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FREE RADICAL REACTIONS WITH ALPHA-TOCOPHEROL AND N-STEAROYL TRYPTOPHAN METHYL ESTER IN MICELLAR SOLUTIONS

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The rate constants have been measured for one-electron oxidation by N_3^- and Br_2^- of N-stearoyl tryptophan methyl ester (STME) and α-tocopherol (α-T) in micelles of sodium dodecyl sulphate (SDS) or tetradecyl trimethyl ammonium bromide (TTAB). Compared with analogous reactions of tryptophan and Trolox C in aqueous solution, the rate constants for oxidation in micellar solution by N_3^- are reduced by 30–70%. The micellar charge increased the rate of oxidation of STME by Br_2^- in TTAB micelles by almost an order of magnitude, compared with the reaction of Br_2^- with tryptophan in aqueous solution. In SDS micelles the rate of oxidation of STME by Br_2^- was reduced more than 30-fold. Quenching of fluorescence from STME in micelles by acrylamide confirmed the accessibility of the indole ring to the aqueous solvent. The rate of repair of the neutral STME radical by α-T in TTAB micelles was found to be accelerated by a factor of at least 27, compared with the similar reaction between Trolox C and tryptophan radicals in aqueous solution.

Key words: α-Tocopherol, free radicals, tryptophan, pulse radiolysis, electron transfer, fluorescence quenching.

INTRODUCTION

α-Tocopherol (Vitamin E, α-T) is essential for the normal physiological function of cellular systems[1] and is found to be localised within the membraneous subcellular fraction of cells[2]. The principal function of α-tocopherol is widely regarded to be that of an antioxidant[3], protecting membranes against peroxidative damage to the unsaturated acyl chains of constituent phospholipids[4]. α-Tocopherol has been shown to act in this respect as a chain-breaking antioxidant[5,6]; lipid peroxy radicals (the propagating species in the free radical chain reaction) are repaired by hydrogen atom transfer from the phenolic hydroxyl group of the α-tocopherol. The resulting radical of α-T residing in the membrane may then be itself repaired by ascorbate in the aqueous phase[7,8]. Rapid reaction techniques have been used to directly demonstrate one-electron oxidation of α-T or Trolox C (a water soluble analogue) not only by alkylperoxy radicals[9], but also by amino acid and protein radicals[10,11], a DNA base radical[12], and by $^{\cdot}OH$[13] and $O_2^{\cdot-}$[14]. It therefore appears that α-T in a membrane may

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have secondary functions, which include scavenging of $O_2^{\cdot-}$, thereby preventing initiation of the peroxidation of lipids, and the repair of protein radicals residing on functional membrane-bound proteins. In order to investigate further the latter proposed role for α-T, a hydrophobic tryptophan derivative, N-stearoyl tryptophan methyl ester (STME), has been synthesised. Free radical reactions involving α-T and STME have been studied by pulse radiolysis in micellar systems as a preliminary model for similar reactions which might occur in biomembranes.

METHODS AND MATERIALS

Materials

DL-α-tocopherol (α-T) was obtained from Fluka and used as received. N-stearoyl-tryptophan methyl ester (STME) was prepared by condensation of stearic acid with tryptophan methyl ester using dicyclohexyl-carbodiimide. It was recrystallised from ethanol, giving m.pt 92–93°C. Mass spectrometry showed the molecular ion at m/e = 484 corresponding to the formula weight for $C_{30}H_{48}N_2O_3$. The infra-red spectrum showed both an amide (1625 cm^{-1}) and an ester (1730 cm^{-1}) absorption. In ethanol the UV maximum was at 280 nm with $\epsilon = 6.25 \times 10^3\ M^{-1}cm^{-1}$. The detergents used were sodium dodecyl sulphate (SDS, specially purified grade for biochemical work, BDH Chemicals Ltd.) and tetradecyl trimethyl ammonium bromide (TTAB, Sigma). Trolox C was a gift from Hoffman La Roche (Nutley, New Jersey, USA). The acrylamide used was BDH "Electran" grade. Water as obtained from a Milli Q water purification system (Millipore Ltd.) and solutions prepared in baked glassware. Other reagents used were Analar grade. STME was found to be insoluble in water, but could readily be solubilised in solutions containing either SDS or TTAB. This was accomplished by injection of a small volume of a concentrated solution of STME in ethanol into the detergent solution.

The resulting ethanol concentration in the final solutions was sufficiently low such that in the pulse radiolysis experiments >90% of the $^{\cdot}OH$ radicals reacted with either N_3^- or Br^-, based on published rate constants. α-Tocopherol was solubilised in detergent solutions in a similar manner.

Fluorescence measurements

Steady state fluorescence measurements were made with a Schoeffel RRS1000 spectrofluorimeter. In the quenching experiments, corrections for absorption of the exciting light by the quencher were applied as described previously[15]. Fluorescence decay times were measured with an Edinburgh Instruments M199 single photon counting fluorometer, employing a thyratron gated discharge lamp filled with hydrogen gas. Deconvolution and least squares fitting of the fluorescence decay was accomplished by conventional methods[16] on an LSI-II minicomputer. All fluorescence measurements were made in air saturated solutions.

Pulse radiolysis

The pulse radiolysis experiments were made using a Febetron 705 (1.5MeV, 20ns electron pulse) at the University of Salford. Transient absorptions were measured in a conventional spectrophotometric system and digitized by a Datalab 905 transient recorder before being transferred to an Apple II microcomputer for data analysis and storage. Dosimetry was performed with air saturated thiocyanate solutions.

Determination of critical micelle concentrations

The critical micelle concentration (cmc) of each detergent solution, containing the same buffer and salt concentration as in the other associated experiments, were measured by the surface tension method, using a tensiometer (White Instruments Ltd.).

RESULTS AND DISCUSSION

Reactions of inorganic radicals with N-stearoyl tryptophan methyl ester (STME) and α-tocopherol in micelles

The second order rate constants for reaction of $Br_2^{\cdot-}$ and $N_3^{\cdot}$ with STME and α-T in micelles composed of either SDS or TTAB are shown in Table I. All of the values quoted were obtained from linear plots of first order rate constants for product radical formation or $Br_2^{\cdot-}$ decay (as specified in Table I) versus solute concentration. An example of such a plot for the reaction of $Br_2^{\cdot-}$ with STME in TTAB micelles is shown in the inset to Figure 1. These experiments were performed in solutions containing 0.1M phosphate buffer, pH7.0 ± 0.2, detergent (2 mM) and N_3^- (0.1 M) or Br^- (0.1 or 0.4 M). The critical micelle concentrations (CMC's) under these conditions were 0.126 mM (0.4 M NaBr), 0.25 mM (0.1 M NaBr) and 0.32 mM (0.1 M NaN_3) for TTAB and 0.70 mM (0.1 M NaN_3) for SDS. The second order rate constants for reaction of $N_3^{\cdot}$ with STME are the same for STME solubilized in TTAB or SDS micelles. This is to be expected from the neutral character of the $N_3^{\cdot}$ radical. At the concentration of azide used in these experiments (0.1 M), formation of $N_6^{\cdot-}$ from the reaction:-

$$N_3^{\cdot-} + N_3^- \rightleftharpoons N_6^{\cdot-} \qquad (1)$$

is expected to be negligible on the basis of the published association constant of 0.33 M^{-1} [17]. Comparison of the rate constant for reaction between $N_3^{\cdot}$ and STME in micelles of SDS or TTAB with that for reaction of $N_3^{\cdot}$ with tryptophan in aqueous solution shows that the reactivity of the STME is decreased by approximately 60% due to its location in either micellar phase. In contrast, the reactivity of $Br_2^{\cdot-}$ radical anions with STME shows a very marked dependence on micellar charge. In the

TABLE I

Second order rate constants for reaction of inorganic radicals with solutes in micellar solution, in units of 10^8 M^{-1} s^{-1}. Solutions contained 2 mM detergent, phosphate buffer (0.1 M, pH7.0) and 0.1 M NaN_3 or NaBr.

Solute	Radical	SDS	TTAB	H_2O[a]
STME	$N_3^{\cdot}$	16.1 ± 0.7[b]	16.4 ± 2.2[b]	41 (ref. 19)
	$Br_2^{\cdot-}$	< 0.2[c]	55.5 ± 3.3[b]	7.7 (ref. 18)
α-tocopherol	$N_3^{\cdot}$	17.7 ± 1.6[d]	21.6 ± 1.2[d]	30 (ref. 20)
	$Br_2^{\cdot-}$	——	7.2 ± 0.5[c,e]	3.8 (ref. 10)

[a] Values either for Trolox C or tryptophan in aqueous solution, pH 7 from the references quoted.
[b] Measured by formation of the STME radical at 520 nm.
[c] Measured by decay of $Br_2^{\cdot-}$ at 380 nm.
[d] Meausred by formation of the tocopherol radical at 440 nm.
[e] Solution contained 0.4 M NaBr.

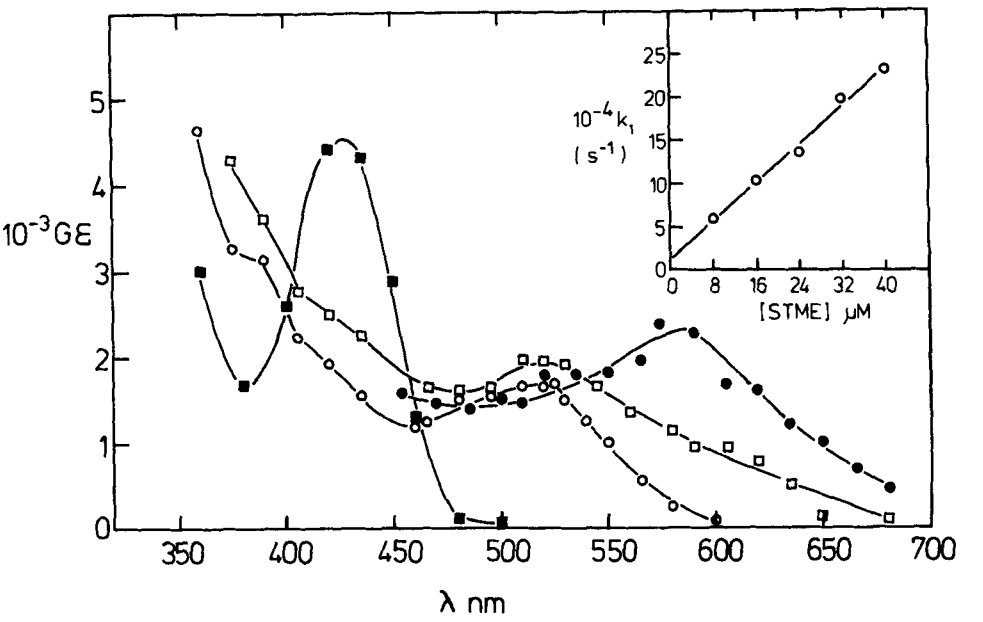

FIGURE 1 Transient absorption spectra from pulse radiolysis of STME and α-tocopherol in N_2O saturated solutions containing TTAB (2 mM), NaBr (0.4 M) and phosphate buffer (0.1 M). The dose per pulse was approximately 2 Gy for the experiments with α-T, and between 5 and 10 Gy for those with STME.
■:- α-tocopherol (200 μM) at pH 7.0, 50 μs after the pulse. (Vertical scale reduced by a factor of 2)
○:- STME (150 μM) at pH 7.0, 400 μs after the pulse.
□:- STME (150 μM) at pH 3.0, 60 μs after the pulse.
●:- STME (150 μM) at pH 1.5, 60 μs after the pulse.
Inset:- Effect of STME concentration on the first order rate constant for reaction of Br_2^- with STME in TTAB micelles at pH 7.0. Measured from formation of the STME radical at 520 nm, dose per pulse = 5.2 Gy.

negatively charged SDS micelles, the reaction of Br_2^- was too slow to be measured. This is good evidence for the strong partitioning of STME into the micellar phase. In the positively charged TTAB micelles the reactivity of STME towards Br_2^- is a factor of 7.2 times greater than that of Br_2^- with tryptophan in aqueous solution. Table I also shows that the second order rates constants for reaction of $N_3^\cdot$ with α-T in micelles formed from either SDS or TTAB are similar, and reduced by between 30 and 40% in comparison with the rate for reaction of $N_3^\cdot$ with Trolox C (a water soluble α-T analogue) in aqueous solution. The rate constant obtained in the present work for reaction of $N_3^\cdot$ with α-T in SDS micelles ($k = (1.77 + 0.16) \times 10^9\ M^{-1}\ s^{-1}$) is similar to that reported by Hoey and Butler ($k = (2.4 + 0.21) \times 10^9\ M^{-1}\ s^{-1}$ [11]). The rate constant for reaction of Br_2^- with α-T in positively charge TTAB micelles is enhanced by a factor of approximately 2, if compared with the rate constant for reaction of Br_2^- with Trolox C in aqueous solution.

The variations in rate constants described above due to micellar and radical charge are similar to those reported by Wallace and Thomas[21] for reaction of $H^\cdot$ and e_{aq}^- with solutes in micellar systems. These authors noted that for uncharged reactant species ($H^\cdot$, O_2 or CH_3NO_2) the micellar location of the reactive solute reduced the rate constant by a factor of 2–4, whereas for reaction between charged species (I^- or e_{aq}^-)

and a solute within a micelle of the same charge, the rate contant was reduced by a factor of up to 600. For reactive species and micelles of opposite charge, increases in rate constants by up to 100 were reported.

Transient absorption spectra of the free radicals derived from one-electron oxidation of STME and α-T in TTAB micelles are shown in Figure 1. The spectrum of the radical from α-T contains a peak at 420–440 nm, corresponding to the phenoxyl radical and similar to that previously reported for Trolox C in aqueous solution[10] and for α-T in SDS micelles[9b]. At pH 7, the one electron oxidation of STME in TTAB micelles by Br_2^- leads to the formation of the characteristic peak at 520 nm due to the neutral tryptophan radical[18,22]. The same spectrum was also observed at pH 5 (data not shown), but at pH 3 an absorption at longer wavelengths is noted. At pH 1.5 the peak occurs at ~580 nm, which may be ascribed to the indole radical cation[22]. Whilst an exact pK_a value for the indole radical of STME in TTAB micelles has not been obtained, the present results indicate a $pK_a \leqslant 3$. This is lower than that for tryptophan in aqueous solution ($pK_a = 4.3$), but is expected from the cationic nature of the TTAB micelle[23]. Reduced pK_a values have also been reported for the radical formed by one electron oxidation of trypophan residues in both pepsin[24] and lysozyme[25]. The measured values of $G \times \epsilon$ for the absorption maxima of the STME and α-T radicals in Figure 1 are only about 20–30% of those expected, indicating either reduced extinction coefficients in the micellar environments or incomplete scavenging of the inorganic radicals ($G \simeq 6$ radicals/heV in neutral N_2O saturated solution) under the present experimental conditions, if values for the corresponding extinction coefficients for Trolox C ($\epsilon = 5.4 \times 10^3\ M^{-1}\ cm^{-1}$ at 440 nm[10]) and tryptophan ($\epsilon = 1.9 \times 10^3\ M^{-1}\ cm^{-1}$ at 520 nm[22]) are used. The latter explanation seems more likely.

Quenching of fluorescence from STME in micelles by acrylamide

The results of the previous section show that in micelles the indole ring of STME, being the oxidisable moeity, possesses a reactivity towards inorganic radicals generated in the aqueous compartment approximately one-third that of tryptophan in aqueous solution. The quenching of tryptophan fluorescence by acrylamide has previously been proposed as a method for probing the exposure of the indole ring in various tryptophan derivatives to the aqueous phase in solutions of micelles and proteins[15,26,27]. As a quencher for such studies, acrylamide has the advantages of being uncharged (and so not susceptible to ionic interactions with the micelle) and of being extremely insoluble in hydrocarbon solvents. The latter property makes it extremely improbable that acrylamide will partition into the micellar phase.

Figure 2A shows the steady-state Stern-Volmer plots for quenching by acrylamide of STME (50 μM) in solutions containing either SDS or TTAB (10 mM) and for tryptophan in water. All the solutions were buffered with phosphate (100 mM) to pH 7.0. The plots all show an upwards curvature and may be analysed assuming that quenching occurs by both static and dynamic processes. The constants for the static (K_S) and dynamic (K_D) mechanisms may be obtained as described by Lakowicz[28]. The fluorescence intensity in the absence (F_o) and prescence (F) of a quencher (Q) are related to the quenching constants by:-

$$[(F_o/F) - 1]/[Q] = (K_S + K_D) + K_S K_D[Q] \quad (2)$$

Figure 2B shows the data plotted according to Eq. (2), and the derived constants are presented in Table II. Values for K_S and K_D for tryptophan measured here in

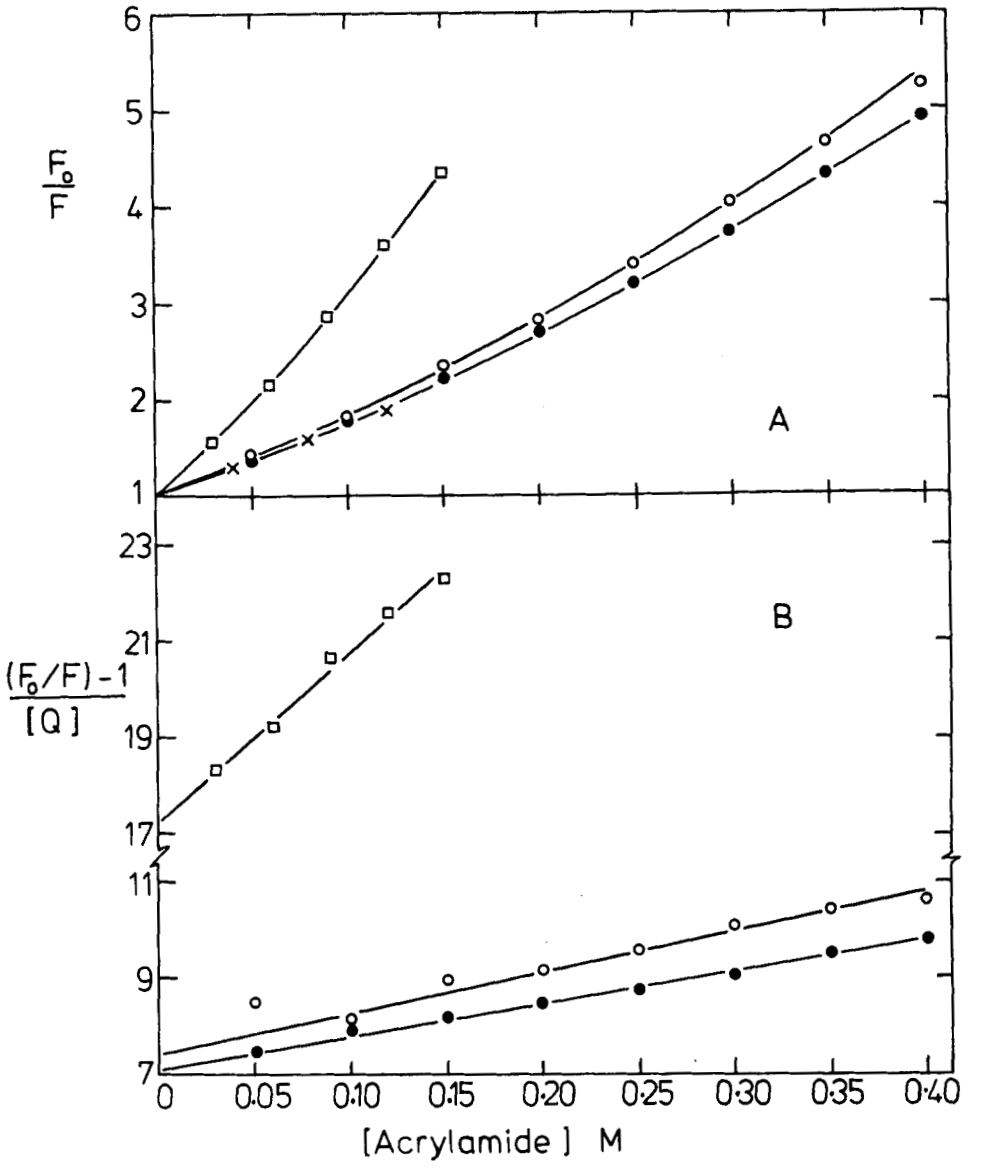

FIGURE 2 A:- Stern-Volmer plots for the steady-state fluorescence quenching by acrylamide of tryptophan in aqueous solution and STME in micelles (λ_{ex} = 300 nm, λ_{em} = 348 nm). B:- data plotted according to Eq. (2), for details see text.
□:- Tryptophan (20 μM) in phosphate buffer (0.1 M, pH 7.0).
●:- STME (50 μM) in solutions containing SDS (10 mM) and phosphate buffer (0.1 M, pH 7.0).
○:- STME (50 μM) in solutions containing TTAB (10 mM) and phosphate buffer (0.1 M, pH 7.0).
×:- values from lifetime data (τ_0/τ) for STME in TTAB micelles (conditions as above, except λ_{ex} = 280 nm).

TABLE II
Quenching constants for STME and tryptophan quenched by acrylamide, calculated from the data in Figure 2.

	K_S (M^{-1})	K_D (M^{-1})	τ_0(ns)	k_q (M^{-1} s^{-1})
Tryptophan	2.3	15.0	2.70	5.6×10^9
STME in TTAB	1.3	6.3	1.98	3.2×10^9
STME in TTAB	—	7.4*	1.98	3.7×10^{9}*
STME in SDS	1.1	6.0	1.14	5.3×10^9

* Values from lifetimes data

phosphate buffered solution are similar to those reported in unbuffered water[15]. The quenching constant for the dynamic component (K_D) is equal to the product of the unquenched fluorescence lifetime (τ_o) and the second order rate constant for quenching (k_q). Fluorescence decays were measured by the single photon counting method and were found to be best fitted as double exponentials, in accord with previous reports[29]. For the present purposes, a quantum yield weighted mean lifetime ($<\tau>$) was calculated from the measured component lifetimes (τ_i) and pre-exponential factors (a_i) from:-

$$<\tau> = \Sigma a_i \tau_i^2 / \Sigma a_i \tau_i \quad (3)$$

The results are included in Table II. For tryptophan in aqueous solution at neutral pH, the value of $<\tau> = 2.7$ ns is in good agreement with previous determinations[29]. The unquenched lifetimes of STME in SDS or TTAB micelles are significantly lower at 1.14 and 1.98 ns respectively. The values of k_q calculated from $<\tau_o>$ and K_D are included in Table II. In TTAB micelles, k_q for quenching of STME is 57% of that for tryptophan in solution, whereas that in SDS micelles is almost the same as for tryptophan in water. The lifetime-derived quenching constant in TTAB micelles from the data in Figure 2A confirms the presence of the dynamic component of the quenching in the steady-state experiments, and gives a value of k_q in reasonable agreement as shown in Table II. Fluorescence lifetimes in SDS micelles were not examined in the presence of quencher because of the short lifetime under these conditions. The results from these quenching experiments with STME and acrylamide confirm that the indole chromophore of STME when incorporated into micelles is highly accessible from the aqueous phase, as found above with the free radical reactions.

Repair of STME radicals by alpha-tocopherol in micelles

The repair of the radical formed by one-electron oxidation of STME by $Br_2^{\cdot-}$ in TTAB micelles by α-T was investigated as a model for the repair of protein radicals in a membrane system. Micelles of TTAB were chosen, since the results in Table I show that in micelles containing both STME and α-T, the STME would be oxidised preferentially by $Br_2^{\cdot-}$. Figure 3 shows the decay of the neutral indole radical of STME at 520 nm in solutions containing TTAB (2 mM) at pH 7.0, STME (150 μM) and α-T (0 to 50 μM). The maximum absorption in the absence of α-T indicates a radical concentration of approximately 1 μM (taking the extiction coefficient to be the same as for tryptophan), corresponding to very much less than an average of one radical per micelle. The rate of decay of the STME radical appears to increase with increasing

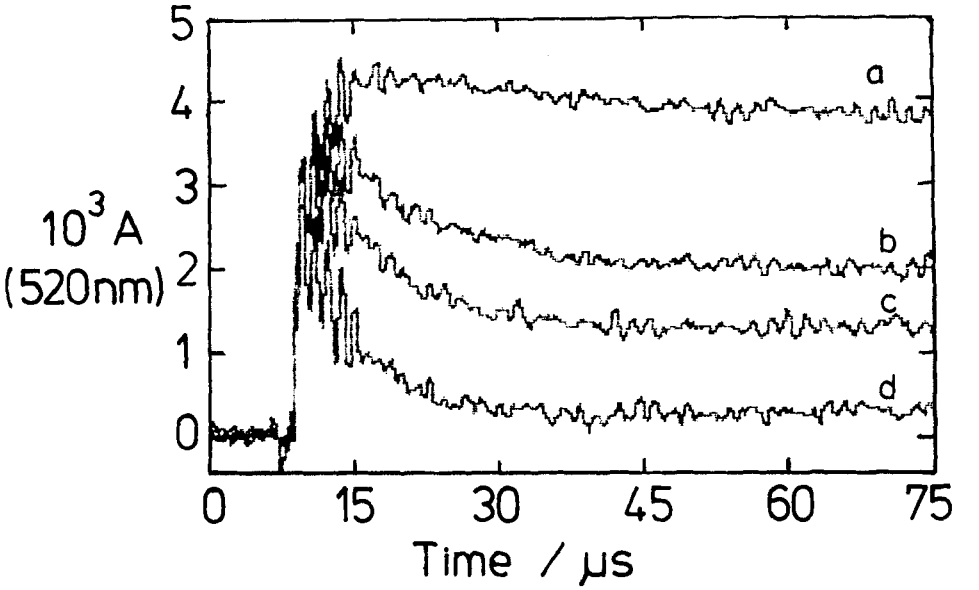

FIGURE 3 Transient decays at 520 nm from pulse radiolysis of N_2O saturated solutions containing TTAB (2 mM), NaBr (0.4 M), STME (150 μM) and α-tocopherol (a:-none; b:-7.5 μM; c:-15 μM; and d:-50 μM). Dose per pulse = 9.5 Gy.

concentration of α-T. The poor signal-to-noise ratio in these experiments, due to the low value of $G \times \epsilon$, precludes a detailed kinetic analysis. However, it may be noted that the half-life for the decay of the STME radical in the presence of 50 μM α-T is ⩽ 10 microseconds. The increase in rate constant for repair of tryptophan radicals by the model compound, Trolox C, in aqueous solution with decreasing pH is shown in Figure 4. The solid line indicates the best fit for a pK curve to the data, with $pK_a = 4.2 \pm 0.1$, in accord with the pK_a of 4.3 for deprotonation of the tryptophan radical cation[22]. At pH values greater than 6, the rate constant for repair is $5.2 \times 10^7\ M^{-1}\ s^{-1}$. The half-life for reaction of the neutral tryptophan radical at pH 7 with 50 μM Trolox C (corresponding to the maximum α-T concentration in Figure 3) would be 270 microseconds. This indicates that in comparison, the repair reaction is increased in rate by a factor of at least 27 when the reacting pair of molecules are confined within the same micelle. It may also be noted that this increase in reaction rate occurs despite the higher apparent viscosity of the micellar interior compared with that of water. Studies of excimer formation[30] and fluorescence depolarization[31] indicate a "microviscosity" within the micelle of approximately 30 centipoise. Whilst "microviscosity" assessed by these methods may only at best be an approximation, it provides a useful basis for comparison.

The results in Figure 3 also show that the repair reaction is incomplete within the range of α-T concentration used, since a residual absorption of the STME radical remains at 75 μs after the pulse. This may be ascribed to the statistical distribution of α-T in the micelles, some not containing a molecule of α-T at the solute concentrations used in these experiments. The fraction of the micelles (F_i) containing i molecules of α-T is related to the average number of α-T molecules per micelle ($\bar{n}$) by the Poisson distribution[32]:-

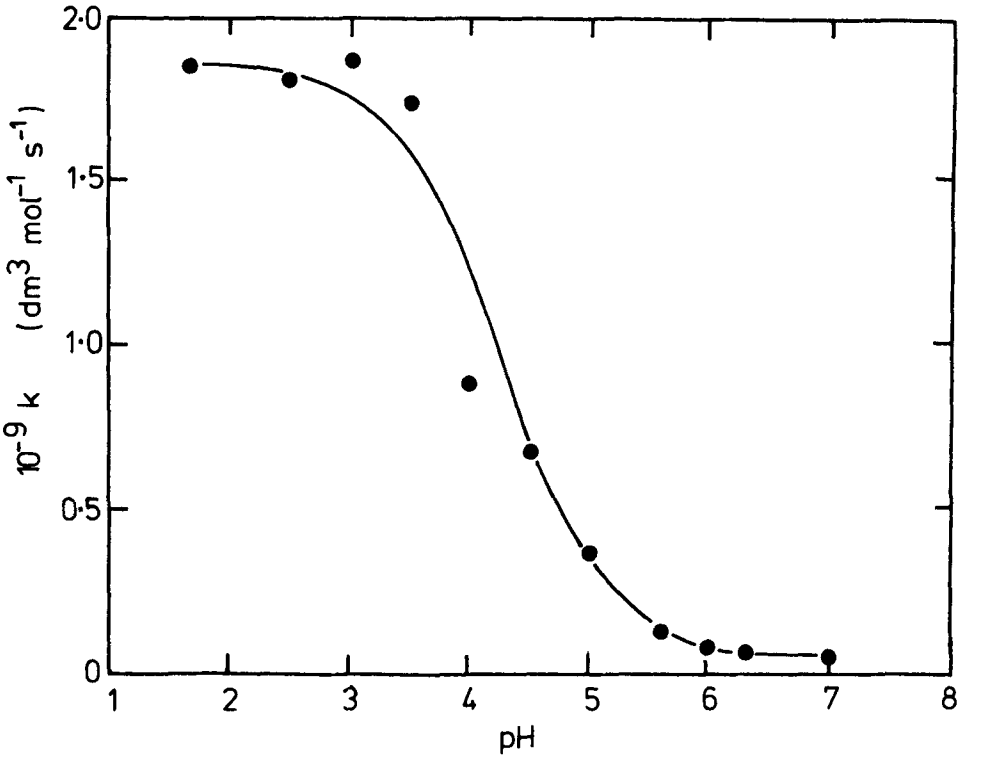

FIGURE 4 Effect of pH on the second order rate constant for repair of tryptophan radicals by Trolox C in aqueous solution.

$$F_i = \frac{(\bar{n})^i e^{-\bar{n}}}{i!} \tag{4}$$

If N is the aggregation number for the micellar assembly, then $\bar{n}$ is simply given by:-

$$\bar{n} = \frac{[\alpha\text{-tocopherol}] \times N}{[\text{detergent}] - \text{CMC}} \tag{5}$$

From Eq. (4), the fraction of micelles *not* containing a molecule of α-T (F_0) is $e^{-\bar{n}}$. If A and A_0 are the absorptions due to the STME radical after completion of the repair reaction in the presence and absence of α-T, then A/A_0 is the experimentally measured fraction of unoccupied (with respect to α-T) micelles. Hence:-

$$\bar{n} = -\ln(A/A_0) = \frac{[\alpha\text{-tocopherol}] \times N}{[\text{detergent}] - \text{CMC}} \tag{6}$$

Figure 5 shows a plot of $-\ln(A/A_0)$ versus α-T concentration. At the concentrations of detergent (2 mM), NaBr (0.4 M) and phosphate (0.1 M) in these solutions, the CMC value was determined as 0.126 mM. A value of N = 161 ± 19 molecules of TTAB per micelle is calculated from the initial slope (up to 20 μm α-T) in Figure 5. This is in good agreement with the aggregation number for TTAB determined by other methods and with the effect of high salt concentrations[33,34]. Curvature of the

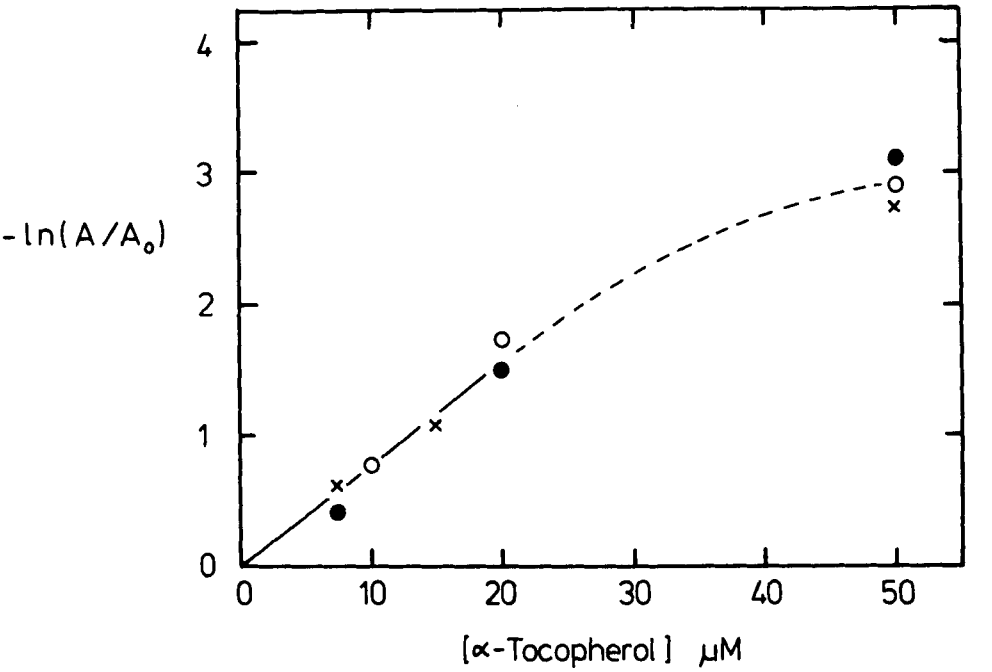

FIGURE 5 Data plotted according to Eq. (6) for the remaining STME radical absorption after repair by α-tocopherol in TTAB micelles. Conditions as in Figure 3. Different symbols represent data from separate experiments.

line in Figure 5 at high concentration of α-T is probably due to perturbation of the micellar structure by the phytyl chain of α-T. This perturbation is expected to be more pronounced than that induced by the unbranched alkyl chain of STME, which is present in these experiments at constant concentration. Changes in lipid bilayer structure and order induced by α-T have previously been observed by a number of techniques[35-37].

In summary, the results show that the micellar charge has a large influence on the rate constant for reaction of Br_2^- with both α-T and STME, whereas the reactivity of the neutral $N_3^\bullet$ radical is unaffected. Compared with the reactivity of related compounds in aqueous solution, the micellar location of both solutes reduces their reactivity with $N_3^\bullet$ by between 30 and 60%. Fluorescence quenching experiments confirm the exposure of the indole ring of STME to the aqueous phase. When both STME and α-T are confined within a single micelle, the rate of repair of the STME radical by α-T is significantly increased.

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