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SPECTROPHOTOMETRIC STUDY ON THE FORMATION OF RADICAL CATION AND DICATION OF THE CAROTENOID ASTAXANTHIN AT ROOM TEMPERATURE

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Radical cations and dications of the carotenoid astaxanthin were prepared at room temperature by chemical oxidation with $FeCl_3$ in fluorinated alcohols 1,1,1-trifluoroethanol and 1,1,1,3,3,3-hexafluoroisopropanol at room temperature. Absorption spectra recorded in the 300-1700 nm range were found to be analogous to those determined recently by other authors for canthaxanthin in CH_2Cl_2 at -70° C. Especially designed experiments have shown the reversibility of dication formation from cation radical. The temporal stability of both cationic species at room temperature indicates a possibility to obtain long-lived radical cations and dications of astaxanthin for spectroscopic studies at low temperature.

INTRODUCTION

The interest in electronic states of carotenoids stems mainly from their significant role in photosynthesis, where they serve as light-harvesting pigments and photoprotectors (Siefermann-Harms, 1985; Koyama, Kuki, Andersson & Gillbro, 1996). Normally, carotenoids are present in the photosynthetic apparatus in the form of neutral molecules. However, several years ago, the formation and accumulation of β-carotene radical cations in Photosystem II reaction centers was detected (Mathis and Rutherford, 1984; Telfer, de Las Rivas & Barber, 1991). The accumulation of β-carotene radical cations is the result of electron withdrawal from β-carotene by photoaccumulated primary electron donor radical $P680^{\bullet+}$ in PS-II reaction centers. Also the proces of nonradiative energy transfer from carotenoid to chlorophyll is thought to proceed with a substantial participation of electron exchange mechanism proposed long time ago (Dexter, 1953), which detailed description requires the knowledge of electronic states in carotenoid ions (Scholes, Harcourt & Fleming, 1997). Both these aspects of carotenoid function in photosynthesis point to the need for information on the properties of carotenoids in their ionized states.

Astaxanthin, the carotenoid investigated in this work, appears in the carapace of some invertebrate marine animals as a pigment in blue carotenoproteins called α-, β- and γ-crustacyanins (Salares, Young, Bernstein & Carey, 1979), where it is non-covalently bound. The absorption maximum of the carotenoid, normally at about 480 nm in organic solvents, appears at 630 nm in these carotenoproteins, which means a 5000 cm^{-1} spectral shift toward the red. Ionic forms of astaxanthin were recently postulated to account for this large bathochromic shift of absorption (Weesie, Verel, Jansen, Britton, Lugtenburg & de Groot, 1997a; Weesie, Jansen, Merlin, Lugtenburg, Britton & de Groot, 1997b).

A non-biophysical reason for intense spectroscopic studies on carotenoids is the search for strongly nonlinear optical materials which, in addition to the prospect of practical applications, leads also to deeper understanding of the electronic structure of conjugated systems. Here, the modification of bond order alternation is the factor of primary importance and this can be achieved by polarizing the polyene chain by unsymmetrical substituents at the end groups (Bublitz, Ortiz, Marder & Boxer, 1997). Another area pertinent to carotenoid ions comprises the studies on the mechanism of electrical conductivity in conjugated polymers, especially in polyacetylene (Roth & Bleier, 1987; Heeger, Kivelson, Schrieffer & Su, 1988). Here, the species leading to electronic conduction are the radical cation and radical anion states of $(CH)_x$ chains, called polarons.

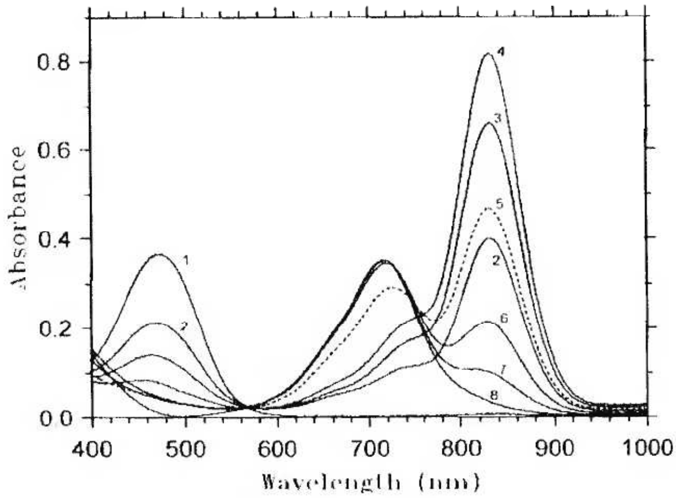

Fig. 1. Absorption spectra of astaxanthin at subsequent stages of oxidative titration with $FeCl_3$ in trifluoroethanol (FET) at room temperature. Spectrum (1) is for 3 μM astaxanthin, supplied with the following concentrations of $FeCl_3$: (2) 1 μM, (3) 2 μM, (4) 3 μM, (5) 6 μM, (6) 11 μM, (7) 25 μM, and (8) 40 μM.

The electronic states of carotenoid ions appear in absorption spectra in the VIS-IR range. However, the low chemical stability of radical cations allows only for recording the transient spectra of radiolytically generated species. As yet, mainly the absorption spectra, sometimes missing the lowest-energy transition, have been available for a long time. Recently, the preparation of relatively stable carotenoid cation radicals and dications by chemical oxidation or electrolysis in CH_2Cl_2 at –70°C was reported (Jeevarajan, Wei, Jeevarajan & Kispert, 1996; Gao, Wei, Jeevarajan & Kispert, 1996).

It is one of the purposes of this paper to examine the possibility of producing stable cation radicals and dications of some long-chain carotenoids in solvents that could serve as glassy matrices for low-temperature studies. Fluorinated alcohols were tried as solvents after checking their ability to form good quality glassy matrix on freezing. This search direction follows the recent report on exceptional properties of hexafluoroisopropanol (HFP) and trifluoroethanol (FET) in stabilizing the radical cations (Eberson, Hartshorn, Persson & Radner, 1996).

MATERIALS AND METHODS

Puriss. grade 2,2,2-trifluoroethanol (FET) and anhydrous $FeCl_3$ were obtained from Fluka and 1,1,1,3,3,3-hexafluoroisopropanol (HFP) from ABCR. Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) was a gift from Prof. G. Britton of Liverpool University.

Absorption spectra were recorded at room temperature in the range 300-1100 nm with Shimadzu UV-160A spectrophotometer for $\approx 10^{-5}$ M deoxygenated solutions in 1 cm, air-tight quartz cuvettes. Measurements farther to the infrared were performed with a home-built spectrometer with InGaAs photodiode as light detector, which allowed for recording the spectra between 700 nm and 1700 nm.

RESULTS AND DISCUSSION

Fig. 1 shows a set of absorption spectra recorded at subsequent stages of titration of astaxanthin with $FeCl_3$ in FET. As can be seen, the decrease of neutral astaxanthin absorption with maximum at 480 nm is accompanied by the growing peak at 831 nm, which, according to literature data (Jeevarajan *et al.*, 1996) should be ascribed to carotenoid cation radical $CAR^{\bullet+}$. In spectra of solutions with CAR completely converted to $CAR^{\bullet+}$, a weak absorption band can be detected at 1280 nm, which is shown in Fig. 2. Thus, in accordance with the known spectra of radiolytically (Bally, Roth, Tang, Schrock, Knoll & Park, 1992) or electrochemically (Jeevarajan *et al.*, 1996) generated carotenoid cation radicals, these two absorption bands shall be attributed to electronic transitions between the ground state (D_0) and the two low-energy excited states of cation radical: $D_0 \rightarrow D_1$ at 1280 nm and $D_0 \rightarrow D_2$ at 831 nm. There is a close similarity in the band shapes and absolute intensities in FET to those in CH_2Cl_2. The position of the weak band at 1280 nm in FET is shifted by 30 nm (which means 180 cm^{-1} in this spectral region) relative to the one at 1310 nm in CH_2Cl_2 (Jeevarajan *et al.*, 1996). The more remarkable difference is the significant shift by 56 nm (760 cm^{-1}) of the strong band ($D_0 \rightarrow D_2$ transition) from 887 nm in CH_2Cl_2 to 831 nm in FET. Since the values of these shifts are correlated with the intensities of both absorption bands, these shifts seem to result from the solvatochromic effects related to the significant difference in refractive indices of both solvents, which are 1.424 for CH_2Cl_2 and 1.291 for FET.

Further increase of $FeCl_3$ concentration, now exceeding the one of carotenoid several times, converts the cation radical to another species with a weaker absorption band at 715 nm (Fig. 1). The formation of the latter band proceeds through quantitative transformation of $CAR^{\bullet+}$, as indicated by the isosbestic point at about 750 nm (Fig. 1). This next oxidation product should be in principle the astaxanthin dication, CAR^{2+}; indeed, the maximum absorption wavelength of this product, 715 nm, is very close to 714 nm reported for canthaxanthin dication in CH_2Cl_2 (Jeevarajan *et al.*, 1996). The results reported in the following support this assignment. As to the lack of the expected hypsochromic absorption band shift in FET relative to CH_2Cl_2, it can be explained by the compensating effect of a bathochromic shift on hydrogen bonding of astaxanthin dication at the two conjugated keto oxygen atoms. Indeed, both solvents FET and HFP are very strong hydrogen bond donors (Eberson *et al.*, 1996). Also the broadening of vibronic bands and the lack of structure in dication spectrum, typical for hydrogen bonded solutes, seems to support this conclusion.

As Fig. 1 shows, it is possible to perform gradual oxidation of astaxanthin in FET through its cation radical into the dication with the final steps corresponding to complete transformation of the carotenoid into its dicationic form. This enables the determination of molar extinction coefficients which, based on the value of $1.25 \cdot 10^5$ M.$^{-1}$ cm^{-1} for neutral astaxanthin, are $2.8 \cdot 10^5$ M.^{1} cm^{-1} for cation radical at 831 nm, and $1.15 \cdot 10^5$ M^{-1} cm^{-1} for the dication at 715 nm in FET. The titration curves are presented in Fig. 3, from which it is clear that the first oxidation step proceeds with much higher equilibrium constant than the next oxidation of cation radical to dication. However, due to the strong overlap of absorption spectra of mono- and dication and to the relatively small equilibrium constant for the second oxidation step, the quantitative description of these dependencies in terms of equilibrium stoichiometry gave rather weak results with significant deviations of fitting curves from experimental points at either the low or the high $FeCl_3$ to CAR ratios (cf. Fig. 3). It seems that the activity of $FeCl_3$ is too low at its low concentrations, and this could result from the presence of small amount of water or other alcohols which became relevant at micromolar concentrations of reagents.

Our experiments showed also that the dicationic forms of astaxanthin in FET are formed reversibly and can be partly converted back to cation radical on addition of excess carotenoid, according to the reaction (Jeevarajan *et al.*, 1996):

$$CAR^{2+} + CAR \rightarrow 2CAR^{\bullet+}$$

However, due to relatively smaller equilibrium constant for the formation of dications, quite large excess of $FeCl_3$ is first needed to generate a substantial amount of dication (cf. also the $FeCl_3$/CAR ratio in Fig. 3). In these circumstances, the addition of free carotenoid needed to completely revert the dication into cation radicals would generate immeasurably intense absorption spectra. Thus, only

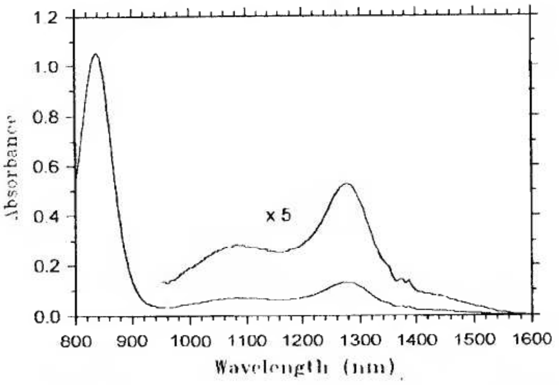

Fig. 2. Absorption spectrum of astaxanthin cation radical in FET showing the two transitions in the near infrared: $D_0 \rightarrow D_1$ at 1280 nm and $D_0 \rightarrow D_2$ at 831 nm.

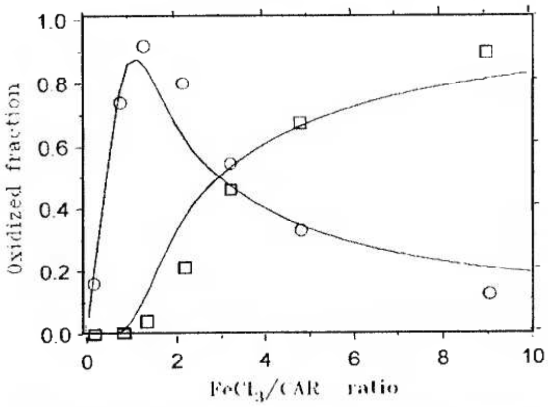

Fig. 3. Titration curves for two-step oxidation of astaxanthin in FET with $FeCl_3$. Experimental data are presented as points: circles for cation radical and squares for dication. Lines are fits calculated with the values of equilibrium constants: 250 M.$^{-1}$ for cation radical and 1 M.$^{-1}$ for dication.

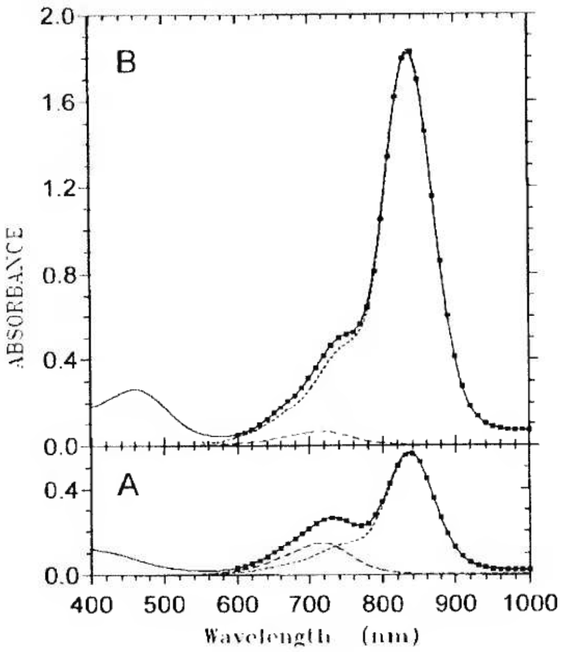

Fig. 4. Panel A: absorption spectrum (continuous line) of 4.4 μM canthaxanthin in FET converted with 13.3 μM $FeCl_3$ into a mixture of cation radical (dots) and dications (dashes), which sum is represented by squares. Panel B: absorption spectrum (continuous line) of the same solution after adding carotenoid to a nominal concentration of 11.6 μM, with contributions from cation radical (dots) and dication (dashes). Both absorption spectra were uniquely decomposed into two components by least squares procedure using the spectra of cation radical and dication determined separately.

a partial conversion of dication into cation radical could be observed, and this is illustrated for canthaxanthin in FET in Fig. 4. In part A of Fig. 4, the absorption spectrum is shown with continuous line for a partially titrated solution containing cation radicals and dications whose individual contributions to the total spectrum are shown with dotted and dashed lines, respectively. The squares represent the sum of both components. After adding the free carotenoid, an absorption spectrum is observed (continuous line in Fig. 4b), which contains a contribution from dication decreased by a factor of 2.25, indicating the reversibility of dication formation as expressed in Eqn. 1. The necessarily large addition of free carotenoid hinders the quantitative assessment of cation radical generated from dication. Nevertheless, the high quality fit of the observed spectrum with the spectra of cation radical and dication (squares in Fig. 4b) points to the conversion of the dications exclusively into cation radicals, with no indication of any other products. This further supports the spectral assignments given above.

Very similar results were also obtained for astaxanthin in neat HFP, both regarding the shapes and the positions of maxima in absorption spectra: the latter are 828 nm for cation radical and 717 nm for dication. However, the temporal stability of both species in HFP was found remarkably lower than in FET, especially for dications. While the half-lifetime of the cation radical was 30 minutes in FET, it was only slightly less (20-25 minutes) in HFP. The dication's half-lifetime was 5-5.5 minutes in FET but did not exceed 3 minutes in HFP.

CONCLUSION

The cation radicals and dications of astaxanthin can be generated in fluorinated alcohols FET and HFP at room temperature. Both oxidized forms were found to be sufficiently stable to perform many-step titration producing a required quantity of radical cations and to determine the spectra and molar extinction coefficients. Although the dication is less stable than the cation radical, its half-lifetime is sufficient to demonstrate the reversibility of its formation and the equilibrium between the cation radical and dication. Since the solvents tested in this work form optically transparent glass on freezing, the results point to the possibility of selectively generating mono- or dications of some carotenoids for long-time spectroscopic studies at low temperatures.

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