (a) 740; (b) 840; and (c) 345 nm.

Table 1
Formation and decay constants of the transients formed in the reaction of β-carotene with halogenated peroxyl radicals

| Radical | Monitoring wavelength (nm) | Formation constant ($k_2$) ($dm^3\ mol^{-1}\ s^{-1}$) | Decay constant[a] ($2k/\varepsilon l$) $s^{-1}$ |
|---|---|---|---|
| $CCl_3OO^{\bullet}$ | 740 | $1.6 \times 10^9$ | $6.3 \times 10^4$ |
| | 840 | $7.2 \times 10^8$ | $9.0 \times 10^2$ |
| | 345 | $4.3 \times 10^8$ | Not observed |
| $CBr_3OO^{\bullet}$ | 740 | $3.3 \times 10^9$ | $9.0 \times 10^4$ |
| | 840 | $7.6 \times 10^9$ | $8.2 \times 10^2$ |
| | 345 | $3.2 \times 10^9$ | Not observed |

[a] Due to the inherent complexity in the microemulsion system it is difficult to give an exact $\varepsilon$.

Surprisingly, in contrast to the earlier observation [34], the absorption peaks in the present study were blue-shifted, not only in hexane medium, but also in an aqueous 2% Triton-X 100 solution. Possibly, this shift is caused by the residence of the radical cation in the inner region of the palisade layer. β-Carotene is present in the oil phase or in the palisade layer, as it is barely soluble in water. The radicals are produced chiefly in the water phase, and these start diffusing through the palisade layer where the reaction occurs and the transients are produced. Along with the surfactant molecules, the palisade layer also contains 1-pentanol, which provides a polar environment to the radicals, causing the remarkable blue shift in their absorption spectra. The bimolecular rate constants for the formation of different transients are given in Table 1.

Most of the above observations, except the blue shift of the transient absorption spectra, were in conformity with the earlier reports [34]. However, the most striking feature of the present study was the appearance of a new absorption band in the UV region with an absorption maximum at 345 nm. This absorption peak was very intense, appeared at a comparatively later time, and was stable up to 5 ms. The oscilloscope traces of the absorption peak are shown in Fig. 2A(c) and B(c). When the solute was kept for 10 min, the solution started to show an absorption in the same region as in the transient spectra, thereby indicating the formation of a stable product. To characterise the product, radiolysis was carried out with a large number of 50-ns pulses and the steady-state absorption and fluorescence spectra were recorded. Fig. 3a shows the steady-state absorption spec-

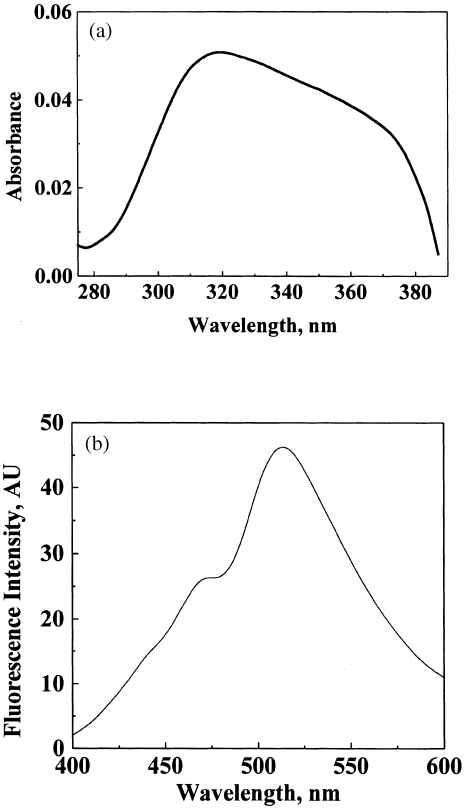


Fig. 3. (a) Steady-state absorption and (b) fluorescence spectra recorded after continuous pulse irradiation of an air-saturated microemulsion solution ($w_0 = 32$) containing $5 \times 10^{-5}$ mol $dm^{-3}$ β-carotene and $5 \times 10^{-2}$ mol $dm^{-3}$ $CCl_4$. Cumulative dose 4.8 kGy (thiocyanate dosimetry).

trum obtained from the microemulsion solution containing $10^{-4}$ mol $dm^{-3}$ β-C and $5 \times 10^{-2}$ mol $dm^{-3}$ $CCl_4$ with a cumulative irradiation dose of 4.8 kGy as measured by thiocyanate dosimetry on a single pulse. A broad peak (Fig. 3a) beginning at 280 nm and culminating at 380 nm with the absorption maximum at approximately 305 nm was observed. The spectral characteristics indicated a strong resemblance to those due to retinol. In order to confirm this, the product was excited at 315 nm, and a strong fluorescence spectrum with a maximum at 525 nm (Fig. 3b) was obtained. Previously, the fluorescence spectrum of retinol has been studied extensively by several groups. These revealed that there was no mirror-image relationship in the fluorescence spectrum, and a definite energy gap between the absorption and emission spectra was reported. Hudson et al. [35] have explained this by postulating the presence of a low lying $^1A_g$-state, which leads to an internal conversion after the initial $\pi-\pi^*$ transition takes place, thus accounting for the anomalies in the spectral lifetime. The fluorescence characteristics of the present product matched exactly with those previously reported [35], the difference between the absorption and emission maxima being $> 150$ nm. Final confirmation of the product composition was arrived at by carrying out HPLC analysis. For this, the HPLC analyses were carried out with a reverse phase RP-18 column (column details in Section 2.3) using different eluent systems and flow rates. Mixtures of acetonitrile/$H_2O$ in different compositions (5, 10, 12 and 15%) were tried as the eluent system, while the flow rates were varied from 0.5–2.0 ml. It was found that the eluent system, acetonitrile/$H_2O$ (9:1) with a flow rate of 1.0 ml/min could efficiently resolve the peaks of β-carotene and retinol, having retention times of 12.34 and 8.68 min, respectively. Under the same conditions, the reaction mixtures were analysed and the chromatographic profiles compared with those of the standard samples. For estimation of the amount of retinol formed, a standard graph was first plotted using pure retinol, and the peak areas of retinol formed in the reaction were compared. To optimise the maximum yield of retinol, pulse radiolysis was carried out with 200, 400 and 600 pulses (50 ns duration). The best yield of retinol was obtained with 400 pulses (cumulative dose of 4.8 kGy according to thiocyanate dosimetry). While a lower number of pulses led to a very poor yield (5–7%) of the product, a higher dose resulted in the degradation of the retinol produced. A quantitative assay of the product formed was also carried out using the external standard technique and, at best, 16–18% retinol formation was noted under the optimised conditions.

It is known that in the reaction of peroxyl radical with β-C a number of products were identified by systematic product analysis [36–38]. According to Mordi et al. [37], the main products in the early stages of the oxidation were 5,6-epoxy-β-carotene, 15,15′-epoxy-β-carotene, diepoxides and a series of β-apo-carotenals and carotenes. As the oxidation proceeds, the longer chain β-apo-carotenals are oxidised to shorter chain carbonyl compounds, β-apo-13-carotenone, β-ionone, etc. The 15,15′-epoxy-β-carotene as the product of peroxyl radical oxidation was first identified by Kennedy and Liebler [36], and has a maximum in its absorption spectrum at 330 nm. In the present study, the product has a broad absorption spectrum peaking at approximately 315 nm, and no other peak at higher wavelengths has been observed. The broadness of the spectrum indicates that it is composed of a number of compounds along with retinol. However, we have concentrated on retinol as one of the components in this product absorption spectrum, and this was confirmed by fluorescence spectroscopy, as well as by HPLC analysis. All the earlier studies confirm that, in addition to other sites, the 15,15′ position is also a site of attack in β-carotene for bulky peroxyl radicals. This possibly explains the formation of retinol in this study. This argument gained further credence by the fact that a similar reaction profile was still obtained with other bulky peroxyl radicals, viz. $CBr_3OO^{\bullet}$, $CHCl_2OO^{\bullet}$, and $CHBr_2OO^{\bullet}$.

In conclusion, the present study shows evidence for the formation of retinol from β-carotene by a non-enzymatic oxidative pathway during its role as an antioxidant. This might also explain the presence of retinol in organs other than the intestine and the liver, as found earlier with mice [20].

## Acknowledgements

The authors are grateful to Dr J.P. Mittal for constant encouragement and support. We are also thankful to Shri V.N. Rao for valuable technical help.

## References

[1] R.K. Boutwell, Selected abstracts on vitamin A in cancer biology, International Cancer Research Data Bank, NCI, Bethesda, Maryland, USA, 1979.
[2] R. Peto, Proc. R. Soc. Lond. B 205 (1979) 111.
[3] M.B. Sporn, D.L. Newton, Fed. Proc. 38 (1979) 2528.
[4] F. Bohm, J.H. Tinkler, T.G. Truscott, Nature Med. 1 (1995) 98.
[5] S.A. Everette, M.F. Dennis, K.B. Patel, S. Maddix, S.C. Kundu, R.L. Willson, J. Biol. Chem. 271 (1996) 3988.
[6] T.A. Kennedy, D.C. Liebler, J. Biol. Chem. 267 (1992) 4658.
[7] G.W. Burton, K.U. Ingold, Science 224 (1984) 569.
[8] A. Bendich, J.A. Olson, FASEB J. 3 (1989) 1927.
[9] N.I. Krinsky, Free Radical Biol. Med. 7 (1989) 617.
[10] O.P. Heinonen, N. Engl. J. Med. 330 (1994) 1029.
[11] R. Peto, R. Doll, J.D. Buckley, Nature 290 (1981) 201.
[12] J.S. Bertram, H. Bortkiewiecz, Am. J. Clin. Nutr. 62 (1995) 1327S.
[13] M. Hanusch, W. Stahl, W.A. Schulz, H. Sies, Arch. Biochem. Biophys. 317 (1995) 423.
[14] T. van Viliet, F. van Schaik, W.H.P. Schreurs, H. van den Berg, Int. J. Vitam. Nutr. Res. 66 (1996) 77.
[15] A. Nagao, A. During, C. Hoshino, J. Terao, J.A. Olson, Arch. Bichem. Biophys. 328 (1996) 57.
[16] X.D. Wang, N.I. Krinsky, G.W. Tang, R.M. Russel, Arch. Biochem. Biophys. 293 (1993) 298.
[17] X.D. Wang, J. Am. Coll. Nat. 13 (1994) 314.
[18] J.A. Olson, O. Hayashi, Proc. Natl. Acad. Sci. USA 54 (1965) 1364.
[19] F.A. Dawe, E.J. Land, J. Chem. Soc. Faraday Trans. I 71 (1975) 2162.
[20] J.E. Packer, J.S. Mahood, V.O. Mora-Arellano, T.F. Slater, R.L. Wilson, B.S. Wolfenden, Biochem. Biophys. Res. Commun. 98 (1981) 901.
[21] S.A. Everett, S.C. Kundu, S. Maddix, R.L. Willson, Biochem. Soc. Trans. 23 (1995) 2308.
[22] K. Kalyansudaram, Photochemistry in Microheterogeneous Systems, Academic Press, New York, 1987, p. 143.
[23] S.S. Atik, J.K. Thomas, J. Am. Chem. Soc. 103 (1981) 4367.
[24] J.M. Furois, P. Brochette, M.P. Pileni, J. Colloid Interface Sci. 97 (1984) 352.
[25] R. Wolf, P. Luisi, Biochem. Biophys. Res. Commun. 89 (1979) 209.
[26] T. Mukherjee, in: S.A. Ahmed (Ed.), Atomic and Molecular Cluster Physics, , 1997, p. 299 (New Delhi).
[27] E.M. Fielden, in: J.H. Baxendale, F. Busi (Eds.), The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis, Riedel, Dordrecht, 1982, p. 49.
[28] M.R. Cholbi, M. Paya, M.J. Alcaraz, Experimentia 47 (1991) 196.
[29] T.N. Das, T. Dhanasekaran, Z.B. Alfassi, P. Neta, J. Phys. Chem. A. 102 (1998) 280.
[30] J.E. Packer, T.F. Slater, R.L. Wilson, Nature 278 (1979) 738.
[31] S. Adhikari, R. Joshi, C. Gopinathan, J. Colloid Interface Sci. 191 (1997) 268.
[32] S. Adhikari, R. Joshi, C. Gopinathan, Int. J. Chem. Kinet. 30 (1998) 699.
[33] S.K. Kapoor, S. Adhikari, T. Mukherjee, Res. Chem. Int. (submitted).
[34] T.J. Hill, E.J. Land, D.J. McGarvey, W. Schalch, J.H. Tinkler, T.G. Truscot, J. Am. Chem. Soc. 117 (1995) 8322.
[35] B.S. Hudson, B.E. Kohler, Chem. Phys. Lett. 14 (1972) 299.
[36] T.A. Kennedy, D.C. Liebler, Chem. Res. Toxicol. 4 (1991) 290.
[37] R.C. Mordi, J.C. Walton, G.W. Burton et al., Tetrahedron 49 (1993) 911.
[38] D.C. Liebler, T.D. McClure, Chem. Res. Toxicol. 9 (1996) 8.