1744 BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 47(7), 1744—1749 (1974) [Vol. 47, No. 7

Chemiluminescence of Succinylfluorescein and Related Compounds. I. Emitting Species Produced by the Reaction of Succinylfluorescein with Oxygen in Aprotic Solvents

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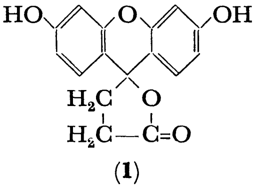
(Received December 24, 1973)

In the course of our studies of the chemiluminescence of xanthene dyes, we found the violet chemiluminescence emission (CL-emission) resulting from the reaction of succinylfluorescein with oxygen in an aerated alkaline aqueous system of a high concentration of an aprotic solvent (DMSO or DMF), an especially intense emission occurring in aerated dry DMSO or DMF with *tert*-BuOK as base. The CL-emission comprised two emission components: an intense violet emission (a peak, at 410 nm) whose spectral distribution was similar to that of the fluorescence band which appeared during the course of the reaction, and a weak greenish-yellow emission similar to the fluorescence of the dye. By isolating and identifying the violet-fluorescent reaction product with an authentic sample, and by comparing the CL-emission with the fluorescence emission of the isolated product, it was concluded that the violet CL-emission may be attributed to the formation of an excited dianion of 3,6-dihydroxyxanthone. The reaction path leading to the dioxetane intermediate which will be produced by the elimination of a proton from the C atom at the β-position of the dye, followed by the oxygenation *via* the caged radical pair, was discussed as a likely mechanism for the formation of the excited dianion.

In the previous paper,[1)] the blue spectral component of the chemiluminescence (with a peak at 480 nm) observed in the reaction of such an xanthene dye as eosin Y with hydrogen peroxide in an alkaline solution was found to correspond to the fluorescence emission (FL-emission) of a blue-fluorescent product isolated from the reaction mixture; we concluded that the blue-fluorescent species is the primary excited product in the chemiluminescent reaction. Further study by means of a kinetic analysis of the blue-fluorescent product provided additional support for that conclusion.[2)] However, the quantity of the isolated product was too small for it to be weighed or identified, so a question as to the identification of the product remained unanswered.

From the findings that several xanthene dyes other than eosin Y, uranin, eosin R, erythrosin B, 2′,7′-dichlorofluorescein, rhodamine B, and even rhodamine S, which is not phthalyl, exhibited a similar blue emission with a production of an analogous blue-fluorescent species, we presupposed that the excited product might be a xanthone compound since excited species generated in many chemiluminescent systems have been reported to be carbonyl compounds,[3)] thus we expected that a similar blue emission would arise from the oxidation of succinylfluorescein (**1**).



(**1**)

When succinylfluorescein was oxidized with hydrogen peroxide in an alkaline solution, however, a violet emission appeared with a peak at 410 nm in addition to a (weak) greenish-yellow emission. The violet emission was also found to appear from the oxidation of the dye with oxygen in an aerated alkaline aqueous system of a high concentration of such aprotic solvents as dimethylsulfoxide (DMSO) and N′,N′-dimethylformamide (DMF). Especially, an intense emission appeared in aerated dry DMSO (or DMF), with potassium *tert*-buthoxide (*tert*-BuOK) as the base. This paper will describe the chemiluminescent reaction of succinylfluorescein with oxygen in detail.

Experimental

Preparation of Succinylfluorescein (SF).[4)] A 20 g portion of zinc chloride powder was added to a mixture of 11.01 g of resorcinol and 5.00 g of succinic anhydride, and then the mixture was heated to 180 °C for 4 hr. The reaction product was extracted with 4% hot hydrochloric acid, and the hot extract was filtered as quickly as possible. The product which precipitated on cooling was purified by repeated recrystallizations from 4% hydrochloric acid. Thin-layer chromatography showed only one spot, indicating the purity of the SF thus prepared.

Preparation of an Authentic Sample of 3,6-Dihydroxyxanthone.[5)] After fluorescein had been chlorinated with phosphorous pentachloride, the produced fluorescein chloride was hydrolyzed by heating it in a concentrated aqueous sodium hydroxide solution to give 2,4,2′,4′-tetrahydroxybenzophenone. With the dehydration of the benzophenone on heating, 3,6-dihydroxyxanthone was obtained. The purity of the prepared sample was confirmed by the thin-layer chromatographic method.

The Chemiluminescent Systems. Two systems were employed to investigate the chemiluminescence emission (CL-emission) of SF with oxygen.

DMSO (or DMF)-$NaOH_{aq}$ System: After 10—20 mg of the prepared SF had been dissolved in 10 ml of a mixture of DMSO (or DMF) and $NaOH_{aq}$, a portion of the reaction mixture (about 3 ml) was quickly transferred into a quartz cell (10×10×45 mm) which had been placed in a thermostat with circulating water at a constant temperature (40 °C). The volume ratio of DMSO to $NaOH_{aq}$ and the concentration of $NaOH_{aq}$ for the system employed are noted in the experimental results.

DMSO (or DMF)-tert-*BuOK System*: 6 mg of SF and 60 mg of *tert*-BuOK were dissolved in 10 ml of DMSO (or DMF) and 10 ml of *tert*-buthanol (*tert*-BuOH) respectively (the concentrations of SF and *tert*-BuOK were about 2.1×

10^{-3} and 5.3×10^{-2} M respectively), and when necessary, the two solutions were diluted with these solvents. The luminescent reaction was initiated by vigorously stirring 0.5 ml of the *tert*-BuOK solution (in *tert*-BuOH) into 2 ml of an aerated SF solution in DMSO (or DMF) in a quartz cell ($10 \times 10 \times 45$ mm) which had been placed in a thermostat.

The measurements of the intensity *versus* the reaction time and the spectrum of the CL-emission, and the absorption spectra of reaction mixture and the isolated product were carried out by a method essentially identical with those described in the previous paper.[1]

Results

The CL-Emission Spectrum of SF in Aerated–NaOH$_{aq}$ System. The CL-emission spectra of SF with oxygen in the system of DMSO–2 M NaOH$_{aq}$ (3: 1 v/v), measured at different reaction times, are shown in Fig. 1, together with the fluorescence spectra (FL-emission) measured at the initial and final stages of the reaction. The results indicate that the CL-emission comprises two emission components: a weak greenish-yellow emission whose spectral distribution is similar to that of the fluorescence of SF (a peak at 555 nm), and an intense violet emission similar to the fluorescence band which appears during the course of the reaction (a peak at 410 nm). The intensity of the greenish-yellow emission at 555 nm showed a rapid decay, whereas that of the violet emission at 410 nm initially rose to a maximum and then showed a slight decay, followed by a long-lived steady glow, as is illustrated in Fig. 2.

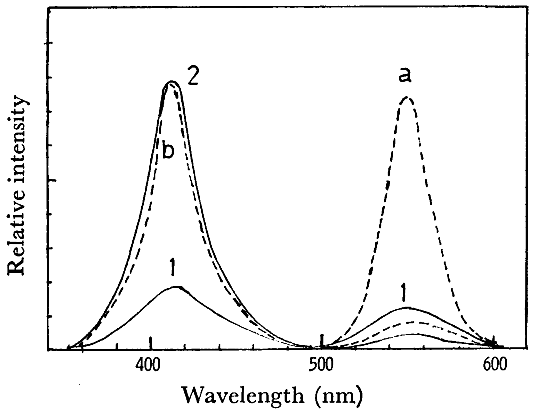


Fig. 1. CL-emission spectra of SF with oxygen measured at different reaction times; 1: after 1 min, 2: after 10 min, and FL-emission spectra of the reaction system measured at initial stage (a), and final stage (b) (DMSO–2 M NaOH$_{aq}$ system).

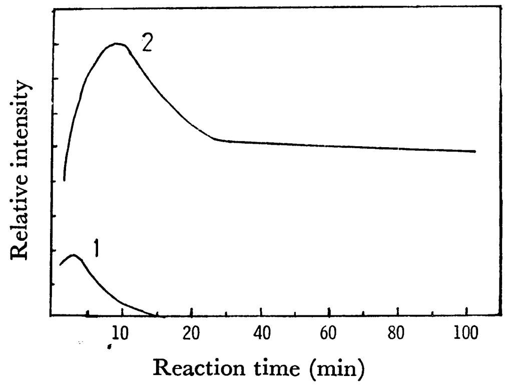


Fig. 2. Changes in the intensities of two components of CL-emission of SF in DMSO–2 M NaOH$_{aq}$ system, 1: violet emission component, 2: greenish-yellow component.

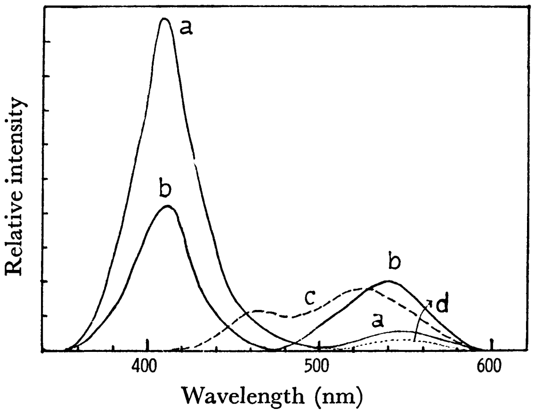


Fig. 3. CL-emission spectra of SF measured in DMSO– (a), DMF– (b), dioxane– (c), ethanol– (d), and methanol–2 M NaOH$_{aq}$ (3: 1 v/v) system (d).

The following two findings are noticeable. When nitrogen was bubbled into the luminescing system to remove the oxygen, the emission disappeared. Such xanthene dyes as uranin, eosin Y, erythrosin B, 2′,7′-dichlorofluorescein, rhodamine B and rhodamine S, which chemiluminesce as a blue emission upon oxidation with hydrogen peroxide, neither decomposed nor chemiluminesced with oxygen under comparable conditions.

The Effect of Solvent on the Chemiluminescence Emission. In DMSO-NaOH$_{aq}$ systems (0.25 M NaOH), the CL-emission appears only when the solution contains more than 50% DMSO in volume, and the higher the concentration of DMSO, the more intense the light which appears. Figure 3 shows the CL-emission spectra in DMSO–, DMF–, dioxane–, methanol–, and ethanol–2M NaOH$_{aq}$ (3: 1 v/v) systems. The spectrum in DMF is similar, but that in dioxane is dissimilar to the CL-emission spectrum in DMSO, and only a greenish-yellow emission appears in such protic solvents as methanol and ethanol. No emission could be observed in the acetone–, *tert*-BuOH–, and acetonitrile–2M NaOH$_{aq}$ (3: 1 v/v) systems, probably because the high concentration of these solvents prevents the dissolution of sodium hydroxide.

The Requisite Molar-ratio of Sodium Hydroxide to Succinylfluorescein for the Generation of the CL-Emission. Table 1 shows the relative intensities of CL-emissions measured when 10 and 20 mg of SF were dissolved in 10 ml of mixtures of DMSO and NaOH$_{aq}$ of different concentrations (3: 1 v/v). The results indicate that no appreciable emission appears when the molar-ratio of NaOH to SF is less than 3. The same requisite molar-ratio of NaOH to SF was found in DMF–NaOH$_{aq}$ systems.

The Effect of Adding Oxidizing Agent on the CL-Emission. When 0.5 ml of a 0.05 M aqueous solution of potassium ferricyanate was added to the luminescing DMSO–2M

Table 1. Relative intensities of CL-emission in DMSO–$NaOH_{aq}$–SF systems of different concentrations of NaOH and SF

[SF]=6.8×10^{-3}M		[SF]=3.4×10^{-3}M	
[NaOH] (M)	Relative intensity	[NaOH] (M)	Relative intensity
1/2	190	3/160	41
1/4	140	9/800	6
1/8	16	7/800	0
1/16	10		
1/40	8		
9/400	8		
3/160	0		
1/80	0		

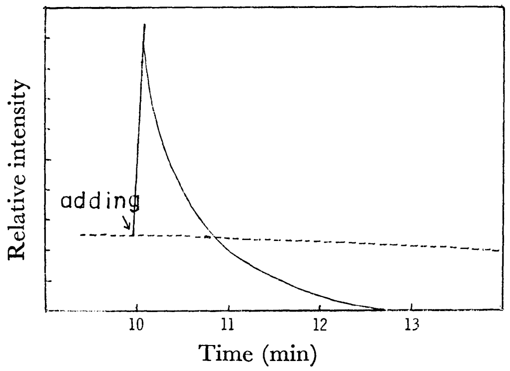


Fig. 4. Effect of adding $K_3Fe(CN)_{6aq}$ on the CL-emission intensity *versus* time; broken line shows intensity *versus* time when oxidizing agent was absent, and full line shows that after adding the agent (40 °C).

$NaOH_{aq}$ (3: 1 v/v) system, the intensity of the emission rapidly increased, then it vanished with a fast decay. A typical example of the effect of adding an oxidizing agent is illustrated in Fig. 4, where the dotted line shows an intensity *versus* reaction time curve measured when the oxidizing agent was absent, while the solid line shows the curve obtained after the agent was added. A similar effect was observed by adding aqueous solutions of bromine and potassium permanganate to the system. No emission, however, appeared when these agents were added to a deaerated system or to a system where the molar-ratio of NaOH to SF was less than three.

The Isolation and Identification of the Violet-fluorescent Product. During the course of the luminescent reaction in a DMSO–2M $NaOH_{aq}$ (3: 1 v/v) system, the absorption peak at 510 nm decreased; simultaneously a new absorption peak appeared at 370 nm, as is shown as the solid line curve in Fig. 5. The change in the absorption spectrum seems to be attributable to the decomposition of SF and the formation of a species. In an aerated aqueous solution of sodium hydroxide (containing no aprotic solvent), the absorption spectrum also changed, as is shown by the dotted line in Fig. 5. From the results, we can expect that the same reaction product may be formed in the two systems since the new absorption peak appeared at the same wavelength (370 nm). This was confirmed by the findings that thin-layer chromatography (Silica gel G, $CHCl_3$–CH_3COOH, 1: 1) showed the same spot at R_f 0.14 (ascending method) and that paper chromatography (Toyo Filter Paper No. 50, 20% aqueous ethanol–5% aqueous ammonia, 1: 1) showed the same spot at R_f 0.30 (the descending method) for the products in the two systems. A large amount of the (violet-fluorescent) product could be prepared by the following method. Four grams of SF and 10 g of sodium hydroxide were dissolved in 300 ml of aerated water, and then the reaction mixture was kept at 40 °C with stirring in an open flask for three days. The residual reaction mixture was acidified with hydrochloric acid, and the precipitated crude brownish product was filtered off and adsorbed on a column of Mallinckrodt Silicic Acid (100 mesh). The column was eluted with ether; then, after the removal of the solvent, white needles were obtained. Recrystallization from 20% aqueous ethanol gave 50 mg of a pure product, which did not melt below 300 °C. The analytical data of the product are listed in Table 2. These data support the idea that the product is 3,6-dihydroxyxanthone. The product was further identified by its position on thin-layer chromatography (R_f 0.14,

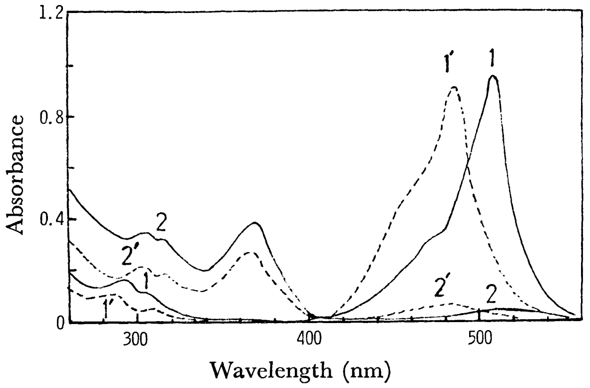


Fig. 5. Absorption spectra of reaction mixture in DMSO–2 M $NaOH_{aq}$ system (1: initial stage, 2: final stage) and those in 1/4 M $NaOH_{aq}$ system (1′: initial stage, 2′: final stage).

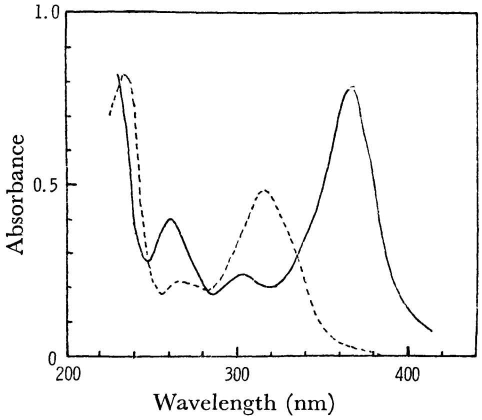


Fig. 6. Absorption spectra of reaction product; ——: dianion in KOH–H_2O system,– – – –: neutral molecule in H_2O (in KOH_{aq}: $\lambda_{max}=368$ nm, $\epsilon=3.99\times10^4$; 304 nm, 1.05×10^4; 263 nm, 1.93×10^4; 225 nm, 4.49×10^4, in H_2O: $\lambda_{max}=315$ nm, $\epsilon=2.40\times10^4$; 267 nm, 1.02×10^4; 237 nm, 4.00×10^4).

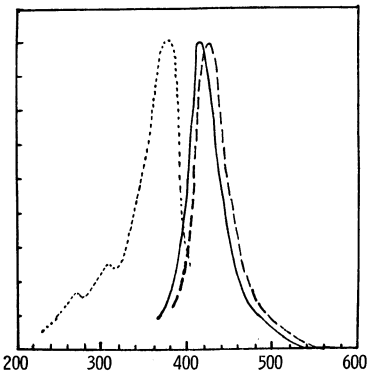


Fig. 7. FL-emission spectra of reaction product; ——: in DMSO-*tert*-BuOK system, ----: in H_2O-KOH system (exciting at 480 nm), and excitation spectrum for the FL-emission peak at 410 nm in DMSO-*tert*-BuOK system (······).

Table 2. Analytical data for the reaction product

Elemental analysis		Mass (M^+)	NMR (δ, ppm)
Found (%)	Calcd for $C_{13}H_8O$(%)		
C 68.29	68.42	228	8.07 (d, J=9.0 Hz)
H 3.74	3.53		6.94 (dd, J=1.5, 9.0 Hz)
			6.88 (s)

(The NMR spectrum was taken in DMSO-d_6 with a JEOL MH-100 type spectrometer at 100 MHz using TMS as an internal standard.)

the ascending method) as well as by its position on paper chromatography (R_f 0.30, the descending method) compared with those of an authentic sample. The absorption and fluorescence spectra of the product in different media are illustrated in Figs. 6 and 7 respectively. These spectra also match those of the authentic sample.

*Emission Characteristic in the DMSO (or DMF)-*tert*-BuOK System.* The intensity of the CL-emission of SF in an aerated dry DMSO (or DMF)-*tert*-BuOK system was about 150 times greater than that in a DMSO-2M $NaOH_{aq}$ (3: 1 v/v) system. The spectra of the CL-emissions measured in the DMSO-, DMF-, and acetonitrile-*tert*-BuOK systems are illustrated in Fig. 8. The observed intense peak at 410 nm indicates that the CL-emission in the systems can be attributed to the formation of the excited dianion of 3,6-dihydroxyxanthone.

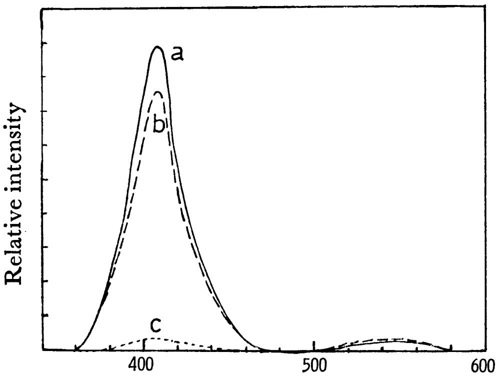


Fig. 8. CL-emission spectra of SF in DMSO- (a), DMF- (b), acetonitrile-*tert*-BuOK system (c)

The dependence of the molar-ratio of *tert*-BuOK to SF on the generation of the CL-emission in a DMSO-*tert*-BuOK system was investigated. The results, which are summarized in Table 3, indicate that no appreciable emission is generated when the molar-ratio of *tert*-BuOK to SF is less than 3.

Table 3. Dependence of molar-ratio of *tert*-BuOK to SF (ρ) on the generation of CL-emission in aerated dry DMSO-*tert*-BuOK system

[SF] (M)	[*tert*-BuOK] (M)							
	1.6×10^{-2}		2.6×10^{-3}		1.2×10^{-3}		6×10^{-4}	
	ρ	emission	ρ	emission	ρ	emission	ρ	emission
1.68×10^{-3}	6.8	G	1.57	N	—	—	—	—
8.4	12.6	G	3.15	G	1.43	N	—	—
5.6	18.9	G	4.7	G	2.14	N	—	—
4.2	25.2	G	6.3	G	2.86	N	1.43	N
2.1	50.4	G	12.6	G	5.7	G	2.86	N
1.05	100	G	25.2	G	11.4	G	5.7	U
5.25	—	—	50.4	G	22.8	G	11.4	G

(G: generated, N: did not generate, U: undefined)

The absorption spectrum of SF in an aerated DMSO solution showed no appreciable peak in the visible region. By adding a *tert*-BuOK solution (in *tert*-BuOH), a strong absorption at about 525 nm appeared, probably because of the structural change in the dye. (In a deaerated system, the absorption at 525 nm was found to be not so strong that oxygen would participate in the reaction which leads to the structural change.) During the course of the reaction, the spectrum changed as is shown in Fig. 9. It is of interest to find that a new absorption peak at 370 nm increases gradually and that the isobestic point appears at 364 nm. It seems reasonable to speculate that the main product is 3,6-dihydroxyxanthone, so the intense emission can be observed in a DMSO-*tert*-BuOK system. Figure 10 shows the intensity of the CL-emission and the change in the absorbance at 525 nm *versus* the reaction

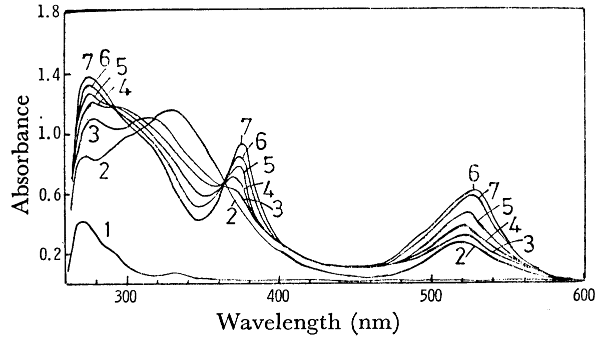


Fig. 9. Change of absorption spectrum of reaction mixture in DMSO-*tert*-BuOK-SF system ([*tert*-BuOK] $=2.6\times10^{-3}$ M, [SF]$=5.25\times10^{-5}$ M); 1: before adding *tert*-BuOK, 2→7: after adding *tert*-BuOK.

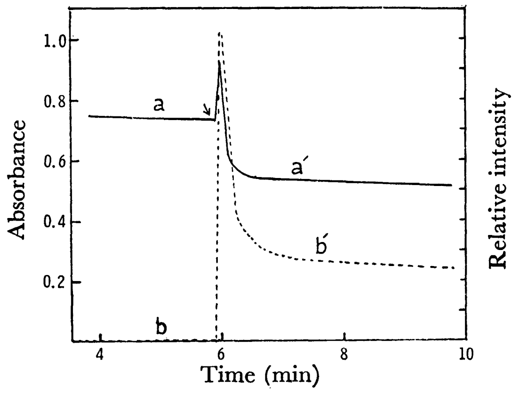


Fig. 10. Changes in the absorbance of reaction system at 525 nm (——a) and CL-emission intensity at 410 nm (----b) when adding 0.05 ml of a 6.6×10^{-3} M solution of *tert*-BuOK in *tert*-BuOH in 2 ml of a 6.8×10^{-5} M solution of SF in DMSO ($\rho=2.3$; *tert*-BuOK deficient system), and those (——a′ and ----b′) after adding 0.5 ml of the *tert*-BuOK solution ($\rho=23$; *tert*-BuOK sufficient system).

time measured when 0.05 ml of a 6.6×10^{-3} M solution of *tert*-BuOK was added to 2 ml of a 6.8×10^{-5} M solution of SF (the molar-ratio of *tert*-BuOK to SF is 2.3; it is a *tert*-BuOK deficient system) and also those measured when 0.5 ml of a *tert*-BuOK solution was added again to the system to make a *tert*-BuOK sufficient system (the molar-ratio is 23). The absorbance at 525 nm was found to increase to a maximum without any emission in the *tert*-BuOK deficient system (the peak at 370 nm did not appear in the system), whereas the absorbance decreased with emission in the *tert*-BuOK sufficient system.

The characteristics of the emission intensity *versus* the reaction time, which is strongly dependent upon the molar-ratio of *tert*-BuOK to SF, are so complicated that no kinetic analysis of the emission intensity could be made in the present study.

When such protic solvents as water, methanol, and ethanol were added drop by drop (each drop was about 0.02 ml) to the luminescing system (2.5 ml), a decrease in the intensity was found upon each addition. On the other hand, when a 0.05 M solution of benzoyl peroxide in DMSO was added, drop by drop, to the system, a bright flash appeared, with a lowering of the intensity of the steady glow, at each addition. Typical examples of the effects of adding a protic solvent and oxidizing agent are illustrated in Figs. 11 and 12 respectively. Neither an appreciable lowering of the intensity nor a flash was observed when a drop of DMSO was added to the luminescent system.

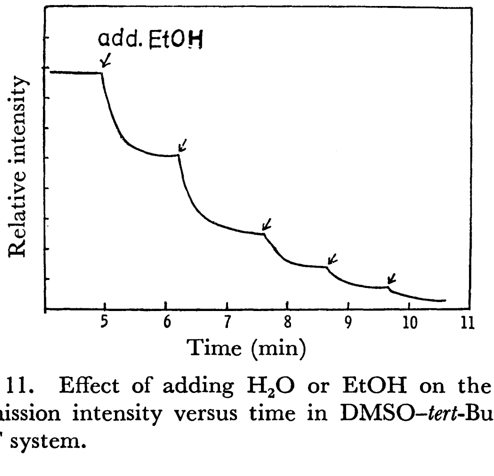


Fig. 11. Effect of adding H_2O or EtOH on the CL-emission intensity versus time in DMSO-*tert*-BuOK-SF system.

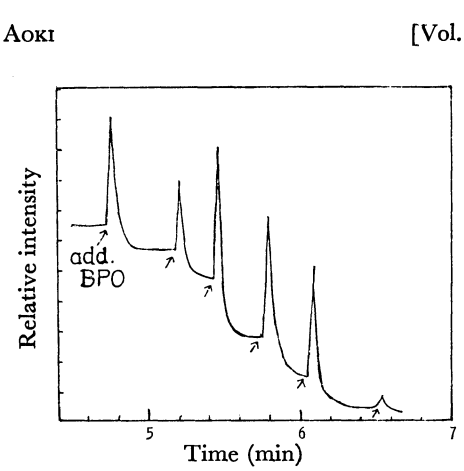
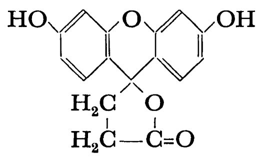


Fig. 12. Effect of adding benzoyl peroxide on the CL-emission intensity *versus* time in DMSO-*tert*-BuOK-SF system.

Discussion

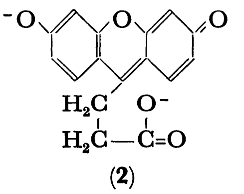
The appearance of a strong absorption peak at 525 nm upon the addition of NaOH or *tert*-BuOK to an aerated solution of SF may be attributed to the following structural change in SF:



(1)

Lactone form of a neutral molecule of SF in DMSO

$\downarrow$ 2 *tert*-BuOK or 2 NaOH and O_2



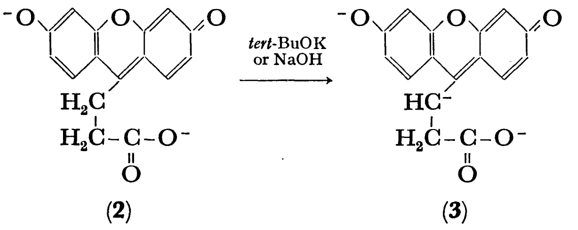
(2)

Quinoid form anion of SF in an aerated alkaline solution

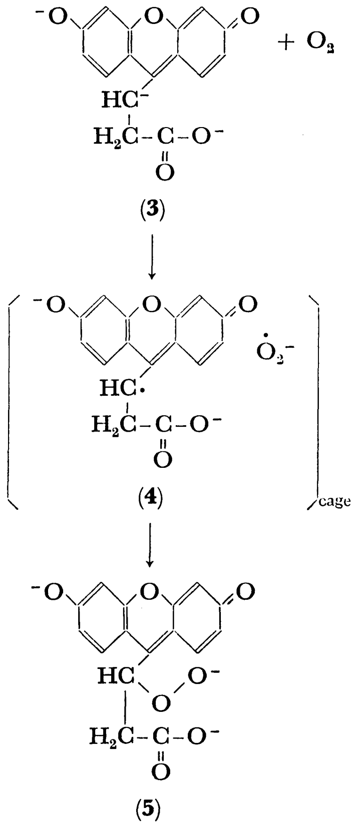
A further reaction of SF with *tert*-BuOK will be necessary to initiate the luminescent reaction, because no appreciable emission appeared when the molar-ratio of *tert*-BuOK to SF was less than 3.

It has been reported that *tert*-BuOK prevents the reaction of DMSO with *tert*-BuOK under the experimental conditions employed here.⁶⁾

A reasonable step may be the following elimination of a proton from the C atom at the β-position:



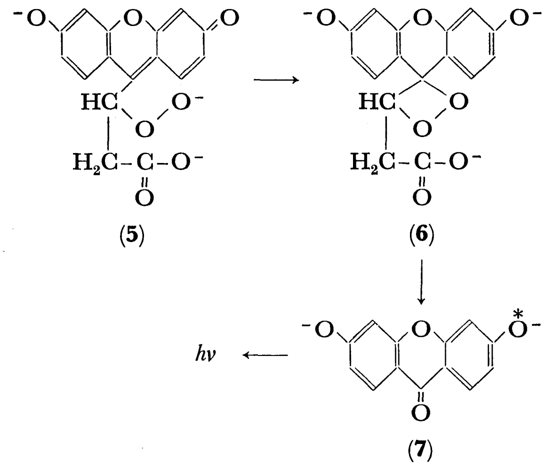
Therefore, one possible mechanism for the reaction of **3** with oxygen may be the following oxygenation *via* a caged radical pair, **4**:⁷⁾



The experimental finding that the CL-emission rapidly increased upon the addition of an oxidizing agent will support this scheme.

The experimental results also indicate that the CL-emission is attributable to the formation of the excited dianion of 3,6-dihydroxyxanthone (**7**); thus, the reaction path leading to the dioxetane intermediate, **6**, may be the most probable mechanism for the formation of **7**, as has been proposed by many investigators.⁸⁾

A finding similar to our finding that no appreciable emission arises in a protic solvent system has also been reported in the chemiluminescent reaction of photinusruciferin with oxygen;⁹⁾ also, a sharp rize in the intensity at the initial stage followed by a slight decay and then a long-lived steady glow, has been demonstrated for the CL-emission of 3,3′-tetrahydro-1,1′-biisoquinolinium iodide with oxygen.¹⁰⁾



The authors wish to express their thanks to Professor Teruo Matsuura of the Faculty of Engineering, Kyoto University, for his useful discussion, to Mr. Tatsuyuki Yamakawa for his measurements of the intensity and the spectrum of the reaction system, to Miss Kyoko Toya for the elemental analysis, and to Mr. Kozo Matsumoto of Nagoya University and Dr. Nobutaka Suzuki of Mie University for their measurements of the MS and NMR spectra and for their helpful discussions.

This work was supported in part by a Scientific Research Grant of the Ministry of Education.

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