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Fluorescence properties of carminic acid in relation to aggregation, complex formation and oxygen activation in aqueous food models

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The fluorescence of carminic acid is moderately dependent on pH in acidic and neutral aqueous solution and has, at pH 2·0 in 1·0 M NaCl at 25°C, a quantum yield 0·00133 ± 0·00019 and an emission lifetime of 0·093 ± 0·010 ns, as determined by the fluorescence phase and modulation method. The fluorescence is quenched by iodide (diffusion controlled reaction), and the quantum yield decreases for increasing carminic acid concentration, suggesting a molecular stacking in fast equilibrium, as further evidenced by pressure quenching of emission. The fluorescence of carminic acid in dilute aqueous solution is increased by aluminium as a result of formation of a 1 : 1 complex with a stability constant of (6·8 ± 1·8) × 10^3 litres mol^{-1} (1·0 M NaCl with pH 4, 25°C), a fluorescence quantum yield of 0·022 ± 0·004 and an emission lifetime of 2·1 ± 0·1 ns. An excited state kinetic analysis based on the photophysical characterization shows an electronic deactivation path in acidic solution for carminic acid which does not involve oxygen activation. The fluorescence of carminic acid, declines rapidly in alkaline solution with concomitant increase in photooxidation of carminic acid, and a photoreactive triplet state, capable of oxygen activation, becomes of increasing importance at higher pH.

INTRODUCTION

The major colouring substance of cochineal is carminic acid (Lloyd, 1980), a β-*C*-glycosyl derivative of anthraquinone (Fig.1), which is weakly fluorescent in aqueous solution (Sahlin *et al.*, 1983). Although carminic acid and carminic acid 'lakes' are finding increasing technological use as natural food pigments (Madsen *et al.*, 1993), only a few studies of the solution chemistry of carminic acid have appeared (Schwing-Weill, 1986; Jørgensen & Skibsted, 1991). This is particularly true for the photochemical and photophysical properties of carminic acid and carminates (Baykut *et al.*, 1983). Notably, light absorption characteristics and photoluminescence properties of natural pigments should be considered, not only in relation to the process of optimizing colouring properties, but also in relation to a possible prooxidative effect in foods, beverages and cosmetics (Gutteridge & Quilan, 1986). As part of our research on the coupling between oxidation of different food components, we have undertaken a detailed investigation of the photophysical properties of carminic acid in aqueous solution in order to provide part of the knowledge required for a rational and safe use of carminates in foods. The present investigation of carminic acid includes an exploration of chemical equilibria in very dilute solutions, which are of relevance as models for pigmented foods, and, for the study of which, fluorescence spectroscopy is expected to be superior to other analytical methods including absorption spectroscopy.

MATERIALS AND METHODS

Materials

Dihydrorhodamine 6G and rhodamine 6G (chloride salt) were from Molecular Probes (Eugene, OR), Glycogen Type II from oyster was from Sigma (St. Louis, MO), and other chemicals were of analytical grade. Carminic acid p.a. (Merck, Darmstadt, Germany) was

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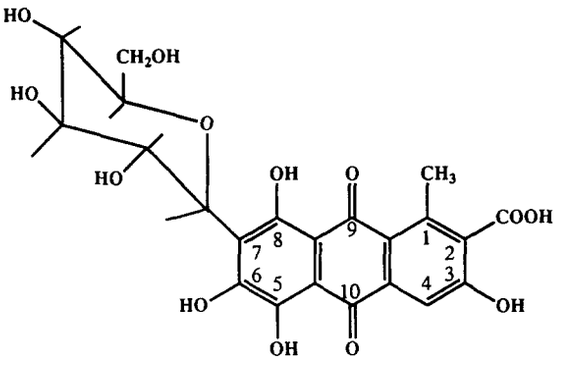

Fig. 1. Carminic acid.

purified by ion–exchange chromatography prior to use: 20 ml of an aqueous solution (35 mg of carminic acid in 100 ml of 0·01 M HCl 0·99 M NaCl) were applied to a column (20 cm long, i.d. 2.5 cm) of DEAE Sephadex A-25 (Pharmacia, Uppsala, Sweden) equilibrated with aqueous 0·01 M HCl, 0·99 M NaCl, which was also used as the eluent. The first and last part of effluent carminic acid solution was discharged. The mid-part (*c.* 50% of the total carminic acid) was collected and used for measurements after spectrophotometric standardization at 494 nm (Schwing-Weill, 1986) and appropriate dilution with aqueous 0.01 M HCl 0·99 M NaCl. This purification procedure removed impurities, which prior to purification could be detected by thin-layer chromatography (TLC) (silica gel, methyl ethyl-ketone/ethylacetate/formic acid/water (5 : 3 : 2 : 1) as eluent) and by high voltage electrophoresis (HVE) (pH 1·9 : water/acetic acid/formic acid (45 : 4 : 1); pH 3·6 : water/acetic acid/pyridine (470 : 25 : 3); pH 6.5 : water/pyridine/acetic acid (470 : 25 : 1)). The impurities consisted of polymeric substances (detectable both by TLC and HVE, and visible on the Sephadex column) and by trace amounts of non-coloured, fluorescent (λ_{ex} = 250 nm) compounds (up to four different impurities could be detected by HVE at pH 1·9 prior to purification).

Spectrophotometric measurements

These were performed on an HP 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). A DX.17MV stopped-flow spectrofluorimeter from Applied Photophysics (London, UK) in absorbance mode was used to monitor fast reactions.

pH

This was measured with a combination glass electrode (Ingold type 405, Steinbach, Germany, in conjunction with a Metrohm 605 pH meter, Herisan, Switzerland). The electrode was standardized against titrated solutions of hydrochloric acid in 1.00 M NaCl (the same medium previously used for the determination of the pK_a values of carminic acid (Jørgensen & Skibsted, 1991)), employing the definition pH = $-\log [H^+]$ throughout.

Fluorescence measurements

These were carried out with an SLM48000S multi-frequency, phase-modulation spectrofluorometer (SLM Instruments Urbana, IL) with a 450-W Xe arc lamp, SLM MC200 excitation and MC320 emission monochromator mounted with a Hamamatsu R928P photomultiplier tube (Hamamatsu, Shimokanzo, Japan) at right angles to the line of illumination. Emission spectra reported (bandpasses of 4 nm on the excitation monochromator, and 2 nm on the emission monochromator) are corrected for instrument response. Excitation spectra reported (bandpasses of 4 nm on the excitation monochromator, and 2 nm on the emission monochromator) are corrected for lamp spectral characteristics by the method of ratio recording (Parker, 1968) using a solution of rhodamine 101 (Exciton, Dayton, OH; 3 g litre^{-1} ethylene glycol) as quantum counter. Measurements were made in a thermostatted cell holder with centrally illuminated 10 mm × 10 mm quartz cells with right angle observation at the centre. Air-saturated solutions were used for measurements, except where otherwise noted. An SLM HPSC-3K high pressure spectroscopy cell (closed 0.75 ml quartz bottle, Teflon lid for pressure transmission) was used for the high pressure measurements. An SLM low temperature cell was used for the low temperature measurements (77 K in liquid nitrogen). The optically dilute concept of Demas & Crosby (1971) was used for quantum yield determination relative to rhodamine B (Φ_{fl}= 0·66 in ethanol; Parker & Rees, 1960) and quinine sulphate (Φ_{fl} = 0·546 in 0·5 M aqueous H_2SO_4; Melhuish, 1961).

Emission lifetimes

The fluorescence lifetime was determined using the multifrequency phase and modulation technique. In the SLM 48000S, the intensity of the exciting light is sinusoidally modulated by means of a Pockel cell, and the phase shift and relative modulation with respect to the emitted light are determined. Phase and modulation lifetimes (τ_p and τ_m) are then calculated according to (Lakowicz 1983):

$$\tau_p = \frac{\tan \theta}{\omega}$$
$$\tau_m = \frac{\sqrt{(1/m^2) - 1}}{\omega} \quad (1)$$

where Θ is the phase shift in degrees, m is the relative modulation and ω is the angular modulation frequency ($\omega = 2\pi f$). For the lifetime measurements, the exciting light was polarized parallel to the vertical laboratory axis, while the emission was viewed through a polarizer oriented at 54·7° ('magic angle' conditions). Emissions from carminic acid samples were collected through a Schott KV 550 cut-on filter (Mainz, Germany). At each frequency, the phase difference and demodulation was taken as the average of 10 measurements.

Detection of superoxide

The nonfluorescent dihydrorhodamine 6G is reported to react with superoxide anion (Haugland, 1989), yielding the highly fluorescent rhodamine 6G ($\lambda_{em,max}$ = 553 nm). Dihydrorhodamine 6G was dissolved in dimethyl sulphoxide, and subsequently mixed with solutions of carminic acid in 25 mM phosphate buffer (pH 7·2) to yield mixtures 1·6% (v/v) in dimethyl sulphoxide with final concentrations of 0·8 to 32 μM carminic acid and 9 μM dihydrorhodamine 6G. Solutions were illuminated in quartz cells with a 20 mm light path using 436 nm monochromatic light selected by an interference filter from the line spectrum of an Osram HBO 100/2 Hg lamp at 25 ± 0·5°C. The yield of superoxide was determined by measurement of the fluorescence of exposed solutions in comparison with that of standard solutions of rhodamine 6G and expressed as quantum yield (moles of superoxide anion formed relative to moles of photons absorbed by carminic acid). Light intensities (*c.* 4·5 × 10^{-6} einstein s^{-1} $litre^{-1}$) was quantified by ferrioxalate actinometry (Hatchard & Parker, 1956).

Non-linear regression

This was carried out using the multivariate secant iterative method in the PROC NONLIN of the SAS ver. 6.03 software (SAS Institute, Cary, NC).

RESULTS

Fluorescence of carminic acid

The emission of carminic acid in aqueous solution was found to be weak and to have a moderate Stokes shift of 3700 cm^{-1}. The emission is broad and structureless and has little spectral overlap with the absorption (Fig. 2). The excitation spectrum for carminic acid agreed with the absorption spectrum and the emission quantum yield for carminic acid (determined at a concentration of 1·3 × 10^{-5} M in aqueous air-saturated 0·01 M HCl 0·99 M NaCl) was found to be independent of the excitation wavelength in the region 330 ≤ λ_{ex} ≤ 500 nm. The emission quantum yield was determined relative to quinine sulphate (330 ≤ λ_{ex} ≤ 370 nm) and rhodamine B (460 ≤ λ_{ex} ≤ 500 nm), and had the value Φ_{fl} = (1·33 ± 0·19) × 10^{-3} at 25°C. The emission intensity was sensitive to the nature of the solvent, and in aqueous 80% (v/v) glycerol, the fluorescence intensity increased by a factor of four.

The emission was not influenced by the presence of oxygen and was identified as fluorescence from the emission lifetime 0·093 ± 0·010 ns. The lifetime was determined from the phase shift of modulated light (λ_{ex} = 480 nm, 140 ≤ f ≤ 185 MHz) with a suspension of glycogen in distilled water as a lifetime reference (scatter with a lifetime of 0·00 ns). The fluorescence was adequately described as a monoexponential decay, and the lifetime measurement was controlled by the measurement of the lifetime of NADH in aqueous phosphate buffer, which was found to have the value τ = 0·39 ± 0·04 ns, in agreement with the reference value τ = 0·4 ns (Lakowicz, 1983).

The fluorescence of carminic acid was moderately dependent on pH in acidic and neutral aqueous solution, whereas the fluorescence intensity declined in alkaline solution. The fluorescence intensity at different solution pH was determined as a relative quantum yield (Fig. 3). For aqueous solution, carminic acid is a tribasic acid (cf. Fig.1), and the distribution between the four acid/base forms, $H_nL^{(3-n)}$, is determined by (n = 0, 1, 2, 3):

$$F_n = \frac{\beta_n[H^+]^{-n}}{\sum_{n=0}^{3} \beta_n[H^+]^{-n}} \qquad (2)$$

in which $\beta_0 = K_{a,1}K_{a,2}K_{a,3}$, $\beta_1 = K_{a,1}K_{a,2}$, $\beta_2 = K_{a,1}$ and β_3 =1, where $K_{a,1}$, $K_{a,2}$ and $K_{a,3}$ are the three acidity constants of carminic acid (Jørgensen & Skibsted, 1991). For a given wavelength of irradiation and for solutions with moderate light absorption, the observed fluores-

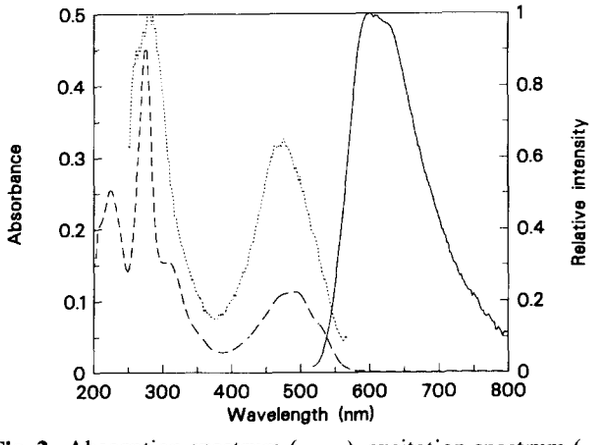

Fig. 2. Absorption spectrum (– – –), excitation spectrum (· · ·; λ_{em} = 590 nm), and emission spectrum (—; λ_{ex} = 490 nm) of dilute aqueous solution of carminic acid (*c.* 10^{-5} M in 1 cm cell) in aqueous 0·01 M HCl 0·99 m NaCl at 25°C.

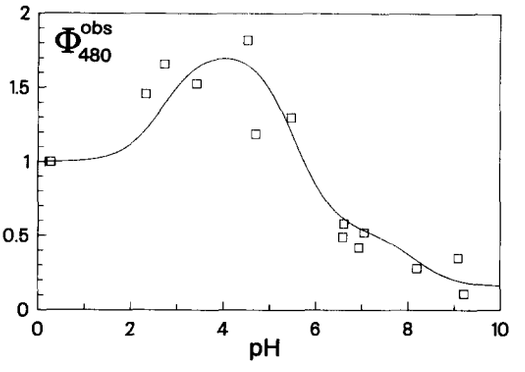

Fig. 3. pH dependence of carminic acid fluorescence in aqueous 1·00 M chloride at 25°C. The observed fluorescence quantum yield is expressed relative to the yield in strongly acidic solution. The full line is calculated according to eqn (4) by nonlinear regression analysis, using ground state pKa values, providing the relative fluorescence quantum yield of the four acid/base forms of carminic acid (Table 1).

cence quantum yield depends on the quantum yield of the four acid/base forms according to

$$\Phi_{irr}^{obs}\varepsilon_{obs}lC_{TOTAL} = \sum_{n=0}^{3} \Phi_{irr}^{H_nL^{(3-n)-}}\varepsilon_{H_nL^{(3-n)-}}C_{TOTAL}F_n l \quad (3)$$

in which C_{TOTAL} is the total concentration of carminic acid, and l is the length of the light path. Equation (3) can be rearranged to yield:

$$\Phi_{irr}^{obs} = \frac{\sum_{n=0}^{3} \Phi_{irr}^{H_nL^{(3-n)-}}\varepsilon_{H_nL^{(3-n)-}}F_n}{\sum_{n=0}^{3} \varepsilon_{H_nL^{(3-n)-}}F_n}$$

$$= \frac{\sum_{n=0}^{3} \Phi_{irr}^{H_nL^{(3-n)-}}\varepsilon_{H_nL^{(3-n)-}}\beta_n[H^+]^n}{\sum_{n=0}^{3} \varepsilon_{H_nL^{(3-n)-}}\beta_n[H^+]^n} \quad (4)$$

A value of 1·00 was assigned for the observed quantum yield in strongly acidic solution, and the resolution of the observed fluorescence quantum yields relative to this value into relative quantum yields for each acid/base form of carminic acid (Table 1) was performed by nonlinear regression analysis, using eqn (4), the independently determined pK_a values and molar absorptivities of each form (Table 1) and the restriction $\Phi^{H_3L} \leq 1{\cdot}0$. The dependence of the fluorescence intensity on pH is satisfactory described by eqn (4), using ground state pK_a values, showing that the acid/base equilibrations in the excited state of carminic acid are slow processes as compared to electronic relaxation. It is furthermore seen, that the form H_2L^-, in which the carboxyl group is deprotonated, has the highest fluorescence quantum yield, and that the diphenolate form L^{3-} shows very little, if any, fluorescence.

Fluorescence quenching

Iodide was found to quench the fluorescence of carminic acid. The decrease in fluorescence intensity of carminic acid in aqueous 0·010 M HCl 0·99 M NaCl, in which chloride was substituted with iodide ($[I^-] \leq$ 0·30 M), can be described by the Stern–Volmer equation:

$$\frac{\Phi_0}{\Phi} = 1 + K_Q[I^-] \quad (5)$$

From the result presented in Fig. 4, a quenching constant $K_Q = 1{\cdot}27 \pm 0{\cdot}09$ litre mol^{-1} (25°C) was determined.

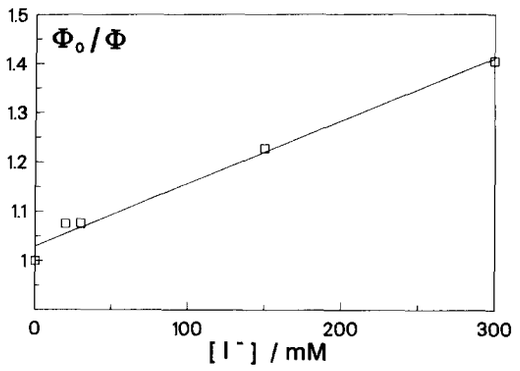

Fig. 4. Iodide quenching of carminic acid fluorescence at 25·0°C plotted according to the Stern–Volmer equation; Φ_0 is quantum yield in the 0·010 M HCl 0·99 M NaCl reference medium, and Φ is the quantum yield in the presence of iodide.

Concentration effect on fluorescence

The light absorption by carminic acid in acidic aqueous solution deviate negatively from Lambert–Beer's law (Fig.5(A)), and it has been suggested that the observed non-linearity is due to an intermolecular condensation reaction between the carboxylic group and a phenolic group (Schwing-Weill, 1986). As may be seen from Fig. 5, which shows absorbance and fluorescence data for carminic acid in aqueous 0·01 M HCl 0·99 M NaCl, deviation from linearity is more significant for fluorescence (intensity at emission maximum at 600 nm, following excitation at 490 nm) than for absorbance (absorption maximum at 494 nm). The observed fluorescence intensity (I_{irr}^{fl}), corrected for attenuation of the excitation at the centre of the optical cell (Lakowicz, 1983) according to:

$$I_{irr}^{fl} = I_{irr}^{obs}10^{A_{irr}/2} \quad (6)$$

is expected to be proportional to concentration (c), provided that self-absorption (absorption of emission by the fluorescent compound) is negligible, and provided that the following approximation is valid (I_{ex}^0 is the intensity of light used for excitation; A_{irr} and ε_{irr} are the absorbance and molar absorptivity at the wavelength of excitation, respectively; and l is the light path):

$$I_{irr}^{fl} \approx (\Phi_{irr}I_{ex}^0 2{\cdot}30\varepsilon_{irr}l)C \quad (7)$$

The corrected fluorescence intensity deviates significantly from linearity and since absorption of emission at $\lambda_{em,max}$ is negligible, other effects such as dimerization

Table 1. Relative fluorescence quantum yields and photooxidation quantum yields of carminic acid and conjugate bases[a] in aqueous 1·00 M NaCl at 25°C

	pK_a[b]	$\varepsilon_{H_nL^{(3-n)-}}^{480}$[c]	$\Phi_{480}^{H_nL^{(3-n)-}}$[d]	$\Phi_{H_nL^{(3-n)-}}^{ox}$[e]
H_3L	2·81 (carboxylic)	6 000	1·00 ± 0·12	$2{\cdot}1 \times 10^{-5}$
H_2L^-	5·43 (phenolic)	7 400	1·77 ± 0·13	$1{\cdot}9 \times 10^{-5}$
HL^{2-}	8·10 (phenolic)	5 600	0·53 ± 0·11	$6{\cdot}5 \times 10^{-5}$
L^{3-}		5 100	0·16 ± 0·14	$4{\cdot}3 \times 10^{-4}$

[a] Carminic acid is considered to be a tribasic acid in aqueous solution: H_3L.
[b] pK_a = −log(K_a/mol litre^{-1}) for $H_nL^{(3-n)-}$ $H_{(n-1)}L^{(4-n)-}$ + H^+, for n = 3, 2, 1; determined in 1·00 M NaCl (Jørgensen & Skibsted, 1991).
[c] From Schwing-Weill (1986).
[d] Fluorescence quantum yields relative to $\Phi_{480}^{H_3L}$ = 1·00 (present investigation).
[e] Photooxidation quantum yield in air-saturated aqueous solution following 436 nm excitation (Jørgensen & Skibsted, 1991).

or polymerization are of importance. For a dimerization reaction for carminic acid:

$$2CA \quad (CA)_2 \tag{8}$$

the equilibrium constant is:

$$K_D = \frac{[(CA)_2]}{[CA]^2} = \frac{\alpha}{2C_{TOTAL}(1 + \alpha^2 - 2\alpha)} \tag{9}$$

where α is the fraction of CA reacted. For the concentration interval, where the approximation of eqn (7) is valid, and neglecting self-absorption, the fluorescence intensity is:

$$I_{irr}^{fl} = I_{ex}^0 \, 2{\cdot}30 A_{irr} \left[\Phi_{irr}^{CA} \frac{\varepsilon_{CA} l C_{TOTAL}(1 - \alpha)}{A_{irr}} + \Phi_{irr}^{(CA)_2} \frac{\varepsilon_{(CA)_2} l \tfrac{1}{2} C_{TOTAL} \alpha}{A_{irr}} \right] \tag{10}$$

Solving eqn. (9) for α, and combining the result with eqn (10) yields

$$I_{irr}^{fl} = C_{TOTAL}(I_{ex}^0 \, 2{\cdot}30 l) \times \left[\Phi_{irr}^{CA} \varepsilon_{CA} + (\tfrac{1}{2}\Phi_{irr}^{(CA)_2} \varepsilon_{(CA)_2} - \Phi_{irr}^{CA} \varepsilon_{CA}) \times \left(\frac{4K_D C_{TOTAL} + 1}{4K_D} - \sqrt{\frac{8K_D C_{TOTAL} + 1}{16 K_D^2 C_{TOTAL}^2}} \right) \right] \tag{11}$$

The dependence of the corrected fluorescence intensity on the total concentration of carminic acid was adequately described by eqn (11), as may be seen from Fig. 5(B). Using nonlinear regression analysis, the dimerization constant was found to have the value 14 ± 1 at pH 2·0 and 25°C; K_D was found to have the value 14·0 ± 0.2 at pH 1·1 in 1·0 M chloride medium, showing that the aggregation is independent of pH in acidic solution. The less significant deviation from Lambert–Beer's law did not provide the basis for a similar numerical analysis. However, concentration jump experiments, in which solutions of carminic acid in 0·010 M HCl 0·99 M NaCl with A_{490} = 2·2 were mixed with equal volumes of the reaction medium, showed that the dimerization equilibrium adjusted itself very fast. An upper limit of 2 ms for the half-life of the equilibration reaction of eqn (8) was established for the actual temperature and concentration conditions, using stopped-flow spectrophotometry. An ester hydrolysis equilibrium, as suggested by Schwing-Weill (1986), is expected to have a half-life for equilibration many orders of magnitude larger than this limiting value, and is also expected to be dependent on pH in the pH region near the pK_a value. Accordingly, in order to account for the deviation from linearity observed both for absorption and fluorescence, other types of aggregation such as molecular stacking should be considered. Stacking of carminic acid in aqueous solution, in which the anthraquinone moieties are held together by hydrophobic interaction, as outlined in Fig. 6, is expected to have almost diffusion-controlled rates. However, in the concentration range used in the present investigation, stacking beyond dimers is expected to be of little importance, and the simple numerical treatment is justified.

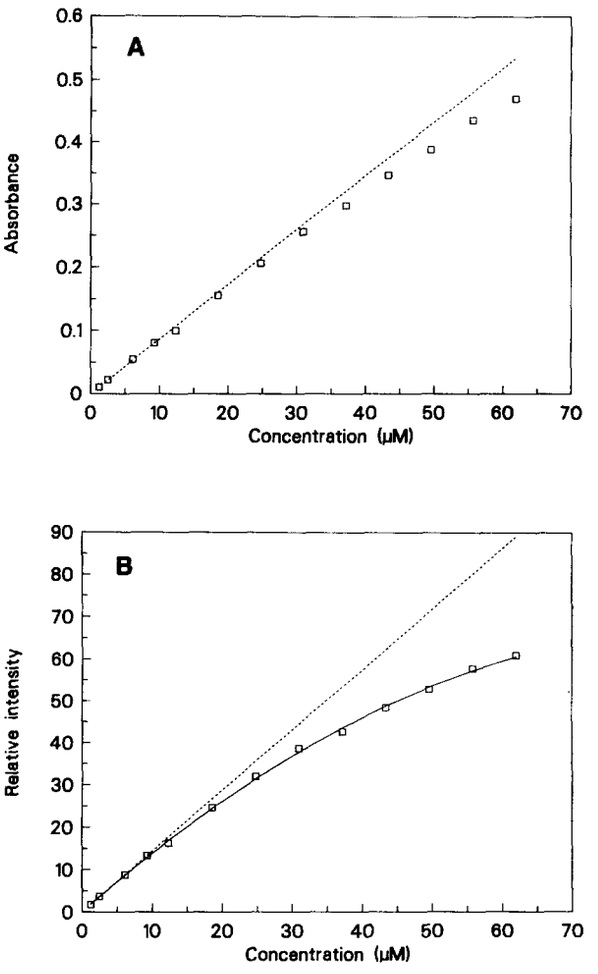

Fig. 5. Concentration effects on absorbance and fluorescence of carminic acid in aqueous 0·010 M HCl 0·99 M NaCl at 25·0°C. (A) Absorbance at absorption maximum at 494 nm. Dotted line indicates linear dependence. (B) Corrected fluorescence intensity in arbitrary units at emission maximum at 600 nm, following 490 nm excitation. Dotted line indicates linear dependence. The full line is calculated according to eqn (11) which has the mathematical form $y = ac + (b - a) \times [(4Kc + 1)/4K - ((8Kc + 1)/16\ K^2)^{1/2}]$, yielding the following values for regression parameters $a = (1{\cdot}46 + 0{\cdot}02) \times 10^6$, $b = (8{\cdot}2 \pm 0{\cdot}2) \times 10^4$, and $K_D = 14{\cdot}1 \pm 0{\cdot}2$ litre mol^{-1}. The small value of b compared to a shows that the fluorescence intensity of the dimer is weak compared to the fluorescence intensity of the monomer. A similar experiment at pH 1·0 gave the regression parameters: $a = (1{\cdot}19 \pm 0{\cdot}02) \times 10^6$; $b = (1{\cdot}8 \pm 0{\cdot}6) \times 10^5$; $K_D = 14{\cdot}0 \pm 0{\cdot}1$.

Pressure effect on fluorescence

In order to provide further experimental support for a molecular stacking of carminic acid in aqueous solution, the pressure dependence of fluorescence of carminic acid was investigated for hydrostatic pressure up to 3000 atm. The fluorescence intensity of carminic acid was found to decrease with increasing pressure, as may be seen from the experimental results presented in Fig. 7. Considering the dimerization equilibrium of

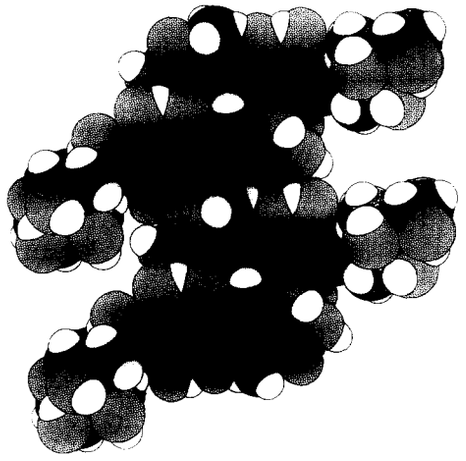

Fig. 6. Stacking of carminic acid in aqueous solution. Anthraquinone moieties are held together by hydrophobic interaction, resulting in negative deviation from Lambert–Beer's law, non-linear dependence of corrected fluorescence intensity on concentration, and pressure quenching of fluorescence.

eqn (8) as model for the molecular stacking, the observed fluorescence intensity is described by eqn (10). The observed quantum yield at elevated pressure can be expressed relative to the value at ambient pressure:

$$\frac{\Phi_p^{obs}}{\Phi_1^{obs}} = \frac{\Phi_p^{CA}\varepsilon_{CA}^{P}(1-\alpha_p) + \Phi_P^{(CA)_2}\varepsilon_{(CA)_2}^{P}\alpha_p{}^1\!/_2}{\Phi_1^{CA}\varepsilon_{CA}^{1}(1-\alpha_1) + \Phi_1^{(CA)_2}\varepsilon_{(CA)_2}^{1}\alpha_1{}^1\!/_2} \quad (12)$$

The subsequent analysis is simplified by assuming that the dimer is nonfluorescent (cf. legend to Fig. 5) and by assuming that pressure effects on absorptivities and fluorescence quantum yields are negligible relative to the pressure effect on the equilibrium constant for the dimerization. From the pressure dependence of equilibrium constants (van Eldik, 1986), applied to the dimerization reaction:

$$\ln(K_p) = -\frac{(\bar{V}(\text{dimer}) - 2\bar{V}(\text{monomer}))}{RT}P + \text{constant} \quad (13)$$

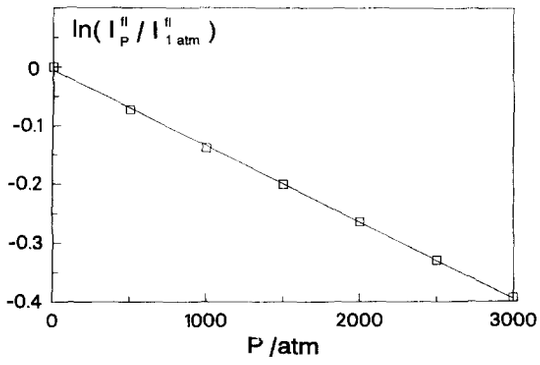

Fig. 7. Pressure dependence of observed fluorescence intensity at 590 nm of carminic acid ($1{\cdot}4 \times 10^{-5}$ M in 1·0 M HCl at 25°C) plotted according to $\ln(I_p^{fl}/I_1^{fl}) = -(\Delta\bar{V}^{app}/RT)P$, yielding an apparent reaction volume of $RT \times 1{\cdot}32 \times 10^{-4}$ atm^{-1}.

it is further seen that a decrease in the monomer fraction $(1 - \alpha_p)$ with pressure, corresponding to an increase in K_p at elevated pressure, is indicative of a contraction of the system on dimerization. The apparent reaction volume ($RT \times 1{\cdot}32 \times 10^{-4}$ atm^{-1}; Fig. 7) obtained by plotting $\ln(I_p^{fl}/I_1^{fl})$ as a function of pressure yields a limit for the reaction volume $\bar{V}(\text{dimer}) - 2\bar{V}(\text{monomer}) < -3{\cdot}2$ ml mol^{-1}. Stacking of carminic acid results accordingly in a *contraction* of the system of more than 3·2 ml mol^{-1}.

Effect of aluminium salts on fluorescence

The addition of aluminium salts to carminic acid solutions changed the absorption spectrum as an indication of complex formation between Al(III) and carminic acid. In moderate acidic solution, this complex formation was accompanied by a significant increase in fluorescence intensity. Copper(II), calcium, and zinc salts were not found to cause a similar increase in the fluorescence yield, and the specific effect of Al(III) should be considered for improvement of sensitivity of the fluorimetric determination of carminic acid. Moreover, the large increase in fluorescence yield also provides a method for identification of the compound formed between Al(III) and carminic acid in dilute aqueous solution as a precursor for the aluminium lakes of cochineal used for food colours. Equimolar solutions of aluminium chloride and purified carminic acid (each $2{\cdot}0 \times 10^{-5}$ M in 1·00 M NaCl with pH adjusted to 4·1, corresponding to the monodeprotonated form of carminic acid) were mixed to yield a series of solutions with pH 4·1 (verified in each solution) with a constant sum of the concentration of the two reactants carminic acid and aluminium:

$$Al^{3+} + n\text{CA} \quad Al(CA)_n^{z+} \quad (14)$$

The free ligand and the complex both contribute to the solution fluorescence, and for solutions, when the approximation of eqn (7) is valid, the fluorescence intensity is

$$I_{irr}^{fl} \approx I_{ex}^{0}\, 2{\cdot}30l\{\Phi_{irr}^{CA}\varepsilon_{irr}^{CA}[\text{CA}] + \Phi_{irr}^{Al(CA)_n}\varepsilon_{irr}^{Al(CA)_n}[Al(CA)_n]\} \quad (15)$$

For a solution with a total concentration, M, of reactants (Al^{3+} plus CA), and a mole fraction X of Al^{3+} and $(1 - X)$ of CA, the difference in fluorescence intensity between the situation where complex formation according to eqn (14) takes place and the situation where the equilibrium of eqn (14) is not established, can be expressed as

$$I_{irr}^{fl}(\text{react}) - I_{irr}^{fl}(\text{nonreact}) \approx I_{ex}^{0}\, 2{\cdot}30l\{\Phi_{irr}^{CA}\varepsilon_{irr}^{CA}[\text{CA}] + \Phi_{irr}^{Al(CA)_n}\varepsilon_{irr}^{Al(CA)_n} \times [Al(CA)_n] - \Phi_{irr}^{CA}\varepsilon_{irr}^{CA}M(1-X)\} \quad (16)$$

The intensity difference depends on the value of the mole fraction X and shows a maximum value for

$$\frac{\partial(I^{fl}_{irr}(\text{react}) - I^{fl}_{irr}(\text{nonreact})}{\partial X} \approx 2{\cdot}30 I_0[\Phi^{Al(CA)_n}_{irr}\varepsilon^{CA}_{irr} - \Phi^{CA}_{irr}\varepsilon^{CA_n}_{irr}]\frac{\partial[Al(CA)_n]}{\partial X} = 0 \quad (17)$$

The maximum difference in fluorescence intensity will coincide with the maximum concentration of $Al(CA)_n$. For the method of continuous variations (Job's method; Vosburgh & Cooper, 1941), as used in the present investigation, the conditions for maximum concentration of $Al(CA)_n$ can be shown to be $n = X/(1 - X)$. From Fig. 8 it is seen that the intensity difference I^{fl}_{irr}(react) − I^{fl}_{irr}(non-react) has a maximum for $X = 0{\cdot}5$, corresponding to $n = 1$ in eqn (14).

The stability constant (K_c) of the Al(CA) complex with 1 : 1 stoichiometry:

$$K_c = \frac{[Al(CA)]}{[Al^{3+}][CA]} = \frac{x}{(c^0_{Al} - x)(c^0_{CA} - x)} \quad (18)$$

was determined at 25°C from a fluorimetric analysis of the concentration of complex (x) in two series of dilute aqueous solutions of carminic acid (pH 4·1; 1×10^{-6} M and 1×10^{-5} M, respectively) with increasing concentration of aluminium (Table 2). For the concentration interval, where the approximation of eqn (7) is valid, the difference in fluorescence intensity between the carminic acid solution with (I^{fl}_{irr}) and without aluminium salt added (I^{fl}_{irr}(CA)) can be shown to be

$$I^{fl}_{irr} - I^{fl}_{irr}(CA) \approx I^{fl}_{irr} - I I^0_{ex}\, 2{\cdot}30\Phi^{CA}_{irr}\varepsilon_{CA}c^0_{CA} \approx I I^0_{ex}\, 2{\cdot}30(\Phi^{Al(CA)}_{irr}\varepsilon_{Al(CA)} - \Phi^{CA}_{irr}\varepsilon_{CA})x \quad (19)$$

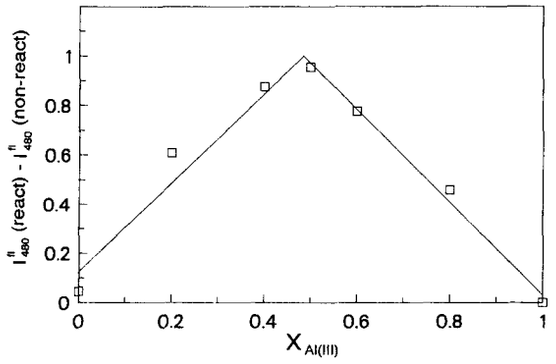

Fig. 8. Application of the method of continuous variation (Job's method), using fluorescence intensity to determine the composition of aluminium/carminic acid complex in weakly acidic solution; $C^0_{Al} + C^0_{CA}$, the sum of the concentrations of the reactants, is held constant at $2{\cdot}0 \times 10^{-5}$ M, and $X_{Al(III)}$ is the mole fraction of aluminium. I^{fl}_{480}(react) − I^{fl}_{480}(nonreact) is the difference between the measured fluorescence intensity and the intensity calculated for the mixture if no reaction had occurred.

Table 2. Determination in dilute aqueous solution at 25°C of the stability constant and the fluorescence quantum yield of the precursor complex (with 1 : 1 aluminium/carminic-acid stoichiometry[a]) to aluminium lakes of cochineal[b]

c^0_{CA} (M)	c^0_{Al} (M)	I^{fl}_{480}[c]	$(I^{fl}_{480} - I^{fl}_{480}(CA))$[d] Observed	Predicted[e]	$\Phi^{Al(CA)}_{480}$[f]
$1{\cdot}0 \times 10^{-6}$		44·3			
$1{\cdot}0 \times 10^{-6}$	$5{\cdot}0 \times 10^{-6}$	45·6	0·11	1·00	
$1{\cdot}0 \times 10^{-6}$	$5{\cdot}0 \times 10^{-5}$	81·2	1·57	1·91	
$1{\cdot}0 \times 10^{-6}$	$5{\cdot}0 \times 10^{-4}$	410	2·56	2·45	
$1{\cdot}0 \times 10^{-6}$	$5{\cdot}0 \times 10^{-3}$	596	2·77	2·58	0·019
$1{\cdot}0 \times 10^{-5}$		203			
$1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-7}$	224	1·31	0·99	
$1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-6}$	253	1·70	1·98	
$1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-5}$	793	2·77	2·89	
$1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-4}$	3 180	3·47	3·45	
$1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-3}$	3 650	3·56	3·58	0·024

[a] Stoichiometry determined by the method of continuous variation, (see Fig. 8).
[b] Aqueous 1·0 M NaCl with pH 4·1
[c] Fluorescence intensity (λ_{ex} = 480 nm) integrated between 520 and 750 nm, in arbitrary units
[d] $(I^{fl}_{480} - I^{fl}_{480}(CA))$ is difference in fluorescence intensity (integrated between 520 and 750 nm) between carminic acid solution, with and without aluminium added.
[e] Calculated according to eqn (26) from parameters determined by nonlinear regression analysis: $K_c = (6{\cdot}8 \pm 1{\cdot}8) \times 10^3$ litre mol^{-1}, and $B = (3{\cdot}4 \pm 1{\cdot}0) \times 10^4$.
[f] Fluorescence quantum yield of Al(CA) calculated from limiting intensity of fluorescence for each series (approximately 97% of the carminic acid is complexed by aluminium): $\Phi^{Al(CA)}_{480} = \Phi^{CA}_{480}(I^{fl}_{480}(\text{limit})/T^{fl}_{480}(CA))$.

Solving eqn (18) for x and combining the result with eqn (19) yields:

$$I^{fl}_{irr} - I^{fl}_{irr}(CA) \approx I I^0_{ex}\, 2{\cdot}30(\Phi^{Al(CA)}_{irr}\varepsilon_{Al(CA)} - \Phi^{CA}_{irr}\varepsilon_{CA}) \times \left[\frac{c^0_{Al}K_c + c^0_{CA}K_c + 1}{2K_c} - \sqrt{\frac{(c^0_{Al}K_c)^2 + (c^0_{CA}K_c)^2 - 2c^0_{Al}c^0_{CA}K_c^2 + 2c^0_{Al}K_c + 2c^0_{CA}K_c + 1}{4K_c^2}}\right] \quad (20)$$

K_c and the quantity $B \approx [I I^0_{ex}\, 2{\cdot}30(\Phi^{Al(CA)}_{irr}\varepsilon_{Al(CA)} - \Phi^{CA}_{irr}\varepsilon_{CA})]$ were determined by nonlinear regression analysis from the intensity data presented in Table 2, and was found to have the value $K_c = (6{\cdot}8 \pm 1{\cdot}8) \times 10^3$ litre mol^{-1} and $B = (3{\cdot}4 \pm 1{\cdot}0) \times 10^4$. The agreement between measured intensities and calculated intensities is satisfactory, when it is considered that the concentration of aluminium is varied over four orders of magnitude.

The fluorescence quantum yield of the aluminium complex of carminic acid was estimated from the fluorescence intensity data used for determination of the complex constant, using limiting intensity for both of the two series of carminic acid solutions with increasing aluminium concentration (footnote *f* of Table 2), and was found to have the value $\Phi^{Al(CA)}_{480} = 0{\cdot}022 \pm 0{\cdot}004$.

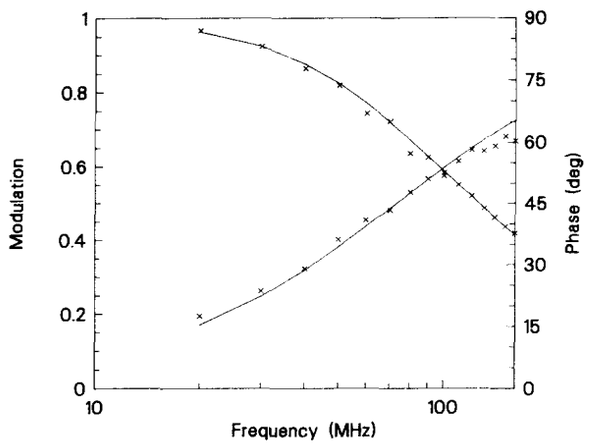

Fig. 9. Multifrequency phase (in degrees) and modulation data for the aluminium/carminic acid complex in aqueous 1·00 M NaCl with pH 4·1 at 25°C. The solid line represents simultaneous fit of demodulation and phase shift data, resulting in an excited state lifetime of $\tau = 2{\cdot}1 \pm 0{\cdot}1$ ns.

The lifetime of the emissive state of Al(CA) was determined from multifrequency phase-shift and demodulation data ($20 \leq f \leq 180$ MHz). As may be seen from the data in Fig. 9, the fluorescence was adequately described as a monoexponential decay, and the emissive state was found to have a lifetime $\tau = 2{\cdot}1 \pm 0{\cdot}1$ ns at 25°C in aqueous 1·0 M NaCl with pH 4·1.

DISCUSSION

Carminic acid is, like certain other anthraquinone glucosides, water-soluble due to the attachment of a hydrophilic sugar moiety to the hydrophobic anthraquinone. However, the deviation from Lambert–Beer's law for carminic acid in acidic aqueous solution and the more significant deviation from linearity between concentration and fluorescence intensity have been used to demonstrate a hydrophobic interaction between the anthraquinone moieties in aqueous solution, resulting in a stacking of carminic acid. The stacking reaction is in fast equilibrium and, from pressure quenching of fluorescence, a negative reaction volume of the stacking reaction has been established. Compared to anthraquinone, the parent compound of the carminates, the absorption spectrum of carminic acid is shifted into the visible region. The low-intensity absorption at longer wavelengths of anthraquinone derivatives has been assigned as a $n \rightarrow \pi^*$ transition, while the more intense and higher energy absorption in the UV region are assigned as $\pi \rightarrow \pi^*$ transitions (Diaz, 1990).Both of these types of transition are spin-allowed populating singlet excited states of increasing energy: $S_1(n \rightarrow \pi^*)$, $S_2(\pi \rightarrow \pi^*)$, $S_3(\pi \rightarrow \pi^*)\ldots$, and for carminic acid at least four $\pi \rightarrow \pi^*$ transitions can be recognized in the absorption spectrum (Fig. 2). The four hydroxyl groups attached directly to the conjugated systems of carminic acid are capable of donating electrons to the carbonyl groups, resulting in a charge transfer transition (Peters & Sumner, 1953). As a result of the extended conjugation in hydroxyl derivatives of anthraquinones, the CT-transitions ($\pi \rightarrow \pi^*$) are of low energy and are mixed with the $n \rightarrow \pi^*$ transition in the visible region. For carminic acid, this is confirmed by the non-Gaussian shape of the absorption band in the visible region and is further confirmed by the sensitivity of the position of this absorption band to deprotonization of the phenolic hydroxyl groups, but not to the deprotonization of the carboxyl group.

The phenolic groups of carminic acid also play important roles in the emissive properties of carminic acid. Carminic acid is a tribasic acid in aqueous solution, and the emission yield shows a maximum around pH 4, corresponding to a maximum concentration of the H_2L^- form of carminic acid with the carboxyl group deprotonated and with both of the phenols nondissociated (Fig.3). The emission decreases in neutral and alkaline solution and is almost completely quenched at pH > 10. Notably, this is not a simple OH^- quenching following Stern–Volmer kinetics (Lakowicz, 1983):

$$\frac{\Phi_0}{\Phi} = 1 + k_Q \tau [OH^-] \qquad (21)$$

since the actual lifetime, $\tau = 0{\cdot}093$ ns, and the decrease to 10% of Φ_0 at pH 9·2 (Fig. 3), where $[OH^-] = 2 \times 10^{-5}$ M, would implicate a quenching rate constant $k_Q = 5 \times 10^{15}$ litre mol^{-1} s^{-1}, five orders of magnitude above the diffusion limit. The presence of phenolate groups in carminic acid lowers the energy of the lowest excited state, as evidenced by the significant red-shifted absorption spectrum in alkaline solution, and the nonradiative deactivation is, according to the energy gap law for weak coupling mechanisms (Englman & Jortner, 1970), expected to increase in efficiency with decreasing energy difference between the lowest excited state and the ground state. An alternative explanation to the lack of fluorescence of carminic acid in alkaline solution is based on the fact that an extension of the conjugate system in the deprotonated form would lower the $S(n \rightarrow \pi^*)$ energy relative to the $S(\pi \rightarrow \pi^*)$ energy (Plotnikov, 1966). Intersystem crossing to the lowest energy triplet $T(\pi \rightarrow \pi^*)$ has a higher probability from a $S(n \rightarrow \pi^*)$ than from a $S(\pi \rightarrow \pi^*)$ singlet state, in effect decreasing the fluorescence due to deactivation via a triplet state (Hirayama & Kobayashi, 1977). Notably, an increased relative population of the lowest triplet state with increasing pH would also explain the observed increase in photooxidation quantum yield for carminic acid with increasing pH (Jørgensen & Skibsted,1991). Assuming that photooxidation is initiated by the spin-allowed reaction between ground state oxygen and carminic acid (CA) in a triplet excited state:

$$^3O_2 + {}^3CA \longrightarrow \text{oxidation products} \qquad (22)$$

an increased photoreactivity is expected to be matched by a decrease in fluorescence. The photooxidation quantum yield for 436 nm excitation is, in Table 1, compared with the relative fluorescence quantum yields for the four acid/base forms of carminic acid, and as may be seen, an increase in photooxidation yield is

paralleled by a decrease in fluorescence yield, in strong support of the suggested excited state mechanism. An important confirmation of the triplet reactivity would be the detection of phosphorescence in alkaline solutions of carminic acid. We found no evidence of solution phosphorescence of deaerated alkaline solutions of carminic acid at room temperature or in a methanol/water glass at 77 K. However, the lack of phosphorescence for the free carminic acid in a 77 K glass is in agreement with the general pattern found for hydroxy derivatives of anthraquinone, where phosphorescence is only seen for 2-hydroxy derivatives (Diaz, 1990).

The photoreaction of carminic acid is linearly dependent on the oxygen partial pressure (Jørgensen & Skibsted, 1991) suggesting a bimolecular reaction with oxygen. The primary step is likely to involve the transfer of an electron from carminic acid to oxygen:

$$CA + O_2 \longrightarrow CA^+ + O_2^- \qquad (23)$$

We tried to detect the superoxide ion during illumination of carminic acid. Although dihydrorhodamine G6 is a sensitive probe for the superoxide radical anion, our results were not very conclusive. By continuous wave photolysis with 436 nm monochromatic light, the formation of rhodamine 6G was significant, as it was twice the formation in non-illuminated mixtures. It should, however, be noted that the estimated quantum yield for superoxide formation ($\Phi(O_2^-) = 2{\cdot}5 \times 10^{-6}$ mol einstein^{-1}) was almost an order of magnitude lower than the quantum yield for photobleaching of carminic acid under similar conditions ($\Phi^{ox}_{H_2L} = 2 \times 10^{-5}$ mol einstein^{-1}, see Table 1). However, the detection of superoxide as a result of photooxidation of carminic acid supports a photooxidation mechanism with an initial electron transfer to oxygen in a triplet state of carminic acid rather than an addition of oxygen to the conjugate system in the singlet state.

The fluorescence lifetime (τ = 0·093 ns) and the fluorescence quantum yield (Φ_{fl} = 0·0013) appears to be the first combined determination for a water-soluble anthraquione derivative and together they yield an estimate of the natural radiative lifetime at ambient temperature (Parker, 1968):

$$\tau_r = \frac{\tau}{\Phi_{fl}} = 7 \times 10^{-8}\ \text{s} \qquad (24)$$

This value is comparable to the value $\tau_r = 2 \times 10^{-8}$ s calculated from $\varepsilon_{max} \approx 6000$ litre mol^{-1} cm^{-1} for the lowest energy transition in carminic acid, using the approximation $\tau_r \approx 10^{-4}/\varepsilon_{max}$ based on the Strickler–Berg equation (Balzani & Carassitti, 1970). The agreement between the experimentally determined radiative lifetime and the radiative lifetime calculated from spectroscopic data supports the suggestion of the lowest energy singlet excited state as the emitting state. The determined value for the actual lifetime is further confirmed by the fact that carminic acid fluorescence is only weakly quenched by iodide. The iodide quenching is, in contrast to the quenching by hydroxide, adequately described by the Stern–Volmer equation, as may be seen from Fig. 4. The quenching constant ($K_Q = k_Q\tau$ = 1·27 litre mol^{-1}) and the lifetime determined under the same set of conditions (τ = 0·093 ns) together yield a quenching rate constant of $k_Q = 1{\cdot}3 \times 10^{10}$ litre mol^{-1} s^{-1}, corresponding nicely to the diffusion limit for a bimolecular process in aqueous solution. The radiative lifetime $\tau_r = 7 \times 10^{-8}$ s corresponds to a first-order rate constant $k_r^{CA} = 1{\cdot}4 \times 10^7$ S^{-1} for radiative deactivation. Combining this value with the fluorescence lifetime ($\tau = (k_r^{CA} + k_{nr}^{CA})^{-1} = 9{\cdot}3 \times 10^{-11}$ s^{-1}, see legend to Fig. 10) yields the value $k_{nr}^{CA} = 1{\cdot}1\ 10^{10}$ s^{-1} for the nonradiative deactivation.

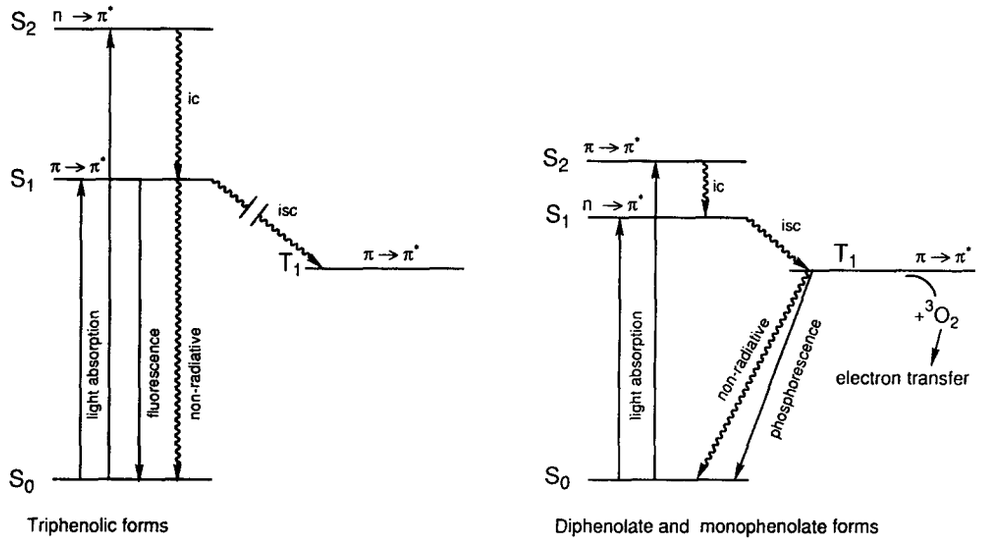

Fig. 10. Excited state diagram for carminic acid in acidic solution (triphenolic form) and in alkaline solution(diphenolate and monophenolate forms). It is suggested that reversal of the energy of $n\pi^*$ and $\pi\pi^*$ excited states leads to different deactivation paths (see text). The fluorescence quantum yield for the triphenolic form, in which the intersystem crossing (isc) is negligible, is related to the first-order rate constant of radiative deactivation (fluorescence), k_r and the first order rate constant of non-radiative deactivation k_{nr} by $\Phi_{fl} = k_r/(k_r + k_{nr})$, where the lifetime of the emissive state is $\tau = (k_r + k_{nr})^{-1}$.

Table 3. Photophysical characterization of emissive state of carminic acid and 1 : 1 carminic acid complex with aluminium in weakly acidic aqueous 1·0 M chloride solution at 25°C

	Φ_{480}^{a}	τ^{b} (ns)	k_r^{c} (s^{-1})	k_{nr}^{d} (s^{-1})
CA	0·001 33 ± 0·000 19	0·093 ± 0·010	$(1·4 \pm 0·3) \times 10^7$	$(1·1 \pm 0·1) \times 10^{10}$
Al(CA)	0·022 ± 0·004	2·1 ± 0·1	$(1·1 \pm 0·2) \times 10^7$	$(4·7 \pm 0·2) \times 10^8$

[a] Fluorescence quantum yield.
[b] Lifetime of emissive state.
[c] First-order rate constant for radiative deactivation.
[d] First-order rate constant for nonradiative deactivation.

Fluorescence spectroscopy has been used to determine the stability constant ($6·8 \times 10^3$ mol litre^{-1}, at pH 4·1 and 25°C) for the 1 : 1 complex formed between aluminium and carminic acid in dilute aqueous solution. From the fluorescence quantum yield ($\Phi = 0·021$) and the emission lifetime ($\tau = 2·1$ ns), an analysis similar to that for the uncomplexed carminic acid yields a rate constant for the radiative deactivation of $k_r^{Al(CA)} = 1·1 \times 10^7$ s^{-1} and of $k_{nr}^{Al(CA)} = 4·7 \times 10^8$ s^{-1} for the nonradiative deactivation of the aluminium/carminic acid complex. The photophysical properties of the emissive state of carminic acid and of the aluminium complex are compared in Table 3, and it is seen that the higher fluorescence yield of the complex is due to the decrease in the rate of nonradiative deactivation of the complex compared to uncomplexed carminic acid. Aluminium has, in general, a high affinity for phenols, and the binding of aluminium to carminic acid helps to identify the phenolic groups as important for the nonradiative deactivation of excited states of carminic acid.

The absence of phosphorescence and the presence of short-lived fluorescence for carminic acid, under the conditions of relevance for food (neutral to slightly acidic conditions) is in agreement with a deactivation from the initially populated singlet states via the lowest singlet state and without any significant inter system crossing to $T(n \rightarrow \pi^*)$ and $T(\pi \rightarrow \pi^*)$ triplet states (Fig. 10). Such triplet states can be either strong oxidants or strong reductants, and anthraquinones which display phosphorescence are in general, found to be effective photosensitizers with concomitant formation of semiquinone radicals (Dearman & Chan, 1965). Carminic acid has previously been found not capable of sensitizing the oxidative degradation of the carotenoid astaxanthin (Jørgensen & Skibsted, 1991) in agreement with the present findings.

From the photophysical investigation of carminic acid, it can be concluded that photooxidation of carminic acid is insignificant and that carminic acid is a poor photosensitizer in the pH region of relevance for food. Photooxidation yields increase in alkaline solution and fluorescence yields decrease, and it is suggested that deactivation via a reactive triplet state populated via intersystem crossing from the initially populated singlet states is becoming of increasing importance for the phenolatic form of carminic acid. The change in deactivation path for the electronic excitation of carminic acid in alkaline solution makes it more exposed to oxidation and, through formation of activated oxygen species, more likely to participate in coupled oxidation reactions at higher pH.

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