

PHOTOCHEMICAL PRODUCTION OF SEMIQUINONE RADICALS OF *o*-METHYLFLUORESC EIN METHYL ESTER IN ETHANOL

SHIGEYA NIIZUMA^a, KOICHI KIKUCHI^b and HIROSHI KOKUBUN^b

^aCollege of Humanities and Social Sciences, Iwate University, Ueda, Morioka 020 (Japan)

^bDepartment of Chemistry, Faculty of Science, Tohoku University, Aoba, Aramaki, Sendai 980 (Japan)

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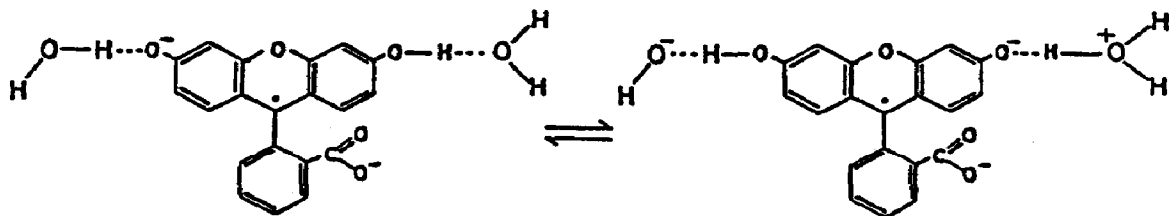
Summary

The mechanism of the formation and the decay of radicals photochemically produced from *o*-methylfluorescein methyl ester (MF) was investigated by continuous light and flash photolysis. Two kinds of radicals were found. One is attributed to the semiquinone radical (MFH·) of MF and the other to the anion radical (MF⁻). The p*K*_a value for MFH· was determined to be 10.5 in ethanol-water (1:1 by volume) at 25 °C. Electron spin resonance and electron nuclear double resonance spectra of MFH· were measured in neutral ethanol and a comparison of the hyperfine splitting constants for MFH· with those for the fluorescein dianion radical (FH⁻²) supports the previously proposed mechanism of the rapid proton exchange between the solvent (water) and FH⁻².

1. Introduction

In basic aqueous solutions containing a reducing agent or in alcohol, fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1(3*H*),9'-[9*H*]-xanthen]-3-one) is reduced on illumination with visible light, yielding a long-lived intermediate (semiquinone) [1 - 3]. This radical has several dissociative protons and its p*K*_a is 9.5 for the monoanion radical and above 13 for the dianion radical [1]. The kinetic behaviour [2, 4] and the electron spin resonance (ESR) signals [5, 6] of these anions have already been examined and discussed in detail.

Previously [6] it has been proposed from an analysis of ESR spectra that fluorescein dianion radical in water exchanges —OH protons rapidly with a solvent molecule:



In this paper it is reported that *o*-methylfluorescein methyl ester (or dimethylfluorescein) (MF), in which two dissociative protons of fluorescein are replaced by methyl groups, is converted into its leuco form via the corresponding semiquinone by photoreduction in ethanol. The mechanism of the photoreduction is established on the basis of the results obtained by continuous light and flash photolysis. The unpaired spin distribution of the semiquinone is obtained from ESR and electron nuclear double resonance (ENDOR) spectra. These results are compared with those for fluorescein.

2. Experimental details

MF was prepared by methylation of fluorescein (Guaranteed Reagent grade, Hikotaro Shudzui & Co.) in methanol with diazomethane in ether and was purified by thin-layer chromatography (using SiO_2 and $\text{CH}_3\text{COOC}_2\text{H}_5$ as the solvent). Dimethylation was confirmed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry. *p*-Phenylenediamine (Extra Pure grade, Tokyo Kasei) was recrystallized twice from ethanol and used immediately after sublimation. Ethanol (Guaranteed Reagent grade, Wakojunyaku) was used as received. Tetrahydrofuran (THF) (Guaranteed Reagent grade, Wakojunyaku) was distilled after refluxing on sodium wire for several hours and was dried on Na-K alloy in vacuum.

$(2 - 5) \times 10^{-5}$ M degassed ethanol solution was employed for photo-reaction and $(1 - 2) \times 10^{-3}$ M for ESR or ENDOR experiments.

The light source for the experiments with continuous light was a Toshiba high pressure mercury lamp (SHL-100UV) with a 464 nm interference filter. The source for ESR and ENDOR measurements was a Ushio superhigh pressure mercury lamp (USH-500D) with a combination of a Toshiba VY-43 cut-off glass filter and a water cell 1 cm thick.

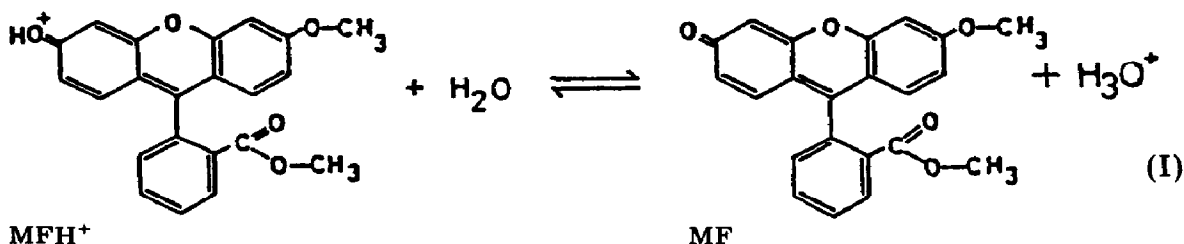
Absorption spectra were recorded on a Hitachi EPS-3T spectrophotometer. Ordinary flash photolysis apparatus (120 J; full width at half-maximum, 10 μs) was employed.

ESR and ENDOR spectra were measured with a JEOL P-10 ESR spectrometer (X-band and 100 kHz field modulation) and a JEOL ES-EDX1 ENDOR spectrometer respectively. The NEC PC-9801 microcomputer and the Hitachi HITAC-8250 Electronic Computer in Iwate University were used for the simulation of ESR spectra and for the calculation of the unpaired spin density respectively.

3. Results and discussion

3.1. The photoreaction in neutral ethanol solution

The absorption spectrum of the cationic form (MFH^+) is shown in Fig. 1(a) [1]. The $\text{p}K_a$ of MFH^+ was determined spectrophotometrically to be 2.6₅ in a water-ethanol (1:1 by volume) mixed solution. This value is close to the $\text{p}K_a$ of the cationic form of fluorescein (Table 1). In neutral ethanol, little protonation is considered to occur on the basis of the absorption spectrum.



When a deaerated solution was illuminated, the absorption band of MF ($\lambda_{\text{max}} = 458 \text{ nm}$) decreased in intensity with an isosbestic point at 383 nm, accompanied by an increase in intensity of a new structureless band with a maximum at 368 nm which was assigned to that of MFH^\cdot , the semireduced form of MF [1, 4, 10 - 13]. In the dark, the absorption of MF recovered at the expense of this new absorption (Fig. 1(b)). The final degree of recovery amounted to half the quantity of MF photochemically consumed. During this recovery, an isosbestic point appeared at 392 nm instead of

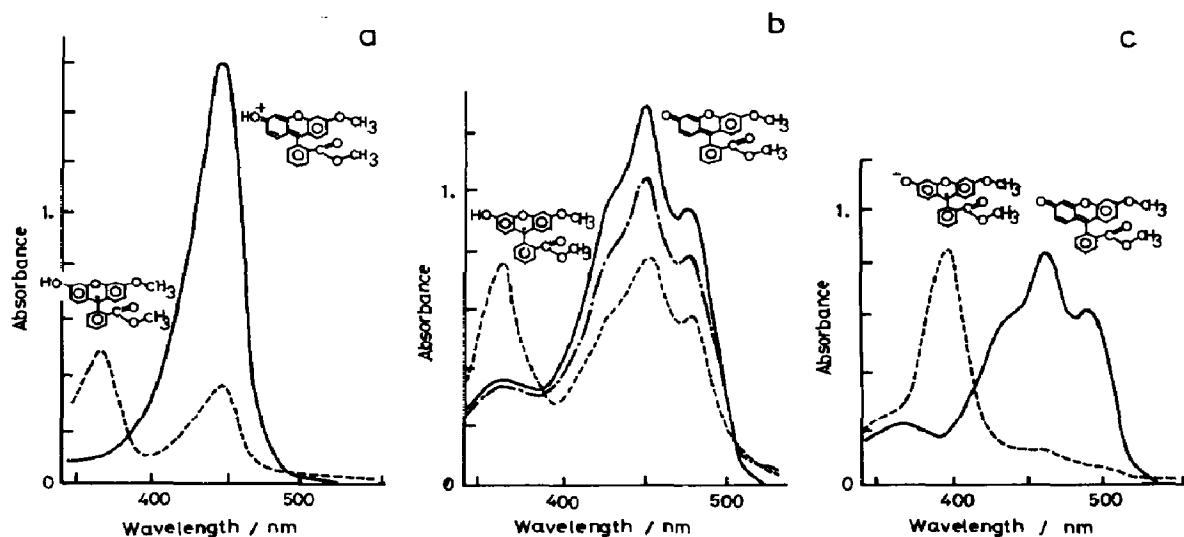
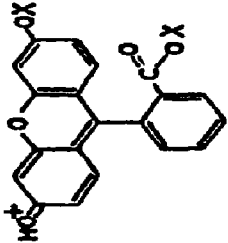
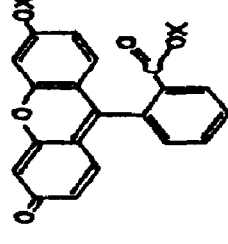
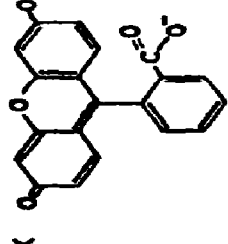
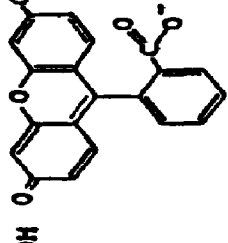
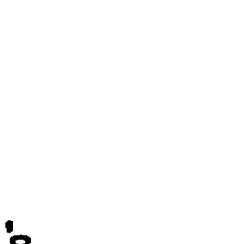


Fig. 1. Absorption spectra of samples. (a) In water-ethanol (1:1) mixture containing 0.02 M H_2SO_4 before (—) and after 100 min (---) illumination. (b) In neat ethanol solution before (—) and after 10 min (---) illumination and after leaving for 22 h in the dark after illumination (---). (c) In ethanol solution saturated with K_2CO_3 before (—) and after 90 min (---) illumination.

TABLE 1

Assignment of the absorption bands (in nanometres) and pK_a values^a: molecules

Solvent					
	Cation	Neutral	Monoanion	Dianion	
Fluorescein ($X \equiv H$)					
Water	437 ^b (2.2) ^b (2.27) ^c	437 ^b , 475 ^b (4.4) ^b (4.32) ^c	455 ^b , 475 ^b (6.7) ^b (6.50) ^c	491 ^b	
Methanol	442	456 ^d , 482 ^d (5.60) ^e	(6.86) ^e	499 ^d , 498	
Methanol-water (1:1)					
MF ($X \equiv CH_3$)					
Ethanol	444	458, 487	—	—	
Acetonitrile	438	453 ^f , 455	—	—	
Ethanol-water (1:1)	(2.6 ₅)				

^aGiven in parentheses.^bFrom ref. 1.^cFrom ref. 7.^dFrom ref. 8.^eFrom ref. 9.^fFrom refs. 4 and 10.

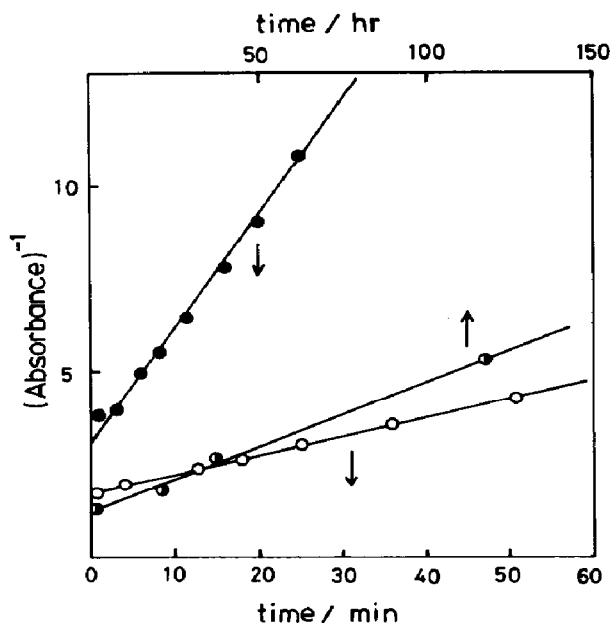


Fig. 2. Plot of the second-order decay of semiquinones in ethanol plus 0.02 M H_2SO_4 (\bullet), in neat ethanol (\circ) and in ethanol saturated with K_2CO_3 (\odot). The time scale is pointed by an arrow.

383 nm. The decay of $\text{MFH}\cdot$ was followed photometrically at 368 nm and a plot of the second-order decay had excellent linearity, as shown in Fig. 2. The following scheme was inferred from these observations:

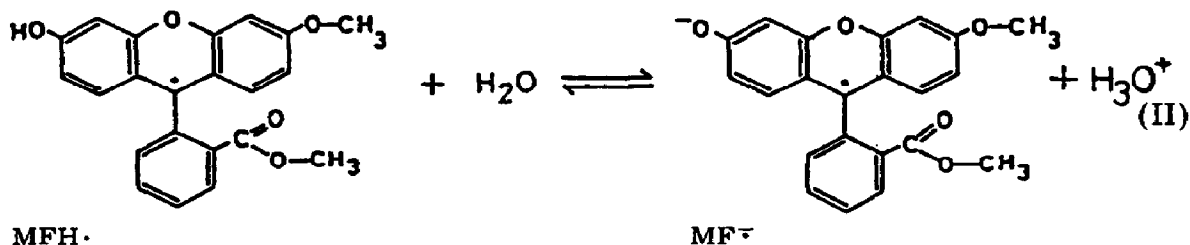


where MF^* is the photoexcited state of MF, RH is ethanol, $\text{R}\cdot$ is the radical formed by abstraction of a hydrogen atom from ethanol and MFH_2 is the leuco form of MF. We confirmed the photochemical formation of MFH_2 by comparing the UV spectrum of the photoproduct with that of leuco MF produced chemically by sodium-amalgam reduction of MF in ethanol. Using the molar extinction coefficients of MF, $\epsilon^{\text{MF}}_{458} = 27\,230 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon^{\text{MF}}_{368} = 7100 \text{ M}^{-1} \text{ cm}^{-1}$, and the stoichiometric relation between $\text{MFH}\cdot$ and MF in process (2), we obtained $31\,060 \text{ M}^{-1} \text{ cm}^{-1}$ as the molar extinction coefficient $\epsilon^{\text{MFH}\cdot}_{368}$ of $\text{MFH}\cdot$, and then $13.5 \text{ M}^{-1} \text{ s}^{-1}$ as the rate constant k_2 of second-order reaction (2). k_2 is comparable with the rate constant of $10 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction of fluorescein semiquinone $\text{FH}\cdot^{2-}$ [5]. In the same way, the apparent second-order rate constant for the corresponding reaction of MF^- is estimated to be $0.169 \text{ M}^{-1} \text{ s}^{-1}$ using $\epsilon^{\text{MF}^-}_{395} = 34\,790 \text{ M}^{-1} \text{ cm}^{-1}$.

3.2. Photoreaction in acidic and basic ethanol solutions

The absorption spectra observed after illumination of a water-ethanol 1:1 mixture containing 0.02 M H_2SO_4 and of a basic ethanol solution

saturated with K_2CO_3 are shown by the broken lines in Figs. 1(a) and 1(c) respectively. The absorption maximum appeared at 370 nm for the basic solution and at 395 nm for the acidic solution. The spectrum of the acidic solution was converted to that of the basic solution by the addition of excess alkali, and vice versa. Therefore the following equilibrium is considered.



The pK_a value of $\text{MFH}\cdot$ was determined to be 10.5 at 25 °C in the water-ethanol (1:1) mixed solution. Accordingly, almost all the semiquinone is in the $\text{MFH}\cdot$ form in the neutral solution. The structures of the molecules and the radicals are depicted in Fig. 1.

Semiquinones in these systems decay with second-order kinetics. Figure 2 shows that the decay in the basic solution is slower than that in the acidic solution. This behaviour is the same as in the case of fluorescein semiquinones [2, 4, 5]. As MF^- has no hydrogen atom to be transferred in the disproportionation process, its decay seems to occur via the trace of $\text{MFH}\cdot$ produced by equilibrium (II). To explain the higher rate constant in acid solution, an intermediate species of the disproportionation reaction such as the electron donor-acceptor complex $\text{MFH}^+\cdots\text{MFH}^-$ might be postulated. The disproportionation reaction would then be promoted remarkably by protons in solution, because the MFH^- portion of the complex is considered to be easily attacked by a proton to give MFH_2 .

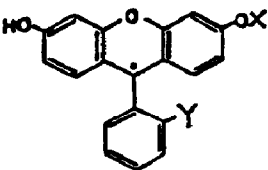
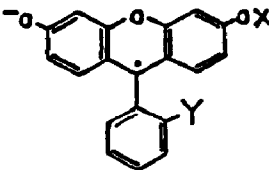
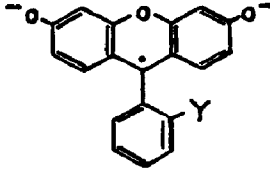
We must comment on the assignment of the absorption bands of fluorescein semiquinone in Table 2. Krüger and Memming have reported that the bands at 355 nm and 394 nm are those of $\text{FH}\cdot^{2-}$ and $\text{F}\cdot^{3-}$ respectively [4, 10, 14]. By comparison with our results regarding the band assignment of dimethylated fluorescein semiquinone, however, they must be assigned to $\text{FH}_2\cdot^-$ and $\text{FH}\cdot^-$ respectively. Our assertion also supports the assignment by Lindquist [1].

3.3. Investigation of elementary processes by the flash technique

The transient absorption shown in Fig. 3 (curve a) was detected by flash photolysis in both the neutral and the basic solutions. This was assigned to the T-T absorption of MF by the triplet energy transfer to anthracene. The spectrum was similar to that of the fluorescein triplet state [1, 4]. A trace of semiquinone ($\text{MFH}\cdot$) was produced in the course of the decay of the triplet state. The decay of T-T absorption at 600 nm and the build-up of the absorption due to $\text{MFH}\cdot$ at 370 nm were simultaneously followed by means of a multichannel oscilloscope and detection system

TABLE 2

Assignment of the absorption bands (in nanometres) and pK_a values^a: radicals

Solvent			
	$FH_2\cdot^-$	$FH\cdot^{2-}$	$F\cdot^{3-}$
<i>Fluorescein</i> ($X \equiv H$, $Y \equiv COO^-$)			
Water	355 ^{b,c} (9.5) ^b	394 ^{b,c,d} (> 13) ^b	—
	$MFH\cdot$	$MF\cdot^-$	—
<i>MF</i> ($X \equiv CH_3$, $Y \equiv COOCH_3$)			
Ethanol	368	395	—
Acetonitrile	363 ^c , 364	408	—
Ethanol-water (1:1)	(10.5)	—	—

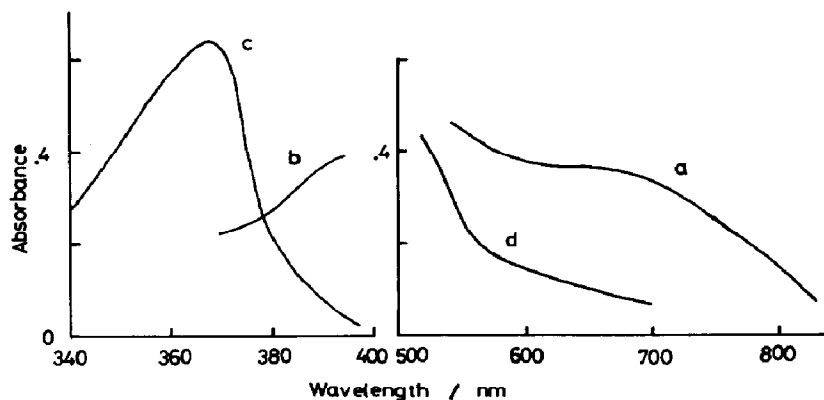
^aGiven in parentheses.^bFrom ref. 1.^cFrom refs. 4 and 10.^dFrom ref. 5.

Fig. 3. Transient absorption spectra. Curve a, T-T absorption spectrum in neutral and basic ethanol; curve b, spectrum observed at the end of a flash for the basic ethanol solution containing 10^{-4} M PDA; curves c and d, spectrum observed at $200 \mu s$ after flashing for both basic and neutral ethanol solutions containing 10^{-4} M PDA.

(Fig. 4(a)). The rates of decay and build-up are the same so the triplet state is a reactive state. The rate constant of triplet decay was $6.8 \times 10^3 \text{ s}^{-1}$ in both neutral and basic solutions.

On the addition of 10^{-4} M *p*-phenylenediamine (PDA), as an electron donor, to the basic solution, the T-T absorption disappeared and a new

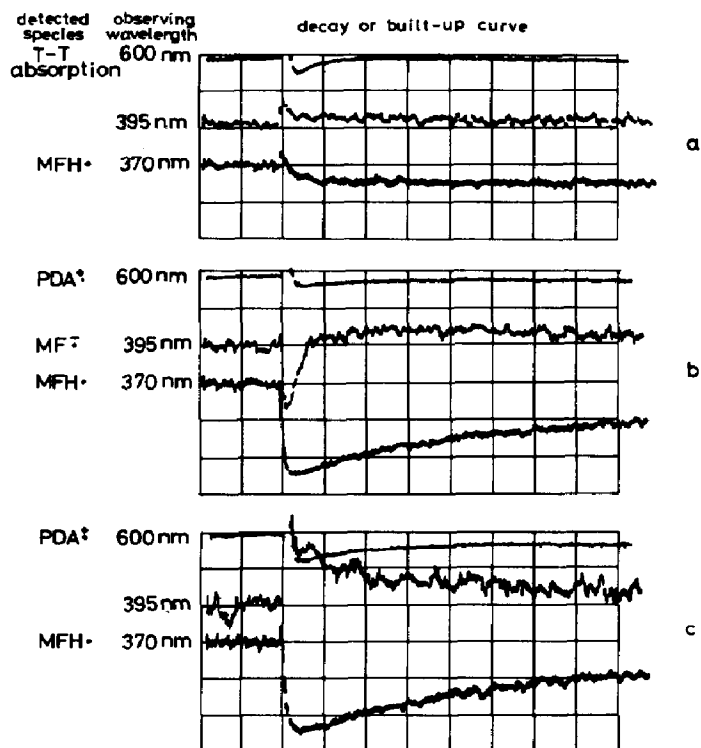
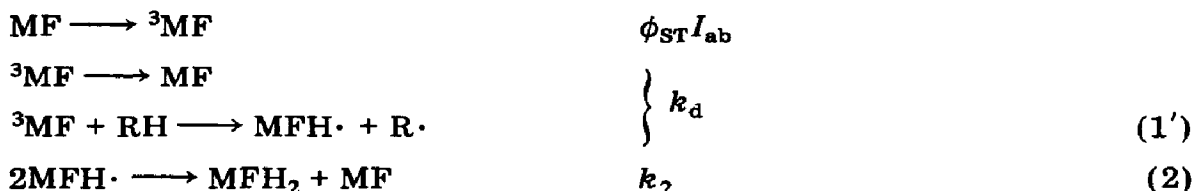


Fig. 4. Oscillograms of decay or build-up of transient species. (a) In the neutral and the basic ethanol. (b) In the basic ethanol containing 10^{-4} M PDA. (c) In the neutral ethanol containing 10^{-4} M PDA. The time scale is $100 \mu\text{s}$ per division for (a) and $200 \mu\text{s}$ per division for (b) and (c).

band which seems to be the absorption of MF^- appeared at the end of the flash (curve b in Fig. 3). At $200 \mu\text{s}$ after flashing the ethanol solution containing 10^{-4} M PDA, the transient spectra shown by curves c and d in Fig. 3 were observed. Spectrum c is similar to that of MFH^\bullet and spectrum d is similar to that of the PDA cation radical [15]. Figure 4(b) indicates the time dependence of the population of these three species that were observed simultaneously at three different wavelengths. From this figure, it is seen that the decrease in MF^- is accompanied by an increase in MFH^\bullet . The time dependence of the absorption at 600 nm is similar to that at 370 nm. In the case of flash photolysis of the neutral solution containing 10^{-4} M PDA, transient spectra similar to those shown in curves c and d of Fig. 3 were observed, but the absorption due to MF^- was not detected at all (Fig. 4(c)). It is thought that the protonation of MF^- occurs rapidly in neutral ethanol.

The above results can be summarized as follows. In neutral ethanol



where ϕ_{ST} and I_{ab} are the rate of intersystem crossing and the rate of photoabsorption respectively. 3MF is the triplet excited state of MF. It has already been evaluated that $k_d = 6.8 \times 10^3 \text{ s}^{-1}$ and $k_2 = 13.5 \text{ M}^{-1} \text{ s}^{-1}$. In basic solution, process (1) may be followed by process (3):



At concentrations of PDA above 10^{-4} M , the photoreduction of MF occurs predominantly through process (4) instead of process (1):



In neutral solution, $MF^{\cdot-}$ may rapidly be protonated by the inverse process of (3) because of the large pK_a value for $MF^{\cdot-}$; $MF^{\cdot-}$ cannot then be detected by the flash method.

3.4. Electronic structure of the semiquinones revealed from ESR and ENDOR spectra

The ESR spectra of the radicals photochemically produced from MF in acidic, neutral and basic ethanol and in THF were measured. All the spectra had triplet lines with a coupling constant of about 0.33 mT. In acidic ethanol, no further resolution was attained. In the other solvent systems, each line of the triplet was split into 8 - 10 lines with a more narrow field modulation, as shown in Figs. 5(a) and 5(b). A similar spectrum was detected for one-electron reduction by contact with metallic sodium in THF (Fig. 5(c)). These facts suggest that the difference in the unpaired spin distribution between structures I and II is so small that it might be difficult to distinguish between them. In a basic environment, $MF^{\cdot-}$ may be stabilized by taking the form of III. This may minimize the difference.

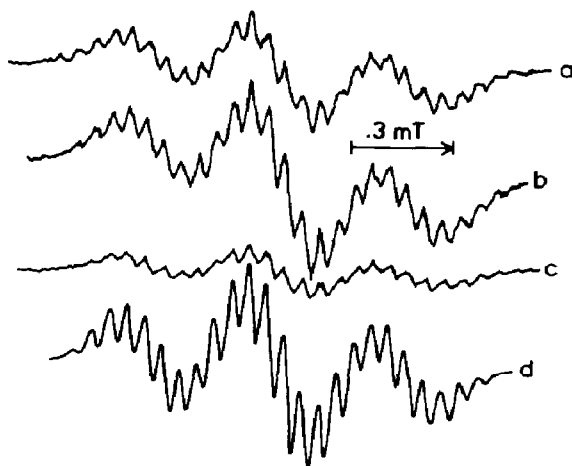
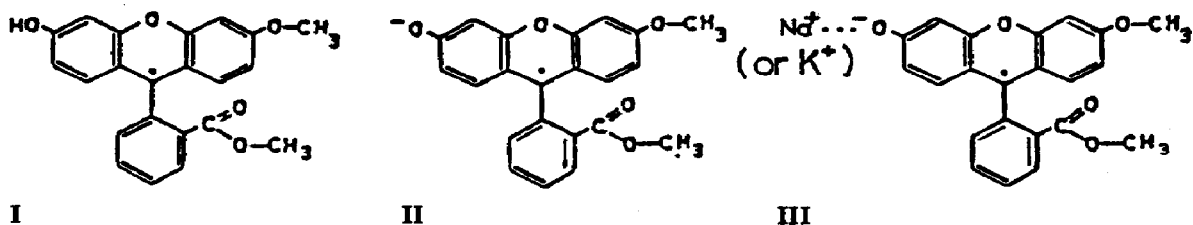


Fig. 5. ESR spectra of semiquinones. (a) Photochemically produced in ethanol. (b) Photochemically produced in THF. (c) Produced by contact with sodium metal in THF. (d) Simulated spectrum.



As the radical has no magnetically equivalent proton and as the resolution of the ESR spectrum is poor, it is hardly possible to analyse the hyperfine splitting by simulation methods. Accordingly, ENDOR measurements were attempted at -50°C for the neutral solution, which had been illuminated at room temperature in advance. Figure 6 shows the ENDOR spectrum. In spite of the high noise level, we can recognize the signals numbered from 1 to 6. The broad line appearing at 18.5 - 18.6 MHz may be assumed to consist of two lines; these were numbered as 5 and 6. ν_{H} in Fig. 6 is the position of the free proton. As the lines marked 4 and 4' are rather broad, we again assumed that they consist of two lines separated by 0.045 MHz. The hyperfine splitting constants determined on the basis of the ENDOR data are listed in the first column of Table 3. The ESR spectrum simulated by using the coupling constants $a_{\text{H}}^{\text{obs}}$ and the number of protons listed in the second column of Table 3 agreed well with the observed data (Fig. 5(d)). The coupling constants $a_{\text{H}}^{\text{calc}}$ calculated by McLachlan's method based on the simple linear combination of atomic orbitals molecular orbital (LCAO MO) method are listed in the third column. The coincidence with observation is quite good.

It is clear that the two protons attached to the carbons at the 1- and the 8-positions are responsible for the largest coupling constant of 0.334 mT and for the next largest value of 0.326 mT. Such a difference between the coupling constants for the 1- and 8-positions, which is impossible in the case of fluorescein semiquinone [6], is caused by the introduction of

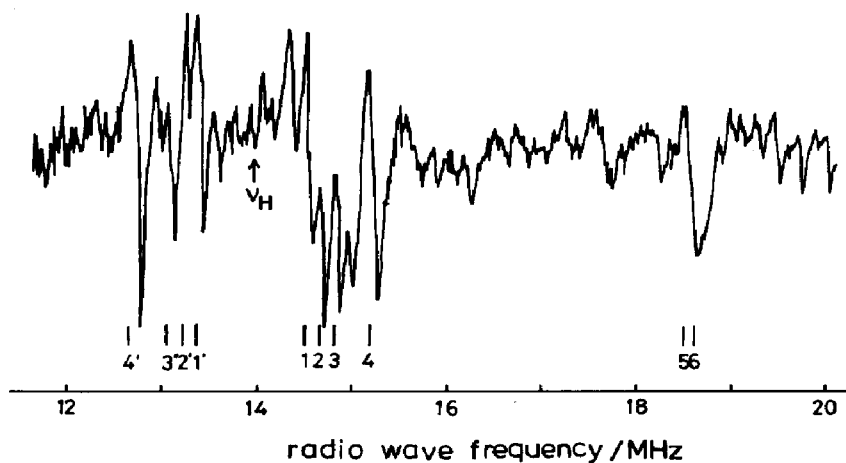
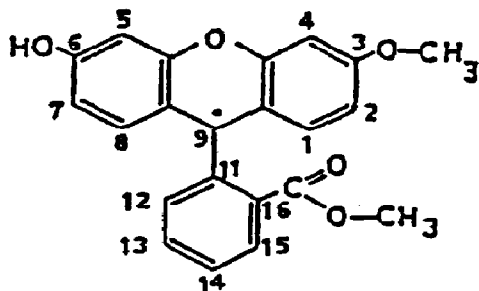


Fig. 6. ENDOR spectrum of MFH· in the neutral ethanol at -50°C ($\nu_{\text{H}} = 13.91$ MHz).

TABLE 3
Hyperfine coupling constants of MFH· radical

$a_{\text{H}}^{\text{obs}}$ (mT)	Number of protons	$a_{\text{H}}^{\text{calc}}$ (mT)	Position ^{a,b}
0.334	1	0.329	1
0.326	1	0.315	8
0.092	1	0.101	4
0.088	1	0.098	2
0.064	1	0.073	5
0.052	3	—	
0.042	1	0.041	7
		0.029	12
		0.027	14

^a $Q = 2.90$ mT was used (Q , McConnell's Q value). The position of the protons in the MFH· radical is shown below:



^bParameters for LCAO MO are in refs. 5 and 6, and

$$\alpha(-\text{OCH}_3) = \alpha_{\text{C}} + 2.25\beta_{\text{CC}}$$

$$\alpha(-\text{OH}) = \alpha_{\text{C}} + 2.0\beta_{\text{CC}}$$

The twisting angle between p_{π} orbitals at the 9- and 11-carbons around the C—C bond axis was assumed to be 55° in the light of the value for fluorescein semiquinone. The $\cos^2\theta$ law was adopted in the calculation.

a methoxy group into the xanthene ring. The discrepancy is even more evident for the couplings of the 2- and 7-protons and for those of the 4- and 5-protons (Table 3). Since the perturbation of the MO of the xanthene ring by the methoxy group (MFH·) is not considered to be very different from that by the hydroxy group (fluorescein semiquinone), this doubtlessly implies that introducing the methoxy group eliminates the protonation and deprotonation mechanism considered for the fluorescein semiquinone anion radical and fixes the spin distribution on the xanthene ring. Thus, it was confirmed that our relation of the observed equality of the coupling constants of the 1- and 8-protons etc. in the dianion radical of fluorescein to the dynamic mechanism was valid.

The value $a_{\text{H}}^{\text{obs}} = 0.0518$ mT with three protons may be attributed to that of three equivalent protons belonging to the methoxy group on the

xanthene frame. The calculated spin density $\rho(-\text{OCH}_3)$ on the methoxy group is 0.006, which seems to be greatly underestimated in comparison with the observed coupling constant.

Acknowledgments

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