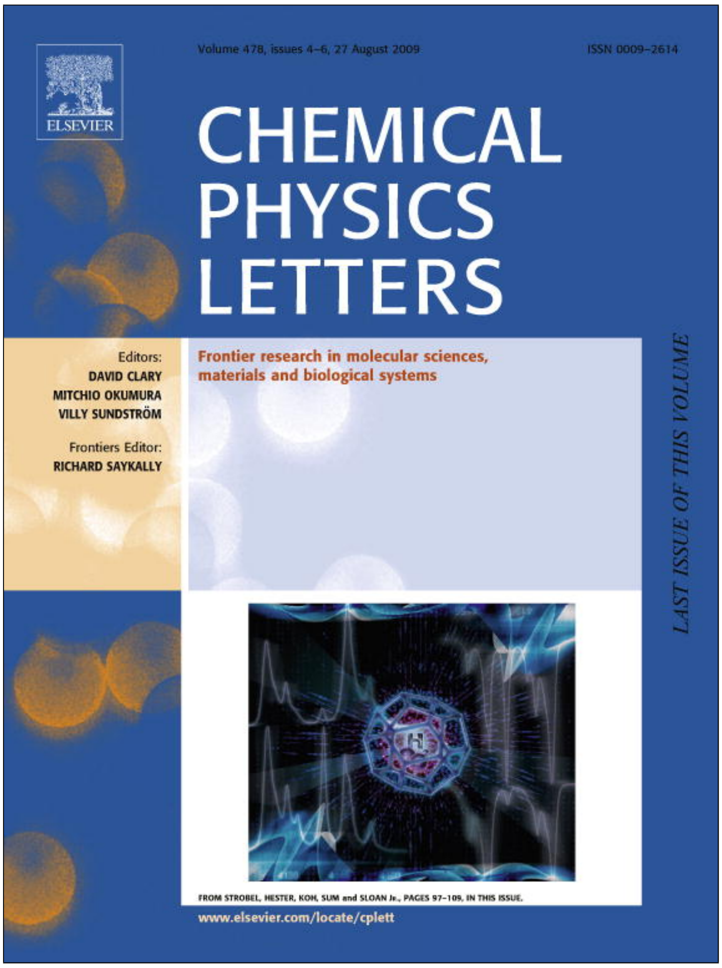
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Electron transfer from all-trans β-carotene to the t-butyl peroxyl radical at low oxygen pressure (an EPR spectroscopy and computational study)

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ABSTRACT

It has been documented that less strongly oxidising radicals, including alkylperoxyl radicals, react with carotenoids via hydrogen atom transfer, thereby generating the neutral carotene radical. In this work, we have studied the reaction between the weakly oxidising *t*-butyl peroxyl radical ($TBHP^{\bullet}$) and β-carotene in dichloromethane under low oxygen pressure. Despite the rather low value of the reduction potential of $TBHP^{\bullet}$, we have succeeded in the formation of the cation radical of β-carotene (β-carotene$^{\bullet+}$). Based on EPR and NIR-IR spectroscopy, the reaction mechanism for this reaction has been assigned to an electron transfer mechanism. This relatively rare mechanism for the reaction between less strongly oxidising species, such as the $TBHP^{\bullet}$ radical and carotenoids has been explained on the basis of DFT and DFT–PCM calculations.

1. Introduction

Of the various classes of pigments that occur in nature, the carotenoids (car) are among the most widespread and important, primarily due to their very varied functions [1]. The antioxidant properties of carotenoids are well known and several mechanisms have been suggested for the protection provided by carotenoids in biological systems [2–6]. Carotenoids are effective quenchers of free radicals, in particular peroxyl radicals [2,7].

It is widely reported that carotenoids react with a wide range of strongly oxidising radicals such as $CCl_3O_2^{\bullet}$, $RSO_2^{\bullet}$, $NO_2^{\bullet}$, and various arylperoxyl radicals via electron transfer producing the radical cation of the carotenoid [8–13]. It has also been reported that with weakly oxidising radicals, such as alkylperoxyl radicals, the reaction proceeds via an hydrogen abstraction process to produce the neutral carotenoid radical [8,10]. In addition, adduct formation with sulphur-centred radicals has also been reported [8]. These three mechanisms can be summarised as follows:

$$\mathrm{CAR} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{CAR}^{\bullet+} + \mathrm{ROO}^{-}\ \text{(Electron transfer)}, \tag{1}$$

$$\mathrm{CAR} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{CAR}^{\bullet} + \mathrm{ROOH}\ \text{(Hydrogen abstraction)}, \tag{2}$$

$$\mathrm{CAR} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{ROO} - \mathrm{CAR}^{\bullet}\ \text{(Addition)}. \tag{3}$$

A hypothesis has been presented by Burton and Ingold [14] and later expanded by Burton [15] which proposed that the addition (reaction 3) of peroxyl radicals to any of the polyene-chain double bonds (Scheme 1, A_I) forms a resonance-stabilised radical adduct which is then followed by the addition of a second peroxyl radical (Scheme 1, A_{II}) leading to non-radical bis-peroxyl adduct. This latter adduct subsequently decomposes (Scheme 1, A_{III}) to carbonyl-containing products by either homolytic or heterolytic cleavage. Addition of a peroxyl radical to the 5,6-double bond (Scheme 1, B_I) followed by elimination of alkoxyl radical (Scheme 1, B_{II}) thus yields non-radical epoxides. Alternatively, the initial carbon centred carotene radical adduct, ROO–CAR$^{\bullet}$, may also react with oxygen reversibly (reaction with O_2, Scheme 1) and lead to the formation of a carotenoid-peroxyl radical. The rate of this reaction depends on O_2 pressure and at high pO_2, the equilibrium of the reaction shifts to the right and the carotenoid, because of autoxidation, forms a peroxyl radical capable of acting as a pro-oxidant.

As mentioned above, less strongly or weakly oxidising radicals react typically via an hydrogen abstraction (reaction 2) process to produce the neutral carotenoid radical. In this work, we present an experimental and modelling study of the relatively rare electron-transfer reaction mechanism (reaction 1) for the reaction between the weakly oxidising peroxy radicals, namely *tert*-butyl peroxyl radical ($TBHP^{\bullet}$) and β-carotene in dichloromethane solution at very low pO_2. This reaction resulted in the formation of the cation radical of β-carotene (β-carotene$^{\bullet+}$)

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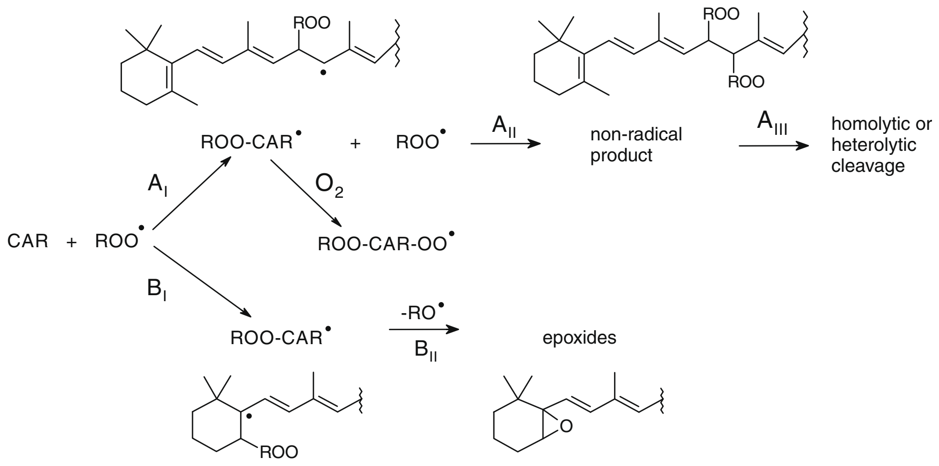

Scheme 1. Pathways of the reactions of carotenoids with peroxyl radicals (ROO·).

$$\beta\text{-carotene} + \text{TBHP}^{\cdot} \rightarrow \beta\text{-carotene}^{\cdot+} + \text{TBHP}^{-}. \quad (4)$$

The stability of both TBHP· and β-carotene·+ radicals under the conditions of the experiments is discussed. In addition, the DFT calculations and the inclusion of solvent effect to substantiate that the studied reaction (4) is feasible is provided.

2. Experimental

2.1. Chemicals

t-Butyl-hydroperoxide (TBHP) and dichloromethane (stored over a molecular sieve) were purchased from Fluka. The PbO_2 was obtained from Lachema Brno (Czech Republic). All trans β-carotene was a gift from the 'Carotenoid Research Laboratory', School of Biological and Earth Sciences, Liverpool John Moores University, Liverpool, England.

2.2. EPR spectroscopy

All the first derivative EPR spectra were recorded using a Bruker SRC 200 D spectrometer equipped with a Bruker variable temperature unit and interfaced with an ASPECT 2000 computer operating at X-band frequencies (≈9.7 GHz). Hundred kHz modulation was used with a Bruker TE_{102} rectangular cavity. For the calibration of the g-factors, a dried DPPH sample characterised by ($g_{DPPH} = 2.0036$) was used. The experimental conditions were as follows: modulation frequency, 100 kHz; modulation amplitude, 1 Gauss; microwave power, 5 mW; gain, 6.3×10^4. Computer simulations of the EPR spectra were performed using the Bruker program SimFonia [16].

2.3. NIR-IR spectroscopy

Optical absorption spectra of the carotenoids were measured with a double-beam Perkin–Elmer near IR–IR spectrophotometer.

2.4. Theoretical calculations

All calculations were carried out using the GAUSSIAN 03 suite of programs [17]. Geometries for β-carotene, β-carotene·+, TBHP− and the TBHP· were optimised using the AM1 Hamiltonian and then subsequently with density functional theory (DFT) using the B3LYP functional coupled with the 6-31G(d) basis set. Inclusion of solvent effect was calculated by PCM (Polarizable Continuum Model) method using the B3LYP/6-31+G functional. EPR g-factors were calculated by a semi-empirical PM3 (Parameterized Model number 3) method. Bond order calculations were performed using the HYPERCHEM [18] or MOPAC [19] packages of programs.

2.5. Generation of TBHP·

During all the experimental steps, TBHP, CH_2Cl_2 as well as their reaction mixtures were kept in ice and de-oxygenated. 0.28 ml of TBHP was added to 5 ml of de-oxygenated dichloromethane using a microsyringe. 0.35 ml of this mixture was then injected into a cylindrical EPR tube (4 mm i.d.), which was equipped with a three way stopcock [20]. To generate TBHP·, a small amount of PbO_2 was added with a spatula and the mixture immediately flushed for 3 s with a stream of nitrogen using a thin glass capillary. At all times the solution was kept in ice. The EPR tube containing the reaction mixture was immediately placed in the EPR cavity.

The β-carotene solution (10.7 mg/2 ml dichloromethane) was prepared immediately prior to the experiment. Dichloromethane was bubbled with nitrogen for 5 min and the carotenoid solution was kept in ice. 0.3 ml of β-carotene solution was added with a microsyringe into 0.35 ml of the peroxyl-radical system (made up as described above). This mixture, still kept in ice, was flushed with a stream of nitrogen for 3 s and was immediately subject to the EPR measurements.

3. Results and discussion

3.1. EPR spectroscopy and thermodynamic data

The EPR spectrum of the TBHP·, generated according to the procedure described above, exhibits a well-defined singlet with $g = 2.012$ with peak-to-peak line width of 14 Gauss (Fig. 1). The low temperature (77 K) EPR spectrum of the TBHP·, has a **g** tensor of rhombic symmetry which can be simulated using $g_{1,2,3} = 2.002$, 2.008, and 2.034, respectively, which in turn gives a calculated value for g_{iso} (2.014) for the glass spectrum in good agreement with that obtained for the room temperature spectrum (2.012). The time decay curve of the normalised EPR signal intensity for TBHP· is presented in Fig. 2. From the time dependence of the EPR signal intensity of TBHP· it follows that, at 291 K, the radical species decayed via a first order process with a rate constant of 0.0025 s^{-1} and with a half-life of $t_{1/2} = 277$ s (see Fig. 2). However, at 315 K, the EPR signal of TBHP peroxyl radical disappears within one minute, whilst at 77 K the signal stays unchanged for several hours (spectra not shown).

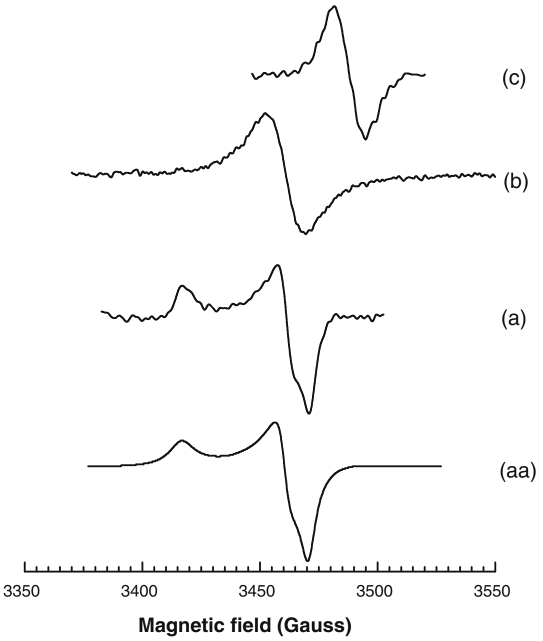

Fig. 1. The EPR spectrum of TBHP· recorded at 291 K (b) and at 77 K (a) and its computer simulation (aa). The EPR spectrum (c) is the product of the reaction between TBHP· and β-carotene and has been assigned to be β-carotene·+.

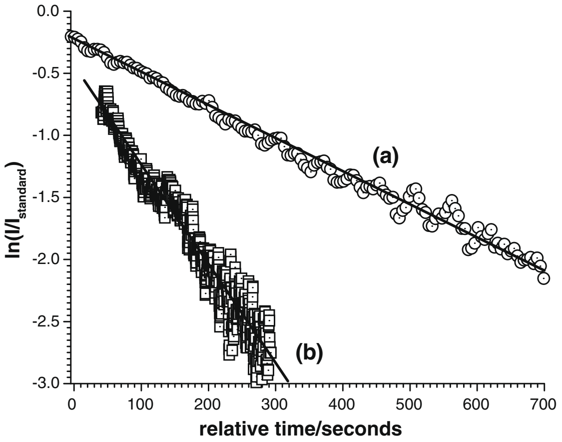

Fig. 2. The decay of EPR signal intensity for the TBHP· (a) and β-carotene·+ (b) radicals.

Despite the above mentioned obstacles associated with the reactivity between TBHP· and β-carotene, we succeeded in observing the product of this reaction, which was assigned to the cation radical of β-carotene. The EPR spectrum of this radical generated *in situ* is presented in Fig. 1c. The spectrum exhibits a symmetrical singlet line with a g-factor 1.998 and peak-to-peak half width of 14 Gauss. The g-value and peak-to-peak half width are characteristic of hydrocarbon cation radicals, which indicate that the signal corresponds to the carotenoid radical cation [21]. This further supports the results of second-moment calculations of the β-carotene cation radical in solution assuming a Gaussian line shape function and spin densities taken from MO INDO calculations which predict a ΔH_{pp} = 15 Gauss. This observation is in good agreement with the electrochemically generated β-carotene cation radical that showed a g-value of 2.0026 and ΔH_{pp} = 14 Gauss [22]. The decay curve of the radical signal is presented in Fig. 2. At 291 K the radical decays via a first order process with a rate constant of 0.0080 s^{-1}, the estimated half-life is $t_{1/2}$ = 90 s.

It has been established that peroxyl radicals are potentially more dangerous than many other types of radicals as they are able to diffuse in biological systems and act as chain carriers by abstracting hydrogen atoms from other organic molecules or by oxidising them [23–26]. Thus, when discussing the reactivity of peroxyl radicals with biological molecules, the important thermochemical parameters for any reaction system containing peroxyl radicals are: (i) ROO–H bond dissociation energies ($ROOH \rightarrow ROO^{\cdot} + H^{\cdot}$) and (ii) the one-electron reduction potential (E^{o}) ($ROO^{\cdot} + e^{-} \rightarrow ROO^{-}$) of the corresponding hydroperoxides. The bond dissociation energy of the weakest hydrogen atom in β-carotene has been estimated to be ~74 kcal/mol [27]. The bond dissociation energy of hydroperoxides is ca. 88–96 kcal/mol. Thus the hydrogen atom transfer from β-carotene to peroxyl radicals is thermodynamically feasible. The reaction mechanism of the system under study (reaction (4)), however, represents a less common type of electron transfer mechanism. The electron transfer between carotenoid molecules and peroxyl radicals in CH_2Cl_2 is dependent on many factors, among which the polarity of solvent, carotenoid structure, one-electron reduction potential of peroxyl radicals and pKa values of the corresponding hydroperoxides are the most important [28]. The reduction potential of TBHP· is approximately 0.43 V, which is a rather low value for the smooth reactivity between TBHP· and β-carotene (the highly reactive peroxyl radicals involving arylperoxyl, acylperoxyl and trichloromethyl radical, all possess reduction potentials >1.1 V).

3.2. Optical spectroscopy

Further evidence supporting the β-carotene cation radical formation comes from optical spectroscopy. Carotenoids usually exhibit an intense absorption in the 380–550 nm range, responsible for their characteristic 'orange' colour. This is a strongly allowed transition from the ground state S_0 ($1^1A_g^-$) to the second excited singlet state S_2 ($1^1B_u^+$) [29,30].

The formation of carotenoid cation radicals can be explained from a molecular orbital (MO) point of view. If one electron is either chemically, electrochemically or photochemically removed from the highest occupied molecular orbital (HOMO) of a neutral carotenoid in its ground state, the second electron stays in a molecular orbital of either g or u symmetry depending on the number of conjugated π-electron double bonds of the carotenoid. Following light absorption, the molecules may undergo a g → u or u → g transition forming an excited state radical cation; the ground state and the two lowest excited states are designated D_0 and D_1 and D_2, respectively [31]. The $D_0 \rightarrow D_2$ absorptions in carotenoids appear in the near-infrared region and similar to $S_0 \rightarrow S_2$ absorptions, exhibit red-shift with increasing number of CC double bonds [29]. It has been well established that λ_{max} values of $D_0 \rightarrow D_2$ transitions occur in the spectral region 800–1500 nm [31]. The energy of this absorption depends not only on the number of conjugated double bonds but also on the extent of delocalisation of the electron charge. Our EPR findings thus can be supported by optical spectra. The absorption spectrum for the product of the reaction β-carotene + ROO· revealed a broad band with λ_{max} at 933 nm (spectrum not shown). This value is in accordance with the values published for the cation radical of β-carotene [31,32].

3.3. Theoretical calculations

DFT calculations have been employed to calculate the bond order of β-carotene in both singlet and triplet states as well as in radical cation form (see Fig. 3). The following conclusions can be drawn from the calculations: (i) the calculated bond orders exhibit change upon formation of the radical cation. A lengthening of the double bond upon loss of an electron to form the radical cation

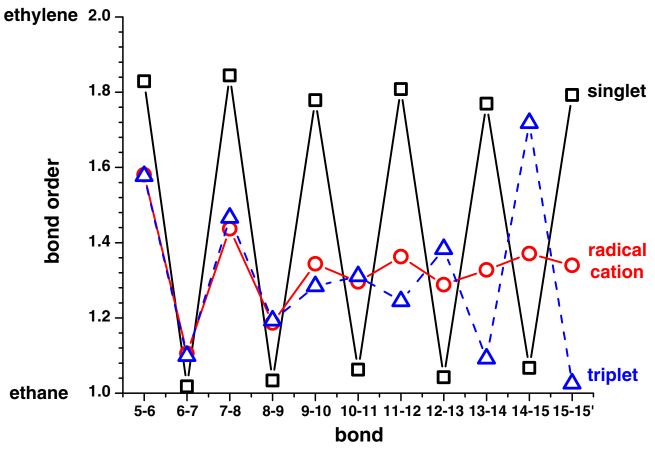

Fig. 3. DFT calculated bond order for β-carotene in singlet state, triplet state and for the radical cation form.

has been noted. The calculations further indicate (ii) cis-isomers possible from rotation around single bonds in β-carotene would not produce a V-shaped molecule; (iii) in both the triplet state and the cation radical form there is an αβ shift in the single/double bonds; (iv) the triplet state produces predominantly cis-isomers in the centre of the chain 15–15′, 13–14 in the above notation.

Geometry optimisation using the PM3 Hamiltonian shows that β-carotene is a π conjugated all-trans planar structure with alternating partially (formally) double and single bonds. At the end of the β-carotene π conjugated system the bond length alternation is greater (1.344 Å vs. the neighbour bond 1.45 Å), whilst in the middle of the molecule the bonds have almost the same length (1.396 Å and 1.39 Å). In the case of β-carotene$^{\cdot+}$ the geometry is similar, however, removal of one π electron from the HOMO orbital causes an increase of the length of partially double bonds while the partially single bonds are shortened. This fact is due to the well known behaviour of the π-HOMO orbital in alternated polyens: the HOMO has the bonding character on partially double bonds and is antibonding on partially single bonds. Finally, it can be demonstrated that calculated PM3 (and also INDO and AM1) values of spin densities and the respective hyperfine splitting constants on hydrogen atoms are relatively small, ca. 0.2 Gauss or less for both radicals, thus hyperfine splitting is not observed in the measured EPR spectra (Fig. 1).

In addition, we also performed PM3 calculation of the g-(iso) factor for both β-carotene$^{\cdot+}$ and the TBHP$^{\cdot}$ neutral radical. The g-values are 2.0275 and 2.0090, respectively. The theoretically obtained β-carotene$^{\cdot+}$ g-factor is larger by 0.0295 in comparison with that obtained experimentally from EPR (1.998). Both theoretical g-values are in reasonable agreement with the experimental data discussed above.

The calculated gas phase energies of the individual constituents of reaction (4) are summarised in Table 1. The reaction energy is 101.6 kcal/mol and this value can also be regarded as the reaction Gibbs energy, since the reaction entropy is negligible. The calculated values imply that the reaction (4) in gas phase is strongly endothermic and thus thermodynamically it is not feasible for it to proceed.

In order to explain the formation of β-carotene$^{\cdot+}$ according to reaction (4), we performed a detailed theoretical study using the DFT method for the calculation of the energy of individual constituents of the reaction. The geometries of the corresponding molecules have been optimised using the semi-empirical PM3 method which is much less demanding of computer time in comparison with more advanced B3LYP method. The cost of the latter is due to very close geometric and electronic similarity between reactants and products. The most probable reason for the reaction proceeding and thus for the formation of β-carotene$^{\cdot+}$ lies in the solvent effect of the polar dichloromethane which should strongly stabilise the ionic products of the studied reaction. The solvation Gibbs energy obtained (Table 1), using the widely applicable theoretical Polarizable Continuum Model (PCM) [33] indicates that the reaction thermodynamics is qualitatively shifted in favour of the formation of products, since the ionic products, especially the anion TBHP^{-} where the negative charge is strongly localised on the peroxo group, strongly polarise the solvent (see the values in Table 1). Though the total reaction Gibbs energy is still positive, +19.6 kcal/mol, we believe that its greatly lowered positive value in the polar solvent allows the formation of the observed β-carotene$^{\cdot+}$ to be both thermodynamic and kinetic feasible.

The effect of solvation on the pre-exponential factor of equilibrium constant of the reaction (4) is difficult to predict accurately, but it seems reasonable to expect that the translational and rotational components of the partition functions for the ions would decrease rather more than those for the neutral molecules. This would lead to an overall lowering of the pre-exponential factor. In a polar solvent such as dichloromethane, such a change would, however, be negligible compared to the change in the exponential factor where, to a first approximation, the exponent would be reduced by the relative permittivity, ε_r, of the solvent. Thus the position of the equilibrium will be controlled by the exponential term $(\exp(-\Delta E/RT))$. Typical values of the relative permittivity $\varepsilon_{r,}$ for some common solvents are: benzene $\sim$ 1.2, dichloromethane $\sim$ 8.9 and water $\sim$ 78.4. Thus the shift in the equilibrium, relative to the gas phase reaction, in favour of the radical cation of β-carotene would be $\sim$3 for benzene, $\sim 1 \times 10^4$ for dichloromethane and $\sim 1 \times 10^{34}$ for aqueous systems. This may explain why the radical cation is observed in dichloromethane but not solvents, e.g. benzene, with lower dielectric constants. Furthermore, this becomes increasingly important in miscible organic/aqueous systems where the limiting value can approach 10^{34} (pure water). Experimental evidence to support this hypothesis is difficult to find, however, there are several reports that the dielectric constant of the solvent can affect the physical/photolytic properties and dynamics of carotenoids. Frank et al. [31] have shown that there is considerable interaction between some carotenoids and solvent molecules via an internal charge transfer state and that halving the dielectric constant related function, $(\varepsilon_r - 1)/(\varepsilon_r + 2)$ (the proportionality factor for the polarisability of the solvent), reduces the lifetimes of excited states by a factor of 10. Similarly, Fournier et al. [34] have discussed the switch from energy transfer to electron transfer, to form the radical cation, when going from non-polar to polar solvents which they partly attribute to differences in reorganisation energy of the solvent.

Our findings are also supported by the conclusions drawn from the experimental work of Das et al. [35], who concluded, that as

Table 1
B3LYP/6-31G*//PM3 gas phase energies of individual constituents of the reaction (4) and their B3LYP/6-31+G* solvation Gibbs energies calculated by PCM model for the CH_2Cl_2 solvent.

	β-Carotene	TBHP$^{\cdot}$	β-Carotene$^{\cdot+}$	TBHP^{-}	ΔE resp. ΔG_{solv} of reaction (4)
E in gas phase [a.u.]	−1557.91	−308.18	−1557.71	−308.22	101.6
Solvation Gibbs energy [kcal/mol]	−7.39	−3.31	−29.92	−63.74	19.7

the polarity of the solvent decreases, alkylperoxyl radicals may be more likely to react by a hydrogen abstraction process, whereas the stronger oxidants may continue to react via electron transfer even at low polarity, if there is a significant difference between the reduction potentials of the peroxyl radical and the substrate.

4. Conclusions

In this work, we have studied the reaction between TBHP$^\bullet$ and β-carotene in dichloromethane under low oxygen pressure. Despite the rather low value of the reduction potential of TBHP$^\bullet$ we have succeeded in the formation of the cation radical of β-carotene (β-carotene$^{+\bullet}$). Based on the EPR and NIR-IR spectroscopy, the reaction mechanism for this reaction has been assigned to an electron transfer mechanism. This relatively rare mechanism for the reaction between weakly oxidising species such as radical TBHP$^\bullet$ and carotenoids has been explained on the basis of DFT theoretical calculations. It has been concluded that there is considerable interaction between molecules of β-carotene and solvent molecules (CH_2Cl_2) via an internal charge transfer state and that halving the dielectric constant related function reduces the lifetimes of excited states by a factor of 10.

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