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Studies of the Chemiluminescence of Several Xanthene Dyes. II. The Chemiluminescence Emission Spectra of Uranine and Eosine

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The emission spectra of uranine and esoine chemiluminescence with hydrogen peroxide were measured photoelectrically and were compared with the fluorescence spectra of the dyes. The apparent spectra did not give the true energy distribution of the emitted light, because self-absorption took place. When the dyes were oxidized with ozonized air, the apparent emission spectra were very similar to those obtained when the dyes were oxidized with hydrogen peroxide. This would indicate that the emitting species are the same in the two reaction systems. The emission from the dye-ozone reaction was intense, and the spectrum could be compared with the fluorescence spectrum by employing a system with a low dye concentration. The coincidence of the two spectra was clearly demonstrated. The chemiluminescence spectrum of uranine at a high dye concentration in the presence of hydrogen peroxide was compared to the "chemi-fluorescence" spectrum of the luminol-dye system. The two systems gave almost the same spectrum. It may be concluded that the emissions from uranine and eosine chemiluminescence are the fluorescence emissions of the dyes and that the emitting species are the excited singlet states of uranine and eosine.

Since organic compounds which emit chemiluminescent light often fluoresce, the chemiluminescence emission spectra can conveniently be compared with the fluorescence emission spectra. The chemiluminescence spectrum is not always the same as the fluorescence spectrum; in luminol chemiluminescence, it has been found[1] that the emitting species is the excited aminophthalic acid ion produced by the reaction. Light emittted by lophin chemiluminescence has also been found[2] not to correspond to the fluorescence of the compound.

On the other hand, the chemiluminescence emission spectra of lucigenin[3] and riboflavin[4] were found to be very similar to their fluorescence emission spectra. The chemiluminescence emission observed when hydrogen peroxide was added to an alkaline solution of uranine or eosine seemed to correspond to the fluorescence of the dye. However, no comparison of the two spectra has yet been made except for a rough observation with the naked eye.[5]

There is a severe restriction in the measurement of the emission spectra; the apparent chemiluminescence spectra of the dyes do not give the true energy distribution, because the dye solutions absorb the emitted light. In a dilute dye solution where the self-absorption is not apreciable, the intensity

1) E. H. White and M. M. Bursey, *J. Am. Chem. Soc.*, **86**, 941 (1964).
2) K. Maeda, H. Ojima and T. Hayashi, This Bulletin, **38**, 76 (1965); E. H. White and M. C. Harding, *J. Am. Chem. Soc.*, **86**, 5686 (1964).
3) A. V. Karyakin, *Optica i. Spektroskopia*, **7**, 122 (1959).
4) B. L. Strehler and C. S. Shoup, *Arch. Biochem. Biophys.*, **47**, 8 (1953).
5) N. N. Biswas and N. R. Dhar, *Z. anorg. u. allegem. Chem.*, **173**, 125 (1928).

of the chemiluminescence is too weak for reliable measurements. Therefore, other, supplementary methods for estimating the emission spectra should be employed together with direct measurements.

Experimental

Measurements of Emission Spectra in Uranine and Eosine Chemiluminescence with Hydrogen Peroxide.—The luminescent system was prepared in the method described previously.[6] The emission spectra were measured photo-electrically with the apparatus shown diagramatically in Fig. 1. A was a hard-glass reaction tube about 20 mm. in diameter; it was placed in a cylindrical copper thermostat, B. Water circulated between the cylinder, B, and a large thermostat through pipes, D, so the reaction tube could be kept at a constant temperature. C was a dark box. Light emitted from the reaction tube was conducted through a convex lens, E, into a electro-spectrophotometer, F. By dialing a wavelength adjuster with a motor, a spectral curve was recorded on G. It took about 2 min. to scan the whole curve. As a result of the decay of the emission intensity during the recording, the recorded spectral curve was corrected by the decay curve which had been measured under the same experimental conditions. In the present work, where the chemiluminescence emission spectra are compared with the fluorescence spectra, no correction was made for the characteristic sensitivity of the photomultiplier, because the fluorescence was measured with the same photomultiplier.

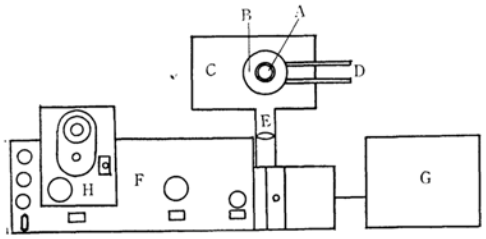

Fig. 1. Diagram of measuring apparatus for the emission spectra of dye chemiluminescence with hydrogen peroxide.

A: Reaction tube B: Thermostat
C: Dark box D: Pipes
E: Convex lens F: Spectrophotometer
G: Selfrecording micro-ammeter
H: Dialing wavelength adjuster

The Measurement of Emission Spectra in Uranine and Eosine Chemiluminescence with Ozone.—When ozonized air was passed through ethanol solutions of uranine and eosine, light was also produced and the apparent emission spectra were similar to those observed when the dyes were oxidized with hydrogen peroxide. The intensity of the emission in this system was more pronounced, although the reproducibility was somewhat poor; hence, the emission spectra could be measured in a solution of such a low dye concentration that self-absorption was not appreciable.

6) I. Kamiya and R. Iwaki, This Bulletin, **39**, 257 (1966).

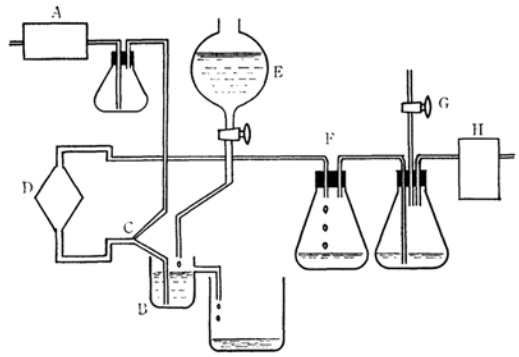

Fig. 2. Diagram of measuring apparatus for the emission spectra of dye chemiluminescence with ozone.

A: Ozonizer B: Beaker
C: Nozzle D: Reaction vessel
E: Sample-solution flask
F: Waste-solution flask
G: Cock H: Pump

The measurement of the emission spectra in the system was carried out with the apparatus shown diagramatically in Fig. 2. Four hundred milliliters of an ethanol solution of uranine or eosine in a flask, E, was dropped into a beaker, B. By pumping out the entire vessel via H, the ozonized air produced in an ozonizer, A, was introduced from a nozzle, C, and reacted with the dye solution. The reacting solution was carried to F through a vessel, D. D is a lozenge-shaped vessel (3.7 cm. wide, 5.4 cm. high) consisting of two parallel glass plates 2 mm. apart, the back of one glass plate being coated with silver. The spectra were measured by inserting D into the A of Fig. 1. As the intensity of emission was practically constant for about 10 min., no correction was needed for the variation with the reaction time. All the measurements were carried out at room temperature because the temperature dependence of the intensity was not appreciable.

Experimental Results

The apparent emission spectra observed in uranine and eosine chemiluminescence with hydrogen peroxide are shown in Figs. 3 and 4. The conentrations of uranine and eosine were 1 g. per 100 ml. and 0.5 g. per 100 ml. respectively. The measurements were carried out in a 60% methanol solution at 60°C. Figures 5 and 6 show the same spectral curves in other solvents, such as ethanol, *n*-propanol, isopropanol and glycerol. It is sohwn that every (apparent) spectrum has the same maximum, at 560 mμ for uranine and at 580 mμ for eosine. These apparent spectra, however, do not give the true energy distribution of the emitted light, because the dye solutions absorb the light. (The absorption spectra of uranine and eosine have maxima at 492 mμ and 517 mμ respectively.) Therefore, no direct comparison of the spectra with fluorescence spectra could be made.

The apparent chemiluminescence spectra observed when ozonized air was passed through ethanol solutions of different uranine concentrations are shown in Fig. 7. The spectra for eosine solutions are shown in Fig. 8. The apparent spectra in a concentrated dye solution are similar to those obtained with hydrogen peroxide. This would indicate that the emitting species is the same in the two reactions. It is very interesting to see that the peak shifts towared a shorter wavelength with a decrease in the dye concentration. In dilute dye solutions, the spectra have maxima at 530 mμ for uranine and at 560 mμ for eosine.

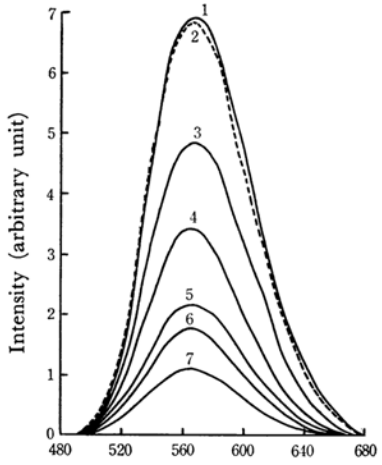

Fig. 3. The emission spectra of uranine chemiluminescence in methanol with hydrogen peroxide at 63°C.

7 ml. of uranine solution (1 g. of uranine in 100 ml. of 60% methanol) + 2 ml. of 2.5 N NaOHaq. + 1 ml. of 20% H_2O_2aq.

1: After 80 sec. 2: After 170 sec.
3: After 241 sec. 4: After 309 sec.
5: After 400 sec. 6: After 470 sec.
7: After 580 sec.

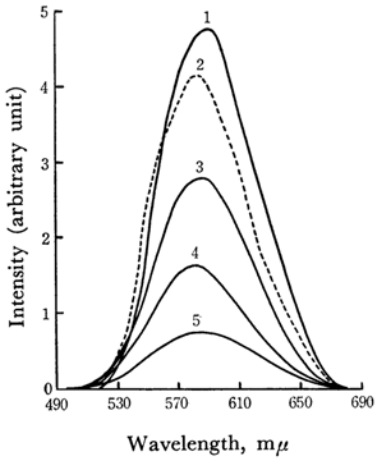

Fig. 4. The emission spectra of eosine chemiluminescence in methanol with hydrogen peroxide at 60°C.

7 ml. of eosin solution (0.5 g. of eosine in 100 ml. of 60% methanol) + 2 ml. of 0.5 N NaOHaq. + 1 ml. of 30% H_2O_2aq.

1: After 43 sec. 2: After 112 sec.
3: After 175 sec. 4: After 236 sec.
5: After 293 sec.

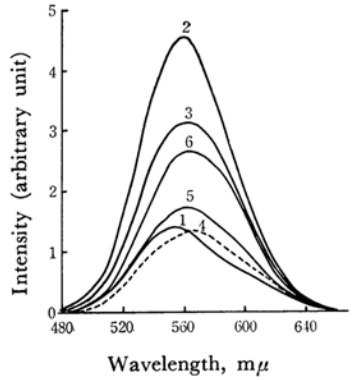

Fig. 5. The emission spectra of uranine chemiluminescence in various solvents with hydrogen peroxide at 57°C.

7 ml. of uranine solution (1 g. of uranine in 100 ml. of solvent) + 2 ml. of 2.5 N NaOHaq. + 1 ml. of 20% H_2O_2aq.

1: Water 2: 60% methanol
3: 60% ethanol 4: 60% *n*-propanol
5: 60% isopropanol 6: 60% glycerine

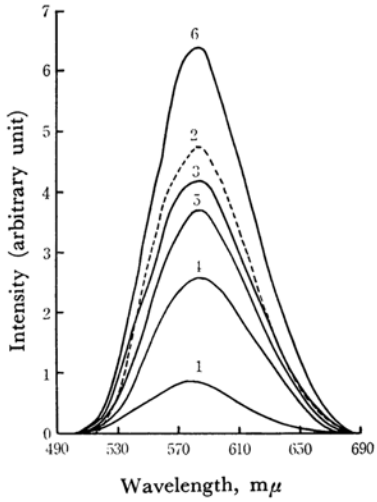

Fig. 6. The emission spectra of eosine chemiluminescence in various solvents with hydrogen peroxide at 60°C.

7 ml. of eosine solution (0.5 g. of eosine in 100 ml. of solvent) + 2 ml. of 0.5 N NaOHaq. + 1 ml. of 30% H_2O_2aq.

1: Water 2: 60% methanol
3: 60% ethanol 4: 60% *n*-propanol
5: 60% isopropanol 6: 60% glycerine

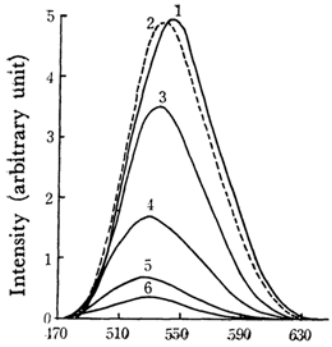

Fig. 7. The emission spectra of uranine chemiluminescence in ethanol with ozone at 12°C.
Concentration of uranine solution in ethanol:
1: 1.33×10^{-2} mol./l. 2: 6.64×10^{-3} mol./l.
3: 3.32×10^{-3} mol./l. 4: 1.66×10^{-3} mol./l.
5: 8.31×10^{-4} mol./l. 6: 4.10×10^{-4} mol./l.

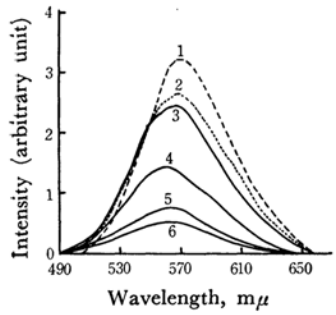

Fig. 8. The emission spectra of eosine chemiluminescene in ethanol with ozone at 12°C.
Concentration of eosine solution in ethanol:
1: 1.44×10^{-2} mol./l. 2: 7.22×10^{-3} mol./l.
3: 3.61×10^{-3} mol./l. 4: 1.81×10^{-3} mol./l.
5: 9.03×10^{-4} mol./l. 6: 4.52×10^{-4} mol./l.

As will be shown later, these maxima correspond to the peaks of the fluorescence of the dyes.

Chemi-fluorescence Emission Spectra in Luminol-Uranine and Luminol-Eosine Systems.—When a dilute hydrogen peroxide solution was added to an aqueous alkaline solution of luminol and a fluorescent dye such as uranine or eosine, a green fluorescence of the dye appeared at the expense of the blue luminescence of luminol. (In such a reaction system, the added dye is almost unoxidized as a result of the low concentration of hydrogen peroxide.) This phenomenon was discovered by Plotnikov[7] and called "Chemi-fluorescence" or "Induced Chemiluminescence." Tamamushi[8] investigated it further and found that both blue light and green light were observed when less than 0.02% of fluoresceine was added to 0.0025% of a luminol solution, and that the blue

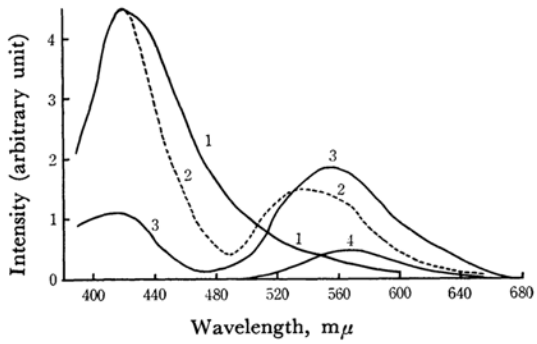

Fig. 9. The emission spectra of luminol and uranine-luminol chemiluminescence at 31°C.
6 ml. of alkaline luminol solution (0.25 g. of luminol and 2 g. of sodium hydroxide in 100 ml. of water) + 21 ml. of uranine aqueous solution of various concentrations + 3 ml. of 10% H_2O_2aq.
Concentration of uranine aqueous solution:
1: 0 g./l. 2: 0.01 g./l.
3: 0.1 g./l. 4: 0.625 g./l.

light changed completely to green light when the dye was more than 0.02%. The light was gradually enfeebled by the further addition of the dye.

With the aid of this phenomenon, the variation in the shape of apparent emission spectra with the dye concentration could be investigated. To initiate the luminol reaction, 3 ml. of 10% of a hydrogen peroxide solution was added to mixtures of 6 ml. of an alkaline luminol solution (0.25 g. of luminol and 2 g. of sodium hydroxide were dissolved in 100 ml. of water) and 21-ml. portions of uranine solutions of various concentrations. The chemi-fluorescence spectra were measured with the apparatus shown in Fig. 1. The reaction was carried out at 31°C; the results are illustrated in Fig. 9. It is shown that the spectrum in a dilute dye solution has two maxima, at 420 mμ and 530 mμ, due to luminol chemiluminescence and uranine chemi-fluorescence respectively. The second peak shifts to longer wavelengths with an increase in the dye concentration, which is very similar to the red shift observed in the apparent chemiluminescence emission spectrum of the uranine-ozone system. This similarity would indicate that the chemiluminescence is the fluorescence of the dye.

Fluorescence Spectra of Uranine and Eosine.—In order to compare the chemiluminescence emission spectra with the fluorescence spectra, the apparent fluorescence spectra were investigated in methanol solutions of uranine and eosine of various concentrations. The spectra were measured with a fluoro-meter attached to the electro-spectro-photometer of Fig. 1 (Fig. 10). The results are shown in Figs. 11 and 12. It is there shown that the peaks of the apparent fluorescence spectra also shift towards longer wavelengths with an increase in the dye concentration.

The chemiluminescence emission spectra o

7) I. Plotnikov, *Umchau*, **42**, 981 (1938).
8) B. Tamamushi, *Naturwiss.*, **28**, 722 (1940).

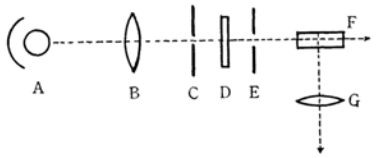

Fig. 10. Diagram of measuring apparatus for fluorescence emission spectra.
A: Mercury lamp B, G: Condencer
C: Iris D: Filter
E: Slit F: Quartz cuvett

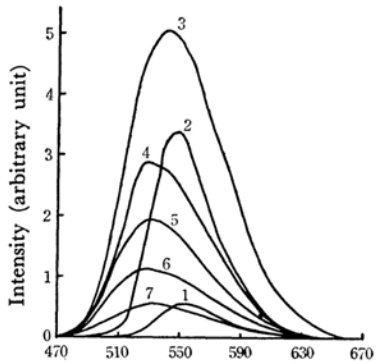

Fig. 11. Fluorescence emission spectra of uranine solutions in ethanol at 28°C.
1: 1.66×10^{-3} mol./l. 2: 4.10×10^{-4} mol./l.
3: 1.03×10^{-4} mol./l. 4: 2.56×10^{-5} mol./l.
5: 1.28×10^{-5} mol./l. 6: 6.41×10^{-6} mol./l.
7: 3.21×10^{-6} mol./l.

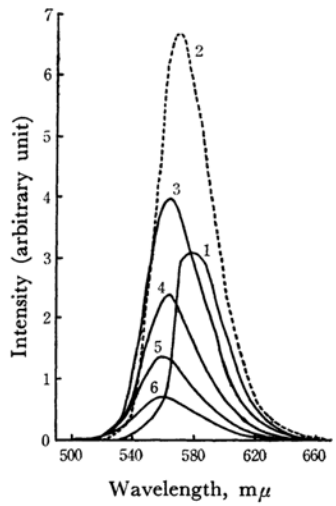

Fig. 12. Fluorescence emission spectra of eosine solutions in ethanol at 12°C.
1: 4.52×10^{-4} mol./l. 2: 1.13×10^{-4} mol./l.
3: 2.32×10^{-5} mol./l. 4: 1.16×10^{-5} mol./l.
5: 5.81×10^{-6} mol./l. 6: 2.90×10^{-6} mol./l.

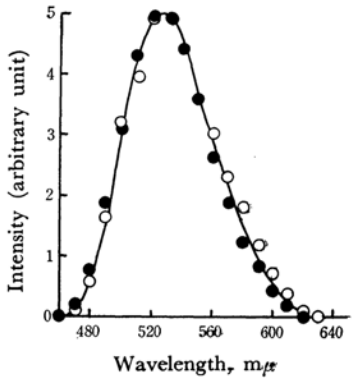

Fig. 13. Fluorescence and chemiluminescence emission spectra of uranine solution in ethanol.
—●— Chemiluminescence (curve 6 in Fig. 7)
—○— Fluorescence (curve 7 in Fig. 11)

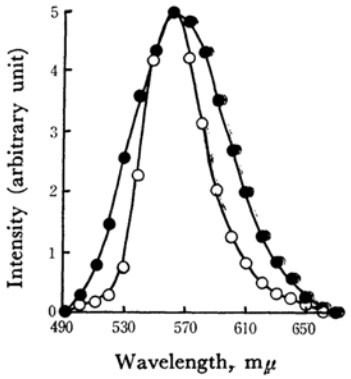

Fig. 14. Fluorescence and chemiluminescence emission spectra of eosine solution in ethanol.
—●— Chemiluminescence (curve 6 in Fig. 8)
—○— Fluorescence (curve 6 in Fig. 12)

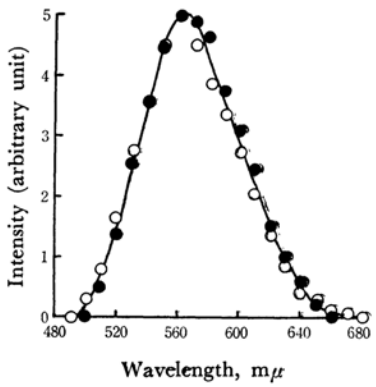

Fig. 15. Chemiluminescence emission spectrum of uranine and compared with the chemi-fluorescence spectrum of uranine-luminol system.
—○— Uranine system (curve 1 in Fig. 3)
—●— Uranine-luminol system (curve 4 in Fig. 9)

Figs. 7 and 8 are compared with the fluorescence spectra of Figs. 11 and 12 in Figs. 13 for uranine and in Fig. 14 for eosine. A correspondence between the two spectral curves is clearly demonstrated, although a slight deviation is found in the eosine system. The same comparison was made between the spectrum of uranine chemiluminescence in the presence of hydrogen peroxide and that of chemi-fluorescence in the luminol-uranine system. This is shown in Fig. 15. The coincidence is apparent.

On the basis of this experimental evidence, it may be concluded that the emissions from the uranine and eosine chemiluminescence are fluorescence emissions of the dyes and that the emitting species are the excited singlet states of uranine and eosine.

Further evidence in support of the present conclusion will be given in a succeeding paper.