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Fluorescence study of the location and dynamics of α-tocopherol in phospholipid vesicles

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The intrinsic fluorescence of α-tocopherol has been used as a tool to study the location and dynamics of the molecule in phospholipid vesicles made of egg yolk phosphatidylcholine using steady-state and time-resolved techniques. From absorption spectra it was concluded that most α-tocopherol molecules are hydrogen bonded, although the aggregates formed are fluorescent. By calculating several fluorescence parameters in different solvents it was concluded that α-tocopherol should be situated in a polar region of the membrane. From the results obtained in measurements of fluorescence quenching and resonance energy transfer it was deduced that the chromanol moiety of the molecule is located in a position close to that occupied by the probes 7-(9-anthroyloxy)stearic acid (7-AS) and 5-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid (5-NS) in the membrane. The lateral diffusion coefficient of α-tocopherol in phospholipid vesicles was calculated through quenching of its fluorescence by the spin probe 5-NS, and a value of $4.8 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1} was found, indicating a very high lateral diffusion of α-tocopherol.

Introduction

α-Tocopherol is known to be a very important component of biological membranes. α-Tocopherol is believed to behave as a potent antioxidant to avoid membrane lipid peroxidation [1,2] and it was also suggested that it might stabilize biological membranes by restricting the molecular mobility of their components [3,4] or by forming complexes with potentially toxic unsaturated fatty acids [5,6]. In order to better understand the molecular mechanism of action of α-tocopherol, it is important to study its interaction with membrane components and specially with lipids. In this way the location and dynamics of α-tocopherol can be known.

From experiments using physical techniques like differential scanning calorimetry [7], fluorescence of probes [7,8], nuclear magnetic resonance [9–11] and Fourier transform-infrared spectroscopy [12] it has been concluded that α-tocopherol has its phenolic group located near the polar moiety of the lipid matrix.

From experiments using model membranes with different phospholipid composition it was concluded that α-tocopherol preferentially partition into the most fluid domain [13].

α-Tocopherol has intrinsic fluorescence and we have tried to exploit this property since this is a very convenient mean of directly observing the molecule. We present in this report our experiments on α-tocopherol location and dynamics in phospholipid vesicles by using steady- and transient-state fluorescence techniques.

Materials and Methods

Egg yolk phosphatidylcholine (EYPC), α-tocopherol, 5-doxylstearate and 16-doxylstearate were obtained from Sigma, Poole, Dorset, U.K. 2-, 7-, 9- and 12-(9-anthroyloxy)stearic acid were obtained from P-L Biochemicals, Gmbh, St. Goar, F.R.G. Organic solvents were obtained from Merck, Darmstadt, F.R.G. Twice distilled and deionized water was used.

Samples for absorption, steady-state excitation and emission spectra and transient-state determinations were made as follows: a film of phospholipid was obtained

Abbreviations: SUV, small unilamellar vesicles; MLV, multilamellar vesicles; EYPC, egg yolk phosphatidylcholine; *n*-NS, *n*-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid; *n*-AS, *n*-(9-anthroyloxy)stearic acid; Mops, 4-morpholinepropanesulfonic acid.

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from evaporation of a chloroform solution and multilamellar vesicles were formed by adding water and vortexing the suspension. These multilamellar vesicles were disrupted to small unilamellar vesicles (SUV) by sonication (Branson, 250). Usually six cycles of 30 s were performed, until obtaining no significant decrease in scatter intensity of the suspension. The vesicles were annealed during 10 min, to eliminate structural defects of the bilayer that would induce their fusion [14] and then low-speed centrifuged to separate titanium particles, arising from the sonication. To this SUV suspension appropriate amounts of α-tocopherol were added from a stock solution in ethanol, the incubation time being one hour in the dark at room temperature. For the low concentration of ethanol used, maximum 3%, no alteration of the bilayer structure is reported [15] and no change in scatter intensity of the sample was found.

Phospholipid vesicles for fluorescence intensity, quenching and resonance energy transfer experiments were prepared as follows: chloroform solutions containing phospholipid and appropriate amounts of α-tocopherol and fluorescent probes were mixed. The organic solvent was evaporated under a stream of nitrogen and the last traces of solvent were removed by a further 5 h evaporation under vacuum. After addition of 0.1 mM EDTA, 10 mM Mops, 100 mM NaCl (pH 7.4 buffer) multilamellar vesicles (MLV) were formed by mixing using a bench vibrator.

Absorption spectra were run on a Shimadzu UV260 or on a Perkin-Elmer Lambda 9 spectrophotometer.

Steady-state excitation and emission spectra were obtained on a Spex III Fluorog spectrofluorimeter. Correction of spectra was performed using a Rhodamine B quantum counter (excitation) and a standard lamp (emission). Excitation and emission bandwidths were 4.5 nm, and 5 × 5 mm fluorescence cells were used, in a right-angle geometry. Excitation and emission wavelengths were 295 and 325 nm, respectively. Fluorescence quantum yields were determined using naphthalene in degassed ethanol as a standard, $\phi_F = 0.21$ [16].

Fluorescence decays were measured using the time-correlated single-photon counting technique. The excitation source was a nitrogen-filled flash lamp (Edimburg Instruments, 199F). Alternated collection of pulse and sample profiles, detected with a Philips XP2020Q photomultiplier, were performed. The decay curves were deconvoluted on a Digital PDP 11/73 computer, employing the modified method of modulating functions [17].

Fluorescence intensity, quenching and resonance energy transfer measurements were carried out on a Shimadzu RF-540 spectrofluorophotometer using excitation and emission wavelengths of 295 and 325 nm, respectively.

In the energy transfer studies, the critical radius of transfer, R_0, was evaluated from Förster's formulation [18] rewritten as

$$R_0 = 0.2108\left(k^2 \phi_D n^{-4} \int_0^\infty I(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda\right)^{1/6}$$

with R_0 in Å, where k^2 is theorientational factor, δ_D the donor quantum yield in the absence of acceptor, n is the refractive index of the medium, $I(\lambda)$ is the normalised fluorescence spectrum, $\varepsilon(\lambda)$ is the molar absorption coefficient ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$) and λ is in nm.

In the above expression the orientational factor for the dynamic regime of transfer $k^2 = 2/3$ was considered [19], this assumption being reasonable considering the low anisotropy of the acceptors ($\equiv$ 0.126, 0.134 and 0.120 for the 2-, 6-, and 12-AS, respectively [20]).

The molar absorption coefficient of the acceptor is $\varepsilon_{365\ nm} = 6.2 \cdot 10^3$ $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ [21]. $\delta_D = 0.34$ (ethanol) was used and $n = 1.40$ [22].

Acceptor surface concentrations were calculated considering an area of 77 $Å^2$ for the phospholipid head group of egg yolk PC [23].

All the fluorescence measurements were carried out in a right-angle geometry, and the eventual errors arising in a steady-state energy transfer study were taken into account, namely: (i) absorption by the acceptor at the excitation wavelength: the consideration of this effect for the highest concentration of acceptor (anthroyloxy probes) implies a correction of 3% in the F/F_0 intensity. In the lower range of concentration, its importance is negligible, (ii) absorption of donor fluorescence by the acceptor (trivial effect): for the highest concentration of acceptor it amounts to a correction of 2.5%, also negligible.

The extent of collisional quenching in a lipid bilayer depends upon the lipid/water partition coefficient and upon the rate of diffusion of the colliding species in the lipid bilayer [24]. Therefore the collisional quenching of fluorescence can be used for calculating the diffusion coefficients for α-tocopherol in lipid vesicles. In a membrane, where quenching occurs only in the lipid phase and partition with the water phase may be significant, the Stern-Volmer relation for collisional quenching is modified, and the following applies:

$$1/k_{app} = \alpha_m(1/k_m - 1/k_m P) + 1/k_m P$$

where k_{app} is the apparent (measured) bimolecular quenching constant in $M^{-1} \cdot s^{-1}$, α_m is the fractional volume of the membrane phase, and P is the partition coefficient, in units of (moles of quencher per liter of phospholipid)/(moles of quencher per liter of water). The partition coefficient is also expressed as mole fractional ratio, i.e. (moles of quencher per mole of phospholipid)/(moles of quencher per mole of water).

A plot of $1/k_{app}$ as a function of α_m gives a straight line with $1/k_m P$ as intercept and $(1/k_m - 1/k_m P)$ as slope. In order to estimate the lateral diffusion coefficient of α-tocopherol in the membrane we have used the Smoluchowski equation as modified for fluorescence quenching in three dimensions measured in the steady state including transient effects [25]:

$$k_m = 4\pi N_A R_{pq} \lambda (D_p + D_q)\left(1 + R_{pq}\gamma/\sqrt{(D_p + D_q)\tau_0}\right)$$

where γ is the quenching efficiency of the fraction of collisional encounters that are effective in quenching, R_{pq} is the sum of the molecular radii of probe plus quencher, N_A is Avogadro's number per millimole, D_p and D_q are the diffusion coefficients of the probe and the quencher, respectively, in the membrane, in units of $cm^2 \cdot s^{-1}$ and τ_0 is the fluorescence lifetime of the probe in the absence of quencher.

From the van der Waals radii we estimate a molecular radius os 4.3 Å for α-tocopherol and 4.6 Å for 5-NS [26]. We have assumed γ to be 1, so that the calculated diffusion coefficient is the smallest possible and would be higher for $\gamma < 1$. See Fato et al. [27], for a detailed discussion on the problems that can be found when trying to estimate γ for this type of system. τ_0 was estimated to be 1.7 ns. Finally, we have assumed D_q to be $2.5 \cdot 10^{-7}\ cm^2 \cdot s^{-1}$ according to Fato et al. [27], who found this value for a similar fluid membrane.

Results

As references to α-tocopherol fluorescence properties are very scarce, and essentially oriented towards their application in analytical determinations, we have first determined some fluorescence parameters of α-tocopherol. Table I shows λ_{max} of the fluorescence emission spectra, quantum yield (ϕ_F) and fluorescence lifetime (τ_F), determined for α-tocopherol in a number of solvents and incorporated into phospholipid vesicles. It is shown that both quantum yield and fluorescence lifetime of α-tocopherol increase as solvents polarity increases. Phenol, a chromophore related to α-tocopherol, shows identical response of its fluorescence parameters to solvent polarity [16,28]. It is interesting to note that λ_{max} and fluorescence lifetime obtained for α-tocopherol in phospholipid vesicles are similar to those obtained in protic solvents (e.g., ethanol).

TABLE I

Fluorescence parameters of α-tocopherol in solution and incorporated into phospholipid vesicles

Medium	λ_{max} [a] (nm)	ϕ_F [b]	τ_F [c] (ns)
Methanol	316	0.43	n.d.
Ethanol	317	0.34	1.8
Acetonitrile	311	n.d.	1.0
Cyclohexane	312	0.16	0.8
EYPC (SUV) [d]	316	n.d.	1.7

[a] Emission fluorescence when excited at 295 nm, obtained from corrected spectra.

[b] Quantum yields determinated considering ϕ_F of naphthalene in degassed ethanol as 0.21[16].

[c] Fluorescence lifetimes determined by the single-photon counting technique.

[d] Small unilamellar vesicles of egg yolk phosphatidylcholine.

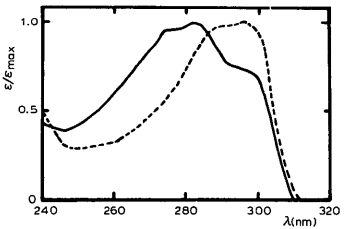

Fig. 1. Absorption spectra of α-tocopherol in *n*-hexane at a concentration of (———) $4.4 \cdot 10^{-5}$ M and (- - - - -) $1.42 \cdot 10^{-4}$ M.

The absorption spectra of α-tocopherol in *n*-hexane is depicted in Fig. 1. It is shown that the maximum of the spectrum at low concentration of α-tocopherol is at 283 nm (predominance of the monomeric form) but it is shifted to 295 nm at a much higher concentration (predominance of the hydrogen bonded form). Experiments made with α-tocopherol in phospholipid vesicles showed that α-tocopherol has a maximum near 295 nm within a wide range of concentration (data not shown), indicating that most α-tocopherol molecules are associated when present in membranes.

Fig. 2 shows the fluorescence intensity of α-tocopherol at increasing concentrations of the molecule. It can be seen that a linear relationship between fluorescence intensity and concentration is obtained for α-tocopherol incorporated in phospholipid vesicles. Identical behaviour was obtained in non-polar (hexane) and polar (ethanol) solvents (data not shown).

The quenching effect of membrane probes 5-doxylstearate (5-NS) and 16-doxylstearate (16-NS) on α-tocopherol intrinsic fluorescence is shown in Fig. 3. The quenching process seems to follow a collisional mechanism as shown by the linearity of the Stern-Volmer plots. It can be seen that 5-NS which has its nitroxide group at carbon-5 quenches α-tocopherol fluorescence much more effectively than 16-NS which has its nitroxide group at carbon-18.

Fig. 4 shows the absorption and emission spectra of α-tocopherol and the absorption spectrum of 6-AS in ethanol and cyclohexane, respectively. We found that

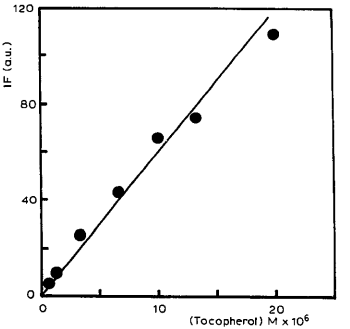

Fig. 2. Fluorescence intensity (in arbitrary units) of α-tocopherol corrected for the inner filter effect vs. concentration of α-tocopherol in multilamellar vesicles made of egg yolk phosphatidylcholine at 25 °C (lipid concentration $0.13 \cdot 10^{-3}$ M).

energy transfer from α-tocopherol (donor), to the fluorescent probes *n*-AS (acceptor), is moderately efficient with a Förster critical radius, $R_0 = 14$ Å (calculated as described under Materials and Methods). We also found an identical spectral overlap between α-tocopherol emission and the anthroyloxy chromophore absorption for all the anthroyloxy acceptor probes used, the same Förster critical radius (R_0) holding for all the donor/acceptor pairs; this implies that an immediate qualitative intercomparison of distances is possible, i.e.,

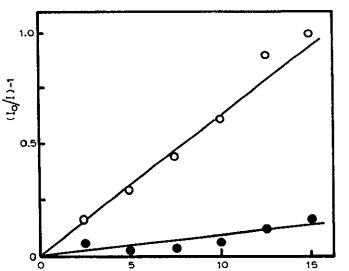

Fig. 3. Stern-Volmer plots of quenching of α-tocopherol fluorescence in egg yolk phosphatidylcholine (EYPC) at 25 °C by (○) 5-NS and (●) 16-NS. Lipid concentration was kept at 0.25 mM and EYPC/α-tocopherol molar ratio was 100:1.

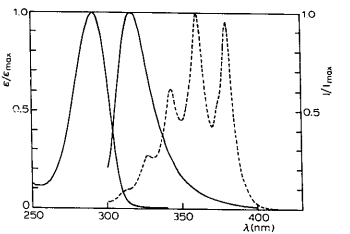

Fig. 4. Absorption and emission spectra of α-tocopherol in ethanol (———, $\varepsilon_{295\,nm} = 3.2 \cdot 10^3$ $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ ($\lambda_{exc} = 292$ nm)) and absorption spectrum of 6-AS in cyclohexane ((— — —), $\varepsilon_{365\,nm} = 6.2 \cdot 10^3$ $dm^3 \cdot mol^{-1} \cdot cm^{-1}$).

the transfer efficiencies (F/F_0) values for the same concentration of the acceptor in the series of probes directly reflects different distances between α-tocopherol and *n*-AS probes. Fig. 5 shows the efficiency of energy transfer from α-tocopherol to different *n*-AS probes differing in the location of their 9-anthroyloxy group. It can be seen that the efficiency of energy transfer follows the order 7-AS > 2-AS > 9-AS = 12-AS.

The calculation of the lateral diffusion coefficient of this molecule when incorporated into phospholipid vesicles can be approached through studies of the quenching of the intrinsic fluorescence of α-tocopherol

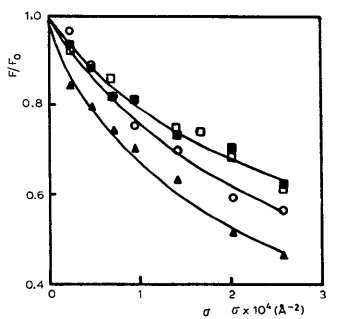

Fig. 5. Relative yield of α-tocopherol fluorescence F/F_0 vs. σ (acceptor surface concentration) of 2-AS (○), 7-AS (▲), 9-AS (■) and 12-AS (□) in egg yolk PC at 25 °C. Each point in the plot represents a different set of experiments. Excitation and emission wavelengths were 295 and 329 nm, respectively.

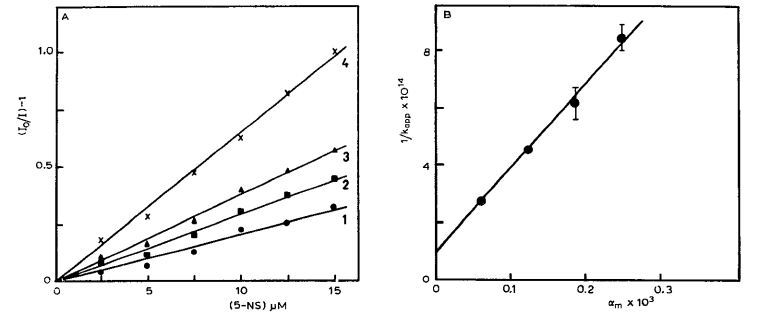

Fig. 6. (A) Stern-Volmer plots of quenching of α-tocopherol fluorescence by 5-NS at 25 °C in egg yolk PC vesicles at different membrane fractional volumes (α_m): $0.25 \cdot 10^{-3}$ (1); $0.1875 \cdot 10^{-3}$ (2); $0.125 \cdot 10^{-3}$ (3); $0.0625 \cdot 10^{-3}$ (4). The α-tocopherol to phospholipid ratio was kept 1:100 (mol/mol). (B) Plot of $1/k_{app}$ vs. membrane fractional volumes (α_m) using values from panel A. Each point represents the average value ± S.E. of three different experiments.

by 5-NS (Fig. 3) as described under Materials and Methods.

Fig. 6A shows Stern-Volmer plots for the quenching of α-tocopherol fluorescence, when incorporated into phospholipid vesicles, by 5-NS, at different lipid concentrations. From these plots apparent bimolecular quenching constants (k_{app}) values were calculated and they were used for the plot shown in Fig. 6B. From the last plot (Fig. 6B) the bimolecular quenching constant in the membrane phase was calculated to be $k_m = 3.4 \cdot 10^9\ M^{-1} \cdot s^{-1}$ and the partition coefficient of quencher was found to be $P = 3.2 \cdot 10^4$ (moles of 5-NS per mole of egg yolk PC)/(moles of 5-NS per mole of water). It is noteworthy that this value for the partition coefficient is very similar to that found by other authors for the same quencher in asophosphatidylcholine bilayers [27] which was $5.9 \cdot 10^4$ expressed in the same units.

In order to calculate the lateral diffusion coefficient of α-tocopherol in the membrane we used the Smoluchowski equation as described under Materials and Methods, and this lead to a lateral diffusion coefficient (D_p) of $4.8 \cdot 10^{-6}\ cm^2 \cdot s^{-1}$.

Discussion

In this study the location and dynamics of α-tocopherol in phospholipid vesicles was investigated by means of its intrinsic fluorescence.

We first determined some fluorescence parameters of α-tocopherol and interestingly noted that the parameters obtained for α-tocopherol in phospholipid vesicles are similar to those obtained in protic solvents. This observation suggests that the chromanol moiety of α-tocopherol should be located in the polar region of the model membrane, in agreement with previous suggestions [9,29].

An important point when considering the fluorescence of α-tocopherol in the membrane is to know whether all the molecules will be fluorescent or if non-fluorescent aggregates may be formed as claimed recently [30]. We found that most α-tocopherol molecules when present in phospholipid vesicles are associated. It could not be discerned from the data presented here whether α-tocopherol molecules are associated (through hydrogen bonding) between themselves or with phospholipid or water molecules. However we clearly show that although α-tocopherol molecules are associated, the aggregates formed are fluorescent, since a linear relationship dependence between fluorescence intensity and a large range of concentration is obtained both in homogeneous media and in membranes.

The location of α-tocopherol in the bilayer has been also approached through the quenching of its fluorescence by membrane probes 5-NS and 16-NS. These probes were used before in a number of similar studies designed to determine the location of chromophores in membranes [31]. We found that 5-NS quenches α-tocopherol fluorescence much more effectively than 16-NS, as should be expected if the chromanol moiety is located near the lipid/water interface.

We have also attempted to study the location of α-tocopherol in phospholipid vesicles by using a set of *n*-(9-anthroyloxy)stearic acid (*n*-AS) probes. The explicit distance dependence (r^{-6}) of electronic energy

transfer (dipolar mechanism) has allowed its application as a spectroscopic ruler for determining distances in biological systems [32, 33]. Two advantages arise from the use of the anthroyloxy family of probes in a systematic study of locations inside a membrane: (i) these probes are known to be located at a graded series of depths inside a membrane [31,34,35] so a precise mapping can be obtained and (ii) considering that the Förster critical radius (R_0) is identical for all acceptors a direct relationship between efficiency and distances between α-tocopherol and *n*-AS can be made. From the data presented here it can be concluded that the chromophore group of α-tocopherol is situated in the membrane in a region between the 9-anthroyloxy group located at carbon-7 and carbon-2, the former being the nearest one. This attribution is again compatible with other previous results. Furthermore, we have found that acrylamide, which is a water-soluble fluorescence quencher, has a very low efficiency of quenching α-tocopherol in phospholipid vesicles, while acrylamide is an efficient quencher of α-tocopherol in ethanolic solution (data not shown). This indicates that although α-tocopherol may have its chromanol group relatively close to the polar part of the bilayer it is not sufficiently exposed to allow acrylamide to reach it (acrylamide being known to have a very low capacity of penetration through phospholipid bilayers) [15]. Hence the conclusion of these studies on the location of α-tocopherol in phospholipid vesicles is that its chromanol moiety lies in a position close to that occupied by 7-AS and 5-NS. The location of the chromanol moiety in the lipid/water interface could be of importance in explaining its mechanism of protection since any oxidizing agent approaching the membrane surface should find reducing protons and hence its introduction in the membrane avoided.

In order to understand the mechanism of action of α-tocopherol in membranes and how it may be active at very low concentrations, it may be very illustrative to know the lateral diffusion coefficient of this molecule when incorporated into phospholipid vesicles. This has been approached through studies of the quenching of the α-tocopherol fluorescence by 5-NS. While Wardlaw et al. [36] have reported a static contribution in the fluorescence quenching of 2-(9-anthroyloxy)palmitic acid by 5-NS, in the present study this effect is supposed to be non-significant as: (i) linear Stern-Volmer steady-state quenching relationships were obtained (see Fig. 6A), (ii) for a quencher concentration (5-NS) of 10^{-5} M and α-tocopherol concentration of $5 \cdot 10^{-5}$ M in SUV of egg yolk PC (10^{-3} M), identical values of τ/τ_0 and ϕ/ϕ_0 were obtained. In this way, the quenching mechanism is essentially dynamic. By using the Smoluchowski equation we found a lateral diffusion coefficient (D_p) of $4.8 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}. This means that α-tocopherol may have high mobility in natural membranes and hence be quite efficient in reacting with oxidizing agents. This value is very similar to the one calculated by other authors [27] for ubiquinone-3 ($5.8 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}) which is a molecule very related in structure to α-tocopherol.

In conclusion two factors may compensate for the low concentration of α-tocopherol in membrane. These are the preferential partitioning in the most fluid domains [13] and a high lateral mobility in the plane of the bilayer.

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References

1 Scott, M.L. (1978) in The Fat-Soluble Vitamins (De Luca, H.F., ed.), pp. 133–210, Plenum Press, New York.
2 De Duve, C. and Hayaishi, O., eds. (1978) Tocopherol, Oxygen and Biomembranes, Elsevier/North-Holland Medical Press, Amsterdam.
3 Witting, L., (1972) Ann. N.Y. Acad. Sci. 203, 192–198.
4 Grams, G.W. and Skins, K. (1972) Biochemistry 11, 606–610.
5 Diplock, A.T. and Lucy, J.A. (1973) FEBS Lett. 29, 205–210.
6 Erin, A.N., Spisni, M.M. Tabidze, L.W. and Kagan, V.E. (1984) Biochim. Biophys. Acta 774, 96–102.
7 Massey, J.B. She, H.D. and Pownall, H.S. (1982) Biochem. Biophys. Res. Commun. 106, 842–847.
8 Schmidt, D., Steffen, H. and Von Planta, C. (1976) Biochim. Biophys. Acta 443, 1–9.
9 Srivastava, S., Phadke, R.S., Govil, G. and Rao, C.N.R. (1983) Biochim. Biophys. Acta 734, 353–362.
10 Perly, B., Smith, J.C.P., Hughest, L., Burton, G.W. and Ingold, K.V. (1985) Biochim. Biophys. Acta 819, 131–135.
11 Wassall, S., Thewalt, J.L., Wong, L., Gorrissen, H. and Cushley, R.J. (1986) Biochemistry 25, 319–326.
12 Villalain, J., Aranda, F.J. and Gómez-Fernández, J.C. (1986) Eur. J. Biochem. 158, 141–147.
13 Ortiz, A., Aranda, F.J. and Gómez-Fernández, J.C. (1987) Biochim. Biophys. Acta 989, 214–222.
14 Lawaczeck, R., Kainosho, M. and Chan, S.I. (1976) Biochim. Biophys. Acta 443, 313–330.
15 Chalpin, D.B. and Kleinfeld, A.M. (1983) Biochim. Biophys. Acta 731, 465–474.
16 Parker, C.A. (1968) in Photoluminiscence of Solutions, pp. 262–268, Elsevier, Amsterdam.
17 Striker, G. (1982) in Deconvolution and Reconvolution of Analytical Signals (Bouchy, M., ed.), pp. 329–354, University Press, Nancy.
18 Förster, Th. (1965) in Modern Quantum Chemistry, Part IIIB, (Sinanoglu, O., ed.), pp. 93–137, Academic Press, New York.
19 Berberan-Santos, M.N. and Prieto, M.J.E. (1988) J. Chem. Phys. 88, 6341–6349.
20 Chefurka, W., Chatelier, R.C. and Sawyer, W.H. (1987) Biochim. Biophys. Acta 896, 181–186.

21 Berberan-Santos, M.N. and Prieto, M.J.E. (1987) J. Chem. Soc. Faraday Trans. 2, 83, 1391–1409.
22 Badley, R.A. (1976) in Modern Fluorescence Spectroscopy (Wehry, E.L., ed.), Vol. 2, pp. 91–168, Plenum Press, New York.
23 Davenport, L., Dale, R.E., Bisby, R.H. and Cundall, R.B. (1985) Biochemistry 24, 4097–4108.
24 Lakowicz, J.R. and Hogen, D. (1980) Chem. Phys. Lipids 26, 1–40.
25 Umberger, J.Q. and Lamer, V.K. (1945) J. Am. Chem. Soc. 67, 1099–1109.
26 Edward, J.T. (1970) J. Chem. Educ. 47, 261–270.
27 Fato, R., Battino, M., Degli-Esposti, M., Parenti-Castelli, G. and Lenaz, G. (1986) Biochemistry 25, 3376–3390.
28 Berlman, J.B. (1965) Handbook of Fluorescence Spectra of Aromatic Molecules, pp. 61–62, Academic Press, New York.
29 Hitchcok, P.B., Mason, R. and Shipley, G.G. (1975) J. Mol. Biol. 94, 297–299.
30 Kagan, V.E. and Quinn, P.J. (1988) Eur. J. Biochem. 171, 661–667.
31 Blatt, E. and Sawyer, W.H. (1985) Biochim. Biophys. Acta 822, 43–62.
32 Stryer, L. (1978) Annu. Rev. Biochem. 47, 819–846.
33 Enng, B.K.K. and Stryer, L. (1978) Biochemistry 17, 5142–5248.
34 Cadenhead, D.A., Kellner, B.M.J., Jacobson, K. and Papahadjopoulos, K. (1977) Biochemistry 24, 5386–5392.
35 Blatt, E., Chatelier, R.C. and Sawyer, W.H. (1984) Photochem. Photobiol. 39, 477–483.
36 Wardlaw, J.R., Sawyer, W.H. and Ghiggino, K.P. (1987) FEBS Lett. 223, (1), 20–24.