Flavin-sensitized Photo-oxidation of Unsaturated Fatty Acids

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Photo-oxidation of unsaturated fatty acids or esters (LH = oleic acid, linoleic acid, methyl linoleate, and linolenic acid) by oxygen proceeds efficiently in the presence of a riboflavin derivative, riboflavin-2′,3′,4′,5′-tetra-acetate (Fl), in acetonitrile by a type II (singlet oxygen) mechanism with a limiting quantum yield ($\varphi = 0.65$) which corresponds to the quantum yield of the intersystem crossing of the flavin. The addition of $HClO_4$—by which Fl is protonated to FlH^+—showed a remarkable inhibitory effect on the Fl-sensitized photo-oxidation of LH, since FlH^+ is photo-reduced by LH to yield the dihydroflavin radical cation $FlH_2^{+\bullet}$ which has no ability to sensitize the energy transfer to oxygen in its ground state.

The photochemistry of flavin and its analogues has been the subject of intense research, since the excited states of flavins can oxidize a variety of substrates such as amino acids, amines, carboxylic acids, and organometallic compounds.[1–6] The reduced flavins, dihydroflavins, are well known to be readily oxidized by oxygen to regenerate the oxidized form.[7] Thus, flavins can act as photocatalysts in oxidations of various substrates by oxygen *via* the photo-reduction of flavins, a so-called type I mechanism.[8] On the other hand, flavins are also able to sensitize photo-oxidations of substrates by the singlet excited state of oxygen ($^1O_2^*$) formed by energy transfer from the triplet excited states of flavins to oxygen in its ground state (type II mechanism).[8] The singlet oxygen $^1O_2^*$ ($^1\Delta_g$) is well known to oxidize a variety of substrates.[8–10] Thus, one of the major points of interest in the flavin-sensitized photo-oxidations may be the balance between the type I and type II mechanisms in which the photo-reduction of flavins and the energy transfer from flavins to oxygen are involved, respectively. Of particular interest has been the flavin-sensitized photo-oxidation of unsaturated fatty acids and esters, in which a sensitive balance between the type I and type II mechanisms has been observed.[11,12] While the riboflavin-sensitized photo-oxidation of methyl linolenate and methyl oleate in an aqueous emulsion has been reported to proceed solely by a type I mechanism,[11] the partial contribution of a type II mechanism has recently been implicated in the riboflavin-sensitized photo-oxidation of phenyl linoleate in methanol.[12] In the latter case, however, the quantum yields are quite small ($\varphi = 10^{-2}$).[12] Thus, there has so far been no report on the efficient flavin-sensitized photo-oxidation of unsaturated fatty acids or esters proceeding solely by a type II (singlet oxygen) mechanism, although other dyes such as methylene blue, erythrosine, and haematoporphyrin are well known to act mainly as sensitizers, effecting the type II (singlet oxygen) photo-oxidation of unsaturated fatty acids and esters.[13–16]

In this study, we report that the photo-oxidation of unsaturated fatty acids or esters by singlet oxygen (type II mechanism) in the presence of a riboflavin-2′,3′,4′,5′-tetra-acetate (Fl) in acetonitrile (MeCN) proceeds efficiently with a limiting quantum yield ($\varphi_T = 0.65$) which agrees well with the quantum yield of the intersystem crossing for the flavin.[17] In addition, we report the accelerating and inhibitory effects of perchloric acid ($HClO_4$) on the photo-reduction of Fl in the absence of oxygen and on the Fl-sensitized photo-oxidation of unsaturated acids (or esters) by oxygen, respectively.

Experimental

Materials.—Riboflavin-2′,3′,4′,5′-tetra-acetate (Fl) was prepared by the reaction of riboflavin with acetic anhydride in pyridine, and purified by recrystallization from ethanol–chloroform.[18] The solubility of Fl in MeCN is much higher than that of riboflavin. Pivalic acid, isobutyric acid, valeric acid, and oleic acid were obtained commercially. Linoleic acid, methyl linoleate, and linolenic acid were purchased from the Nakarai Chem. Co., 99% grade, free from isomers. The concentrations of hydroperoxides in unsaturated fatty acids or esters before use were confirmed to be $< 1.0 \times 10^{-4}$ mol dm^{-3}. Perchloric acid (70%) and sodium iodide were obtained from Wako Pure Chemicals. Potassium ferrioxalate used as an actinometer was prepared according to the literature,[19] and purified by recrystallization from hot water. Acetonitrile used as a solvent was purified and dried by the standard procedure,[20] and stored over calcium hydride under an atmospheric pressure of nitrogen.

Reaction Procedure.—Typically, after an MeCN solution (2.0 cm^3) containing fatty acid (0.10 mol dm^{-3}) and Fl (1.5×10^{-4} mol dm^{-3}) in a square quartz cuvette had been saturated with oxygen, the mixture was irradiated with visible light from a Ushio Model V1-501C Xenon lamp through a Toshiba glass filter L-39 (transmitting light of $\lambda > 360$ nm). Fatty acids (0.2 mol dm^{-3}) are soluble in an aprotic solvent such as MeCN. The total amount of hydroperoxides formed in the photo-oxidation was determined by the standard method (titration by iodide ion);[5,21] the diluted MeCN solution (200–1 000-fold) of the product mixture was treated with excess amounts of NaI, and the amount of I_3^- formed was determined by the visible spectrum (λ_{max} 361 nm, ε 2.50×10^4 dm^3 mol^{-1} cm^{-1}). The concentration of conjugated diene hydroperoxides was determined from the absorption band at 233 nm. The extinction coefficient [$\varepsilon = (2.6 \pm 0.2) \times 10^4$ dm^3 mol^{-1} cm^{-1}],[22] has been reported to be constant, irrespective of various conjugated diene isomers. The electronic absorption spectra were measured using a Union SM-401 spectrophotometer.

The dihydroflavin radical cation ($FlH_2^{+\bullet}$) formed in the photochemical reaction of a protonated flavin FlH^+ with fatty acids in the presence of $HClO_4$ in deaerated MeCN was identified by the e.s.r. measurements using a JEOL-X band spectrometer (JES-ME-LX). The e.s.r. spectra were recorded under a non-saturating microwave power condition with a modulation amplitude 8.0×10^{-2} mT. The further reduction of modulation amplitude has not improved the hyperfine resolution. The *g*-value and the hyperfine coupling constants

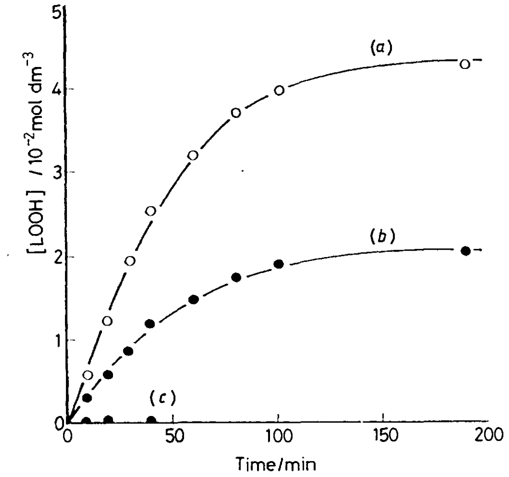


Figure 1. The concentrations of (*a*) total hydroperoxides and (*b*) conjugated diene hydroperoxides formed in the Fl-sensitized photo-oxidation of lineoleic acid (0.10 mol dm^{-3}) in the presence of Fl (9.5×10^{-4} mol dm^{-3}), in an oxygen-saturated MeCN solution under irradiation of light $\lambda > 360$ nm, plotted against the irradiation time, and (*c*) the inhibitory effect of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) on the photo-oxidation.

(h.f.c.) of the e.s.r. spectra were calibrated by using an Mn^{2+} e.s.r. marker. The simulation of the e.s.r. spectrum was performed using a NEC 9801 VM_2 microcomputer.

Quantum-yield Determinations.—A standard actinometer (potassium ferrioxalate)[19] was used for the quantum yield determinations. The actinometry experiments were carried out under the same conditions as those for the photochemical reactions of Fl with fatty acids, both in the absence and presence of oxygen, in MeCN using monochromatized light. The intensities of monochromatized light of λ 442 and 390 nm, used for the photochemical reactions of Fl and FlH^+ with fatty acids in the absence and presence of $HClO_4$ in MeCN, were determined to be 3.9×10^{-6} and 5.0×10^{-6} einstein dm^{-3} s^{-1}, respectively. The quantum yields for the Fl-sensitized photo-oxidation of unsaturated fatty acids were determined from the rates of formation of total hydroperoxides or conjugated diene hydroperoxides by comparison with the incident light intensity absorbed by Fl. Similarly, the quantum yields for the photochemical reactions of Fl and FlH^+ with fatty acids in the absence and presence of $HClO_4$ were determined from the disappearance of the absorption band due to Fl ($\lambda_{max.}$ 442 nm, ε 1.24×10^4 dm^3 mol^{-1} cm^{-1}) and the rise of the absorption due to $FlH_2^{+\bullet}$ (λ_{max} 504 nm, $\varepsilon = 9.40 \times 10^3$ dm^3 mol^{-1} cm^{-1})[6], respectively.

Fluorescence Quenching Experiments.—Fluorescence measurements were carried out on a Hitachi 650-10S fluorescence spectrophotometer. In the quenching experiments, the excitation wavelengths were selected to be 442 and 390 nm, which correspond to the absorption maxima of Fl and FlH^+, respectively. Relative emission intensities at emission maxima (λ_{max} 507 and 490 nm for Fl and FlH^+, respectively) were measured for MeCN solutions of Fl and FlH^+ with a quencher at various concentrations, respectively. No change in the shape of the emission spectrum (only the intensity) was observed on addition of a quencher. The Stern–Volmer relationship [equation (1)] was obtained between the ratio of the emission

$$I_0/I = 1 + K_q[Q] \qquad (1)$$

intensities in the absence and presence of a quencher (I_0/I) and the quencher concentration [Q]. The fluorescence lifetime of $^1FlH^{+*}$ (τ_f) in the presence of $HClO_4$ in MeCN was evaluated to be 1.4 ns with the maximum K_q value, which becomes constant with increasing donor ability in a given series of quenchers ($X\text{-}C_6H_4CH_2OH$; X = *p*-MeO, *p*-Pr^i, *p*-Me, H, *p*-Cl), when the quenching rate constant ($k_q = K_q\tau_f^{-1}$) is assumed to be equal to the diffusion rate constant; 2.0×10^{10} dm^3 mol^{-1} s^{-1}.[4]

Results and Discussion

Fl-*Sensitized Photo-oxidation of Unsaturated Fatty Acids by Oxygen.*—Irradiation of an oxygen-saturated MeCN solution containing Fl (9.5×10^{-4} mol dm^{-3}) and linoleic acid (0.10 mol dm^{-3}) with visible light of $\lambda > 360$ nm resulted in the formation of the hydroperoxides of linoleic acid. The time course for formation of total hydroperoxides and conjugated diene hydroperoxides, determined from iodometry and the absorption band at 233 nm, is shown in Figure 1 [parts (*a*) and (*b*), respectively], where the yields of the hydroperoxides are about twice as large as those of the conjugated diene hydroperoxides. The molar ratio total hydroperoxide:Fl reaches 45 after 190 min of irradiation, after which the photo-decomposition of Fl was observed and thus no further formation of hydroperoxides occurs (Figure 1). Such photo-degradation of flavin analogues due to the intramolecular oxidation of the ribityl side chain of the flavin analogues has been well known for many years.[23] It was confirmed that no photo-oxidation of linoleic acid occurred in the absence of Fl under otherwise identical experimental conditions.

A remarkable inhibitory effect of $HClO_4$ on the Fl-sensitized photo-oxidation is observed as shown in Figure 1(*c*). Flavin analogues are known to be protonated at the N(1) position in a strongly acidic aqueous solution ($pK_a = 0$).[24] In MeCN, the protonation of Fl [equation (2)] occurs much more readily than

$$Fl + H^+ \rightleftharpoons FlH^+ \qquad (2)$$

that in H_2O,[25] and the absorption band due to Fl (λ_{max} 442 nm) in the absence of $HClO_4$ was blue-shifted in the presence of a small excess of $HClO_4$ (λ_{max} 390 nm). Under the experimental conditions in Figure 1(*c*), the absorption band due to FlH^+ (λ_{max} 390 nm) disappeared, accompanied by the rise of a new absorption band at 504 nm, when no hydroperoxides are formed. The identification of the new absorption band as well as the reason for the inhibitory effect of $HClO_4$ on the photo-oxidation of linoleic acid will be discussed later.

The quantum yields for the formation of the total hydroperoxides (φ) and conjugated diene hydroperoxides (φ_{conj}) in the Fl-sensitized photo-oxidation of various fatty acids or esters in the absence of $HClO_4$ in MeCN were determined from their initial rates of formation using monochromatized light of λ 442 nm, and the values at 0.10 mol dm^{-3} fatty acids are listed in Table 1. While no hydroperoxides are produced from fatty acids containing no double bond (isobutyric acid, valeric acid, and pivalic acid), the Fl-sensitized photo-oxidation of unsaturated fatty acids or esters (oleic acid, linoleic acid, methyl linoleate, and linolenic acid) proceeds efficiently. The φ values of unsaturated fatty acids or esters containing a 1,4-diene unit, *i.e.*, linoleic acid, methyl linoleate, and linolenic acid, are larger than the corresponding φ_{conj} values, indicating that both conjugated and non-conjugated hydroperoxides are formed in the Fl-sensitized photo-oxidation. The ratios of conjugated and non-conjugated hydroperoxides are determined from the values of $\varphi_{conj}/(\varphi - \varphi_{conj})$; the ratios are 0, 1.1, 1.1, and 2.1 for oleic acid, linoleic acid, methyl linoleate, and linolenic acid, respectively (Table 1). Such product ratios of hydroperoxides

Table 1. Quantum yields for the formation of conjugated diene hydroperoxides (φ_{conj}) and total hydroperoxides (φ) in the Fl-sensitized photo-oxidation of fatty acids or esters (LH 0.10 mol dm^{-3}) in the presence of Fl (1.5×10^{-4} mol dm^{-3}) in an oxygen-saturated MeCN solution under irradiation of light of λ 442 nm.

LH	φ_{conj}[a]	φ[b]	$\varphi_{conj}/(\varphi - \varphi_{conj})$[c]
Isobutyric acid	*d*	*d*	
Valeric acid	*d*	*d*	
Pivalic acid	*d*	*d*	
Oleic acid	*d*	0.21	0
Linoleic acid	0.16	0.30	1.1
Methyl linoleate	0.16	0.30	1.1
Linolenic acid	0.30	0.44	2.1

[a] Determined from the absorbance at λ 233 nm. The experimental errors are within ±10%. [b] Determined by iodometry. The experimental errors are within ±5%. [c] The value corresponds to the ratio of conjugated and non-conjugated hydroperoxides formed in the reaction. [d] No reaction.

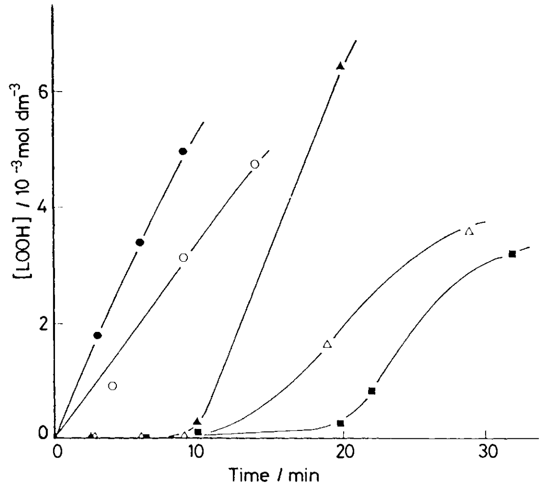
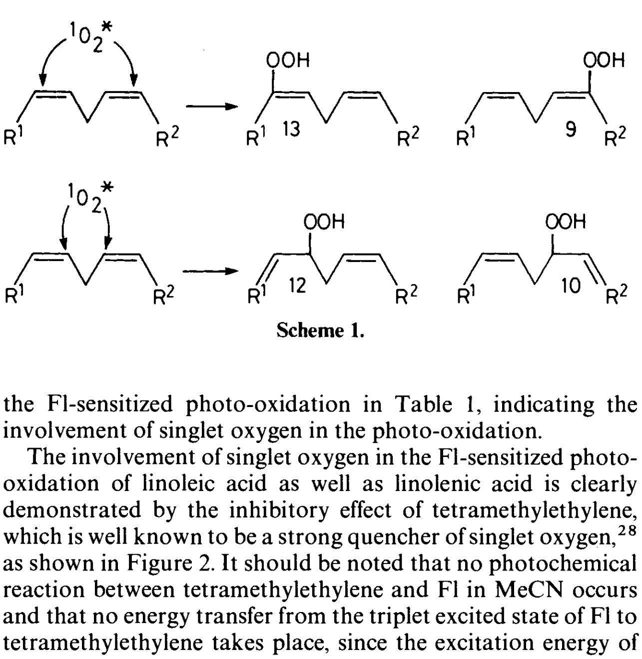


Figure 2. Inhibition of the Fl-sensitized photo-oxidation of linoleic acid and linolenic acid (0.10 mol dm^{-3}) in the presence of Fl (1.5×10^{-4} mol dm^{-3}) in an oxygen-saturated MeCN solution under irradiation of light of λ > 360 nm; no inhibitor for linoleic acid (○) and linolenic acid (●); tetramethylethylene 4.1×10^{-2} mol dm^{-3} for linoleic acid (△) and linolenic acid (▲); 8.1×10^{-2} mol dm^{-3} for linoleic acid (■).

are well known to be characteristic of the oxidation of unsaturated fatty acids and esters by singlet oxygen.[14] In contrast, the free-radical oxidation of unsaturated fatty acids and esters (linoleic acid, methyl linoleate, and linoleic acid) by triplet oxygen is known to produce only the corresponding conjugated hydroperoxides.[26,27] Since the position of the hydroperoxide group in the oxidation by singlet oxygen is the carbon atom located at both sides of the double bond, the number of isomers produced is twice that of the number of double bonds in fatty acids and esters. The double bond shifts to the adjacent position and thereby both conjugated and non-conjugated isomers may be formed for unsaturated fatty acids and esters containing 1,4-diene units, as shown in Scheme 1 for linoleic acid or methyl linoleate. When the attack of singlet oxygen on the double bonds is assumed to be random, the ratios of conjugated and non-conjugated hydroperoxides formed from oleic acid, linoleic acid (or methyl linoleate), and linolenic acid should be 0 (non-conjugated 9,10-isomers), 1 (conjugated 9,13-isomers and non-conjugated 10,12-isomers), and 2 (conjugated 9,12,13,16-isomers and non-conjugated 10,15-isomers), respectively. These product ratios expected from the oxidation by singlet oxygen agree well with those observed in the Fl-sensitized photo-oxidation in Table 1, indicating the involvement of singlet oxygen in the photo-oxidation.



Scheme 1.

The involvement of singlet oxygen in the Fl-sensitized photo-oxidation of linoleic acid as well as linolenic acid is clearly demonstrated by the inhibitory effect of tetramethylethylene, which is well known to be a strong quencher of singlet oxygen,[28] as shown in Figure 2. It should be noted that no photochemical reaction between tetramethylethylene and Fl in MeCN occurs and that no energy transfer from the triplet excited state of Fl to tetramethylethylene takes place, since the excitation energy of tetramethylethylene is much higher than that of Fl. However, there remains the possibility of quenching of the Fl excited state with tetramethylethylene by other pathways. The inhibition of the photo-oxidation by β-carotene, which is known to quench the singlet oxygen with a diffusion-controlled rate, was therefore also examined.[29,30] The quantum yield φ of oleic acid (0.10 mol dm^{-3}) in the presence of β-carotene (2.0×10^{-4} mol dm^{-3}) was reduced to $<10^{-2}$, and the φ values of linoleic acid, methyl linoleate, and linoleic acid (0.10 mol dm^{-3}) were reduced to *ca.* a fifth of the original volumes.

Kinetics and Mechanism of Fl-*Sensitized Photo-oxidation.*—Based on the above results, a type II (singlet) mechanism may be applied to the Fl-sensitized photo-oxidation of unsaturated fatty acids or esters (LH), as shown in equations (3)–(6), where τ

$$\mathrm{Fl} \xrightarrow{h\nu} {}^1\mathrm{Fl}^* \longrightarrow {}^3\mathrm{Fl}^* \; (\varphi_T) \tag{3}$$

$${}^3\mathrm{Fl}^* + {}^3\mathrm{O}_2({}^3\Sigma_g^-) \longrightarrow {}^1\mathrm{O}_2^*({}^1\Delta_g) + \mathrm{Fl} \tag{4}$$

$${}^1\mathrm{O}_2^*({}^1\Delta_g) \xrightarrow{\tau^{-1}} {}^3\mathrm{O}_2({}^3\Sigma_g^-) \tag{5}$$

$${}^1\mathrm{O}_2^* + \mathrm{LH} \xrightarrow{k_R} \mathrm{LOOH} \tag{6}$$

is the lifetime of ${}^1O_2^*({}^1\Delta_g)$, 30 μs in MeCN.[31] With regard to the ene reaction [equation (6)], some multi-step mechanisms have been proposed, although a concerted transformation with simultaneous C–O bond formation and allylic C–H bond breaking is widely accepted.[10] Irrespective of the mechanisms, k_R in equation (6) corresponds to the rate constant for the rate-determining step of the ene reaction between ${}^1O_2^*$ and LH. According to equations (3)–(6), the quantum yield φ for the formation of total hydroperoxides (LOOH) may be given by equation (7), which can be rewritten as equation (8).

$$\varphi = \varphi_T k_R \tau[\mathrm{LH}]/(1 + k_R\tau[\mathrm{LH}]) \tag{7}$$

$$\varphi^{-1} = \varphi_T^{-1}\{1 + (k_R\tau[\mathrm{LH}])^{-1}\} \tag{8}$$

The quantum yields φ for the formation of total hydroperoxides in the Fl-sensitized photo-oxidation of oleic acid, linoleic acid, methyl linoleate, and linolenic acid (LH) were determined at various LH concentrations. The observed quantum yields are much larger than those reported for the

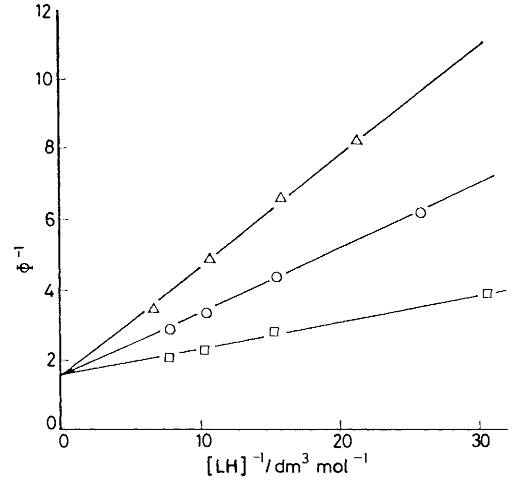


Figure 3. Plots of φ^{-1} *vs.* $[LH]^{-1}$ for the Fl-sensitized photo-oxidation of oleic acid (△), linoleic acid (○), and linolenic acid (□) in the presence of Fl (1.5×10^{-4} mol dm^{-3}) in an oxygen-saturated MeCN solution under irradiation of light of λ 442 nm.

Table 2. Limiting quantum yields (φ_T) and the rate constants (k_R) for the reactions of singlet oxygen with unsaturated fatty acids and esters (LH) obtained from the Fl-sensitized photo-oxidation of LH in an oxygen-saturated MeCN solution at 298 K.

LH	φ_T[a]	k_R[b]/ dm^3 mol^{-1} s^{-1}
Oleic acid	0.65	1.6×10^5
Linoleic acid	0.65	2.8×10^5
Linolenic acid	0.65	6.4×10^5
Tetramethylethylene		4.0×10^7[c]

[a] The experimental error ±0.02. [b] The experimental errors are within ±10%. [c] Ref. 31.

Table 3. Quenching constants (K_q) for the fluorescence quenching of FlH^+ (2.0×10^{-5} mol dm^{-3}) by fatty acids and esters (LH) in the presence of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) in deaerated MeCN at 298 K.

LH	K_q[a,b]/dm^3 mol^{-1}
Isobutyric acid	5.0
Valeric acid	4.7
Pivalic acid	1.8
Oleic acid	15
Linoleic acid	19
Methyl linoleate	14
Linolenic acid	25

[a] The experimental errors are within ±5%. [b] No quenching of $^1Fl^*$ by each LH has been observed in the absence of $HClO_4$ in deaerated MeCN ($K_q \ll 1$).

riboflavin-sensitized oxidation of phenyl oleate and the methyl and phenyl esters of linoleic acid in methanol ($\varphi = 10^{-2}$), which proceed *via* both type I and type II mechanisms.[12] The validity of equation (8) is confirmed by the plots of φ^{-1} *vs.* $[LH]^{-1}$ which gave linear correlations as shown in Figure 3. The intercept on each plot gives the same value ($\varphi_T = 0.65 \pm 0.2$), which agrees well with the reported quantum yield for the intersystem crossing of riboflavin ($\varphi_{isc} = 0.67$).[17] Thus, it can be concluded that the Fl-sensitized photo-oxidation of LH proceeds solely by a type II mechanism [equations (3)–(6)]. The rate constants k_R for the reaction of $^1O_2^*$ with LH are obtained from the slopes and intercepts of the linear plots in Figure 3 and the lifetime of $^1O_2^*$ (τ 30 μs),[31] and the k_R values are listed in Table 2. The k_R value increases with an increase in the number of double bonds in LH; oleic acid < linoleic acid < linolenic acid. The reported k_R value (4.0×10^7 dm^3 mol^{-1} s^{-1})[31] of tetramethylethylene is much larger than those of linoleic acid (2.8×10^5 dm^3 mol^{-1} s^{-1}) and linolenic acid (6.4×10^5 dm^3 mol^{-1} s^{-1}) (Table 2). This may be the reason why tetramethylethylene inhibits the Fl-sensitized photo-oxidation of linoleic acid and linolenic acid so efficiently (Figure 2).

We next examined the photo-reduction of Fl and FlH^+ by fatty acids, in both the absence and presence of $HClO_4$ in deaerated MeCN, respectively, in order to investigate the reason for the absence of a contribution from the type I mechanism (through the photoreduction of Fl by LH) as well as the reason for the dramatic inhibitory effect of $HClO_4$ on the Fl-sensitized photo-oxidation of LH [Figure 1(*c*)].

Photo-reduction of FlH^+ *by Fatty Acids.*—The fluorescence of flavin analogues is known to be quenched by electron-transfer reactions with various electron donors in MeCN.[3,5,32] However, no fluorescence quenching of $^1Fl^*$ in deaerated MeCN has been observed by LH (see footnote in Table 3), indicating that the donor ability of LH is not strong enough to transfer an electron to $^1Fl^*$. This may be the reason why no contribution due to the type I mechanism has been observed in the Fl-sensitized photo-oxidation of LH in which $^3Fl^*$, formed by intersystem crossing from $^1Fl^*$, is quenched exclusively by oxygen to yield singlet oxygen that can oxidize LH.

On the other hand, the electron-acceptor ability of Fl is known to be enhanced by protonation in the presence of $HClO_4$ in MeCN [equation (2)].[7,25] Thus, the fluorescence of the protonated flavin $^1FlH^+$ (λ_{max} 490 nm) in the presence of $HClO_4$ in MeCN was quenched by electron transfer from LH to $^1FlH^{+*}$. The quenching constants K_q of $^1FlH^{+*}$ by various fatty acids are listed in Table 3. The maximum quenching rate constant ($k_q = K_q\tau_f^{-1} = 1.8 \times 10^{10}$ dm^3 mol^{-1} s^{-1}) obtained from the K_q value of linolenic acid (Table 3) and the fluorescence lifetime of $^1FlH^{+*}$ (τ_f 1.4 ns, see Experimental) is about the same as the diffusion rate constant in MeCN (2.0×10^{10} dm^3 mol^{-1} s^{-1}).

Irradiation of a deaerated MeCN solution containing FlH^+ (1.2×10^{-4} mol dm^{-3}), linoleic acid (8.1×10^{-3} mol dm^{-3}), and $HClO_4$ (1.2×10^{-2} mol dm^{-3}) with visible light of λ 390 nm results in the disappearance of the absorption band due to FlH^+ (λ_{max} 390 nm), accompanied by the appearance of a new absorption band at λ_{max} 504 nm, which is the same as that observed in the reaction in oxygen-saturated MeCN, as shown in Figure 4. The new absorption band at λ_{max} 504 nm is about the same as that due to the dihydroriboflavin radical cation (λ_{max} 500 nm).[33] In order to confirm this assignment, an e.s.r. analysis of the product was carried out and the e.s.r. spectrum is shown in Figure 5, together with a computer simulation spectrum with the best-fit e.s.r. parameters, the hyperfine coupling constant (h.f.c.), $a_5(N) = 0.630$, $a_5(H) = 1.411$, $a_8(CH_3) = 0.305$, $a_{10}(N) = 0.477$, and $a_{10}(CH_2) = 0.477$ mT, and the maximum slope width, $\Delta H_{msl} = 0.080$ mT. The simulation spectrum agrees well with the observed spectrum, except for some line intensities, probably because of the neglect of the small h.f.c. due to other protons. The large h.f.c. value due to N(5)H and no appreciable h.f.c. due to C(6)H and C(9)H are characteristic of a dihydroflavin radical cation.[25,24] The e.s.r. parameters show that the product is the dihydroflavin radical cation $FlH_2^{+\bullet}$. The formation of $FlH_2^{+\bullet}$ was observed in the photo-reduction of FlH^+ by other fatty acids in the presence of $HClO_4$ in deaerated MeCN. The quantum yields φ_R for the formation of $FlH_2^{+\bullet}$ in the photo-reduction of FlH^+ by various fatty acids (0.10 mol dm^{-3}) in the presence of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) in deaerated MeCN were determined using monochromatized light of λ 390 nm, and the φ_R values are listed in Table 4.

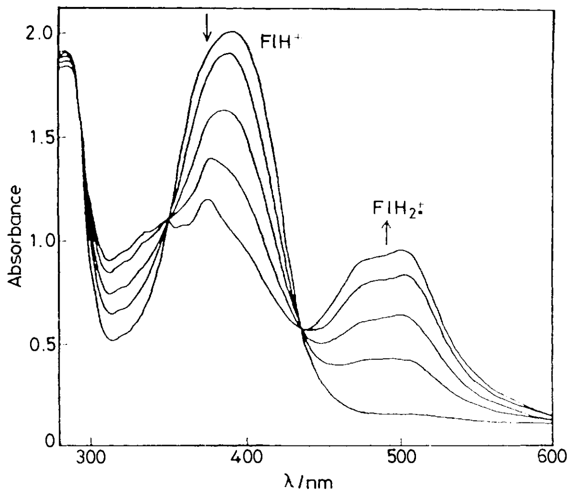
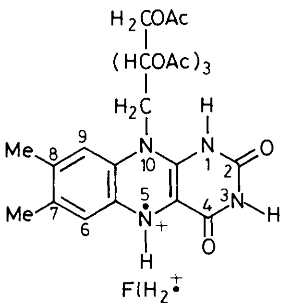


Figure 4. Electronic absorption spectra observed in the photochemical reaction of FlH^+ (1.2×10^{-4} mol dm^{-3}) with linoleic acid (8.1×10^{-3} mol dm^{-3}) in the presence of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) in deaerated MeCN. The spectra were recorded after each 30 s irradiation of light of λ 390 nm.



	h.f.c./mT
$a_5(N)$	0.630
$a_5(H)$	1.411
$a_8(CH_3)$	0.305
$a_{10}(N)$	0.477
$a_{10}(CH_2)$	0.477
ΔH_{msl}	0.080

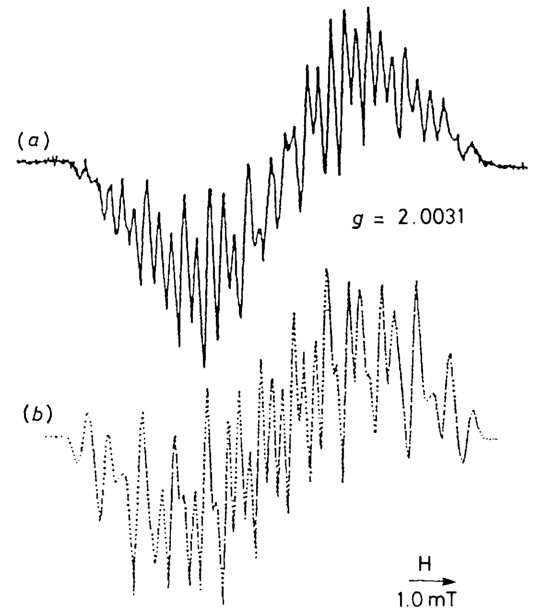


Figure 5. (a) E.s.r. spectrum of $FlH_2^{+\bullet}$ formed in the photo-reduction of FlH^+ by linoleic acid in deaerated MeCN. (b) The dotted line shows the corresponding computer simulation spectrum using the e.s.r. parameters listed above.

Table 4. Quantum yields (φ_R) for the photo-reduction of Fl (1.5×10^{-4} mol dm^{-3}) and FlH^+ (1.2×10^{-4} mol dm^{-3}) by fatty acids and esters (0.10 mol dm^{-3}), in the absence and presence of $HClO_4$ (1.20×10^{-2} mol dm^{-3}) in deaerated MeCN, under irradiation of light of λ 442 and 390 nm, respectively.

	φ_R[a]	
LH	Fl	FlH^+
Isobutyric acid	*b*	3.4×10^{-2}
Valeric acid	*b*	1.7×10^{-2}
Pivalic acid	*b*	1.3×10^{-1}
Oleic acid	2.7×10^{-2}	8.1×10^{-2}
Linoleic acid	4.6×10^{-2}	2.2×10^{-1}
Methyl linoleate	4.6×10^{-2}	2.2×10^{-1}
Linolenic acid	1.8×10^{-2}	1.0×10^{-1}

[a] The experimental errors are within ±5%. [b] $<10^{-3}$.

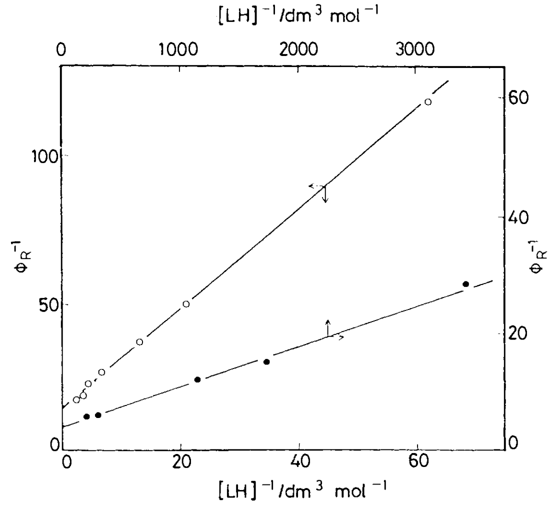


Figure 6. Plots of φ_R^{-1} *vs.* $[LH]^{-1}$ for the photo-reduction of FlH^+ (1.5×10^{-4} mol dm^{-3}) by linoleic acid (LH) in the absence (○) and presence (●) of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) in deaerated MeCN under irradiation of light of λ 442 and 390 nm, respectively.

In the absence of $HClO_4$, photo-reduction of Fl by fatty acids in deaerated MeCN occurs, as demonstrated by the disappearance of the absorption band due to Fl (λ_{max} 442 nm) under irradiation of light of λ 442 nm. The quantum yields φ_R for the photo-reduction of Fl by various fatty acids (0.10 mol dm^{-3}) in the absence of $HClO_4$ in deaerated MeCN were determined using monochromatized light of λ 442 nm, and these values are also listed in Table 4. The φ_R values in the presence of $HClO_4$ are significantly larger than those in the absence of $HClO_4$ (Table 4), in agreement with the enhancement of the electron acceptor ability of FlH^+ compared with that of Fl.[6,32] The φ_R values for the decay of Fl and the formation of $FlH_2^{+\bullet}$ in the photo-reduction of FlH^+ by linoleic acid (LH), in both the absence and presence of $HClO_4$ (1.2×10^{-2} mol dm^{-3}) in deaerated MeCN, were determined at various LH concentrations, respectively. The plots of φ_R^{-1} *vs.* $[LH]^{-1}$ gave a linear correlation as shown in Figure 6; this is in agreement with equation (9), in which φ_∞ is the limiting quantum yield and

$$\varphi_R^{-1} = \varphi_\infty^{-1}\{1 + (K_{obs}[LH])^{-1}\} \qquad (9)$$

K_{obs} is the observed quenching constant of Fl* or FlH^{+*}. From the slopes and intercepts are obtained the K_{obs} values of Fl* and FlH^{+*} by LH as 8.6 dm^3 mol^{-1} and 5.3×10^2 dm^3 mol^{-1}, respectively. The K_{obs} values of Fl* and FlH^{+*} are much larger than the quenching constant K_q of ^{1}Fl* ($\ll 1$ dm^3 mol^{-1}) and ^{1}FlH^{+*} (19 dm^3 mol^{-1}) in Table 3. Thus, the photo-reduction of Fl by fatty acids in the absence and presence of HClO$_4$ in deaerated MeCN may proceed by the reaction of the triplet excited states ^{3}Fl* and ^{3}FlH^{+*}, respectively. In the presence of oxygen, which can quench ^{3}Fl* efficiently, no photo-reduction of Fl can occur, and thereby Fl can act solely as a sensitizer to produce singlet oxygen [equations (3)–(6)]. On the other hand, in the presence of HClO$_4$, the singlet excited state ^{1}FlH^{+*} can be quenched by LH (Table 3), and thus the photo-reduction of FlH$^+$ by LH to yield FlH$_2^{+\bullet}$ can occur by the reaction of ^{1}FlH^{+*} with LH even in oxygen-saturated MeCN. In this case, HClO$_4$ can act as a strong inhibitor on the Fl-sensitized photo-oxidation of LH, since FlH$_2^{+\bullet}$ is stable to dioxygen,[6] and has no ability to sensitize the energy transfer to oxygen in the triplet ground state ($^3\Sigma_g^-$).

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Received 17*th March* 1989, *Paper* 9/01160I