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The Chemiluminescence of Luminol and Acetyl-luminol

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Several acetyl derivatives of luminol were synthesized and their chemiluminescence were studied in water or in DMF-water. The role of the amino group in the chemiluminescence was revealed by comparing the chemiluminescence of luminol (I) with that of *N*-acetyl-luminol (II). Also, it was found that, in the case of the chemiluminescence of I or II in water, the emitting species did not seem to be the corresponding substituted phthalate ion, while in the case of the chemiluminescence in DMF-water, the 3-aminophthalate or 3-acetylaminophthalate ion could be presumed to be the emitting species.

During the course of previous investigation of synthetic approach to the mechanism of chemiluminescence and bioluminescence, the authors found that the chemiluminescence spectra in dimethylformamide (DMF)-water mixtures of several kinds of *N*-acyl derivatives of luminol (I) roughly coincided with the fluorescence spectra of the corresponding chemiluminescence products.1)

Further studies of the role of the amino group in the chemiluminescence of I have since been carried out in the authors' laboratory by comparing the chemiluminescent reaction of I with that of *N*-acetyl-luminol (II) from several points of view. This paper is concerned with syntheses and structures of acetyl-luminols and the acetylaminophthalate ion; these will also be a comparative study of the chemiluminescence of I and II.

Results and Discussion

Preparation and Structures of Acetyl Derivatives. Monoacetyl and diacetyl derivatives of I were prepared by the reactions shown in Fig. 1. The monoacetyl derivative (II) and the diacetyl derivative (III) were obtained by the reaction of I with acetyl chloride in DMF.1) When the same reaction was carried out in pyridine, the diacetyl derivative (IV) was obtained. When I and acetic anhydride were refluxed, the product

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1) Y. Omote, T. Miyake, S. Ohmori and N. Sugiyama, This Bulletin, **39**, 932 (1966).

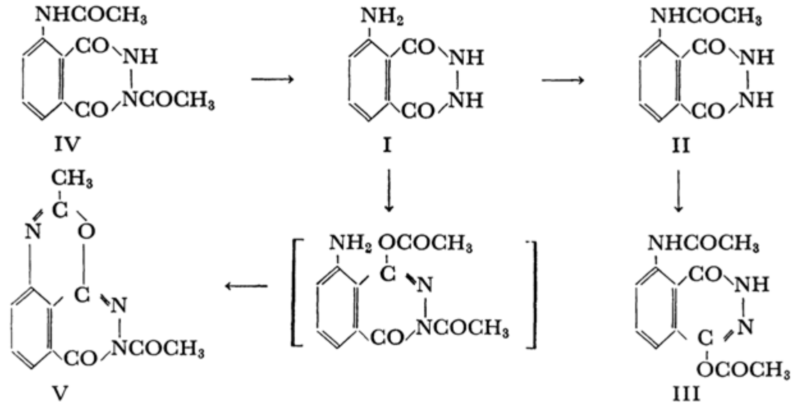

Fig. 1

obtained was proved to be V, which is presumed to be formed by dehydration between amino and acetoxy groups, as is shown in Fig. 1.

The infrared absorption spectra (IR) of II show an amide band at 1530 cm^{-1} which is also recognized in III and IV, but which is absent in I. This fact shows that the newly-formed amide bond is in a trans configuration. Furthermore, the structures of III, IV and V are confirmed by the similarity of their IR spectra to those of the corresponding acetyl derivatives of phthalhydrazide.²⁾ Compounds III and V show a very strong peak at 1690 cm^{-1} characteristic of the C=N stretching vibration. III also has a strong peak at 1760 cm^{-1} characteristic of the C=O stretching of the enol ester. The peak at 1720 cm^{-1} in IV or 1730 cm^{-1} in V presumed to be that of the C=O on the hydrazide ring. The acetyl group on the hydrazide ring was easily hydrolyzed in an alkaline solution. The ultraviolet absorption (UV) and fluorescence of III, IV and V in an alkaline solution were agreed with that of II.

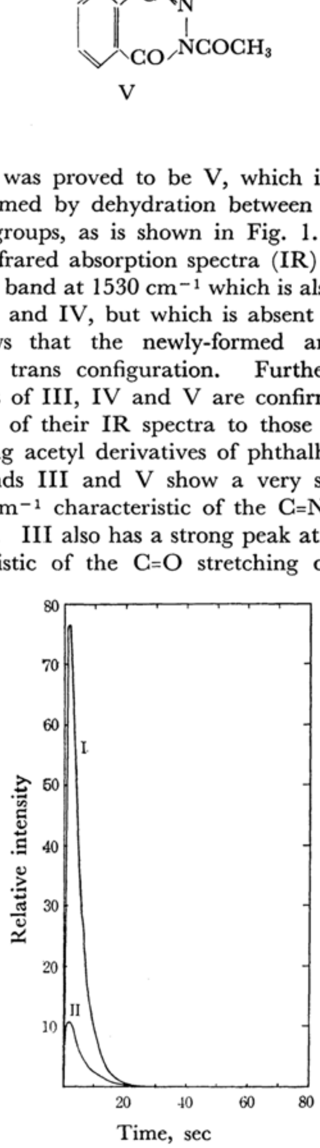

Fig. 2. Chemiluminescence intensity curves of luminol (I) and acetyl luminol (II) in water.
Final concentration of each component : the sample 5×10^{-5}, NaOH 5×10^{-2}, H_2O_2 2.2×10^{-2}, $K_3Fe(CN)_6$ 4.54×10^{-3} mol/l

Chemiluminescence in Water. In the presence of sodium hydroxide, hydrogen peroxide, and potassium ferricyanide, the chemiluminescence of I and II in water was examined. The intensity curves of the chemiluminescence are shown in Fig. 2. It should be noted that an acetyl group decreases the total amount of light emission.

From the standpoint of bioluminescence, it is interesting to examine the effect of protein upon the amino group. If the amino group of I is related to protein, a difference should be observed between the ratio of the total amount of the chemiluminescence of I to that of II in the presence of hemin and the ratio in the presence of hemolgobin, because II has an acetyl group which may prevent

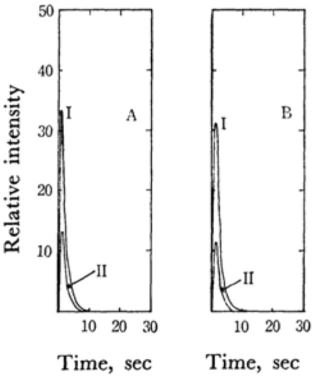

Fig. 3. Chemiluminescence intensity curves of luminol (I) and acetyl luminol (II) in water using hemin (A) or hemoglobin (B) as catalyst.
Final concentration: sample 5×10^{-7}, NaOH 5×10^{-2}, H_2O_2 2.2×10^{-2}, hemin or hemoglobin 2.5×10^{-6} mol/l

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2) H. D. K. Drew and H. H. Hatt, *J. Chem. Soc.*, **1937**, 16.

the protein molecule of hemoglobin from approaching the amino group. However, in both cases, as is indicated in Fig. 3, the total light emission is reduced to about 40% when the amino group is converted into an acetylamino group regardless of the presence or the absence of the protein component. This shows that there is no specific interaction between the amino group in I and the hemoglobin molecule.

White *et al.*³⁾ proved the presence of aminophthalate ion in the oxidation products of luminol and suggested that the excited singlet state of the aminophthalate ion was the light-emitting species in the chemiluminescence. The present authors have synthesized 3-acetylaminophthalic acid as well as 3-aminophthalic acid, and have measured UV and fluorescence spectra under the same conditions as in the chemiluminescence reaction. The results are summarized in Table 1. The chemiluminescence maxima of I and II in water do not precisely agree with the corresponding fluorescence maxima measured after the chemiluminescence reaction; also, they are inconsistent with the fluorescence maxima of 3-aminophthalate and 3-acetylaminophthalate ion respectively. If the emitting species is the excited state of the 3-aminophthalate or the 3-acetylaminophthalate ion, the chemiluminescence maximum should be consistent with the fluorescence maximum of each substituted phthalate ion. Therefore, it is difficult to say that the emitting species is only the 3-aminophthalate ion or the 3-acetylaminophthalate ion.

TABLE 1. FLUORESCENCE AND CHEMILUMINESCENCE IN WATER

		Fluorescence nm	Chemiluminescence nm	UV absorption nm
Luminol (I)	a)	417	465	303, 348
	b)	430		349
3-Aminophthalic acid	a)	438*		305*
	b)	450*		—*
Acetyl-luminol (II)	a)	500	470	263, 326
	b)	490		—
3-Acetylamino-phthalic acid	a)	435		286 (sh.)
	b)	—		—

a) In 0.1 N NaOH aq. 10^{-4} mol/*l*.

b) Under the condition of the chemiluminescence, 5×10^{-5} mol/*l*.

* The same results were obtained for 3-aminophthalic acid hydrochloride.

Chemiluminescence in DMF-Water, In the case of the chemiluminescence in DMF-water (3 : 1 in vol.), hydrogen peroxide was not used.¹⁾

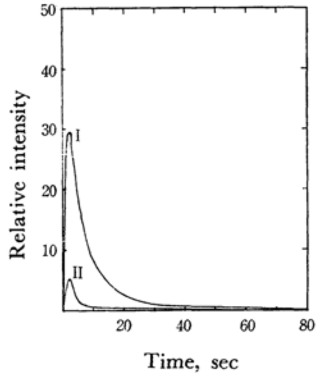

Fig. 4. Chemiluminescence intensity curves of luminol (I) and acetyl luminol (II) in DMF-water.

Final concentration: the sample 7.5×10^{-5}, NaOH 2.5×10^{-1}, $K_3Fe(CN)_6$ 2.27×10^{-3} mol/*l*·

As Fig. 4 shows, the total amount of light emission of I or II decreases compared with that in water. The ratio of the emission of I to that of II in water is about 6 : 1, while that in DMF-water is about 9 : 1. This shows that there is some specific interaction between the amino group and DMF.

In a previous paper¹⁾ the present authors reported the photochemical change of I and II by irradiation with a 365-nm ultraviolet ray. Now the variation in the fluorescence and UV spectra measured before (I and II) and after (I′ and II′) irradiation for 2 hr are shown on the left in Figs. 5 and 6, while the intensity curves of chemiluminescence measured before (I and II) and after (I′ and II′) irradiation are shown on the right in Figs. 5 and 6. A remarkable difference may be seen between Figs. 5 and 6.

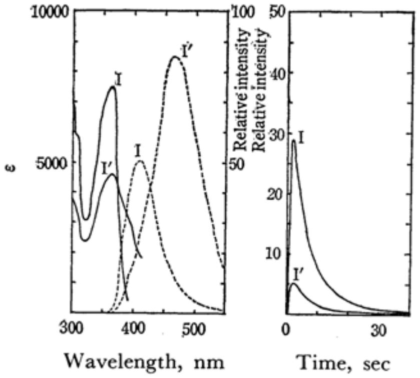

Fig. 5. Change of fluorescence (----) and UV spectra (——) (left) and chemiluminescence intensity curves (right) of luminol, before (I) and after (I′) irradiation for 2 hr.

Final concentration for fluorescence and UV: 10^{-4} mol/*l*; for chemiluminescence, the sample 7.5×10^{-5}, $K_3Fe(CN)_6$ 2.27×10^{-3}, NaOH 2.5×10^{-1} mol/*l*

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3) E. H. White and M. M. Bursey, *J. Am. Chem. Soc.*, **86**, 941 (1964); *J. Org. Chem.*, **31**, 1912 (1966).

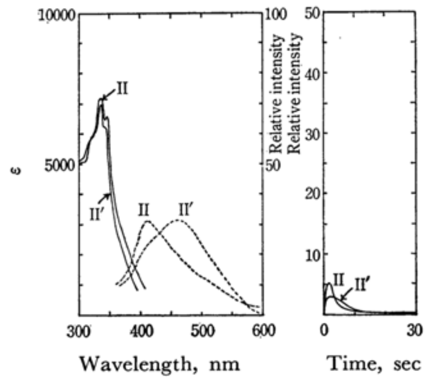

Fig. 6. Change of fluorescence (----) and UV spectra (——) (left) and chemiluminescence intensity curves (right) of acetyl luminol, before (II) and after (II') irradiation for 2 hr.
Final concentration for fluorescence and UV: 10^{-4} mol/*l*; for chemiluminescence, sample 7.5×10^{-5}, $K_3Fe(CN)_6$ 2.27×10^{-3}, NaOH 2.5×10^{-1} mol/*l*

The intensity of the chemiluminescence of I decreases after irradiation for 2 hr, while that of II does not change so much under the same conditions. The difference may be explained by assuming an interaction between the amino group and DMF. In the case of I this interaction may be considered to promote some destruction of the hydrazide ring indispensable to the chemiluminescence. This theory is supported by the fact that, in the absence of DMF, I is stable under irradiation with ultraviolet rays in the air.[4]

Furthermore, it can be pointed out that the photochemical product (I') is neither the intermediate of the chemiluminescence reaction nor 3-aminophthalic acid as is determined by comparing the UV and fluorescence spectra. In the case of II it is not clear why the fluorescence maxima measured before and after irradiation are different, whereas the UV and the chemiluminescence spectra measured before and after irradiation are similar. The fluorescence product (II') was proved to be different from 3-acetylaminophthalic acid by comparison of the UV and fluorescence spectra.

TABLE 2. CHEMILUMINESCENCE IN DMF-WATER

		Fluorescence nm	Chemiluminescence nm	UV absorption nm
Luminol (I)	a)	410	470	279, 293(sh.)
				353
	b)	475		360
3-Aminophthalic acid	a)	460*		347
	b)	470		—
Acetyl-luminol (III)	a)	412	480	333, 346
	b)	500		340
3-Acetylamino-phthalic acid	a)	460		308
	b)	480		—

a) In DMF, 10^{-4} mol/*l*.
b) Under the same condition as that of chemiluminescence, 7.5×10^{-5} mol/*l*.
* 3-Aminophthalic acid hydrochloride gave 453 nm.

As is shown in Table 2, the present work indicates that, in the chemiluminescence of I or II in DMF-water, the aminophthalate ion and the 3-acetylaminophthalate ion may be presumed to be the respective light-emitting species.

Experimental

All melting points are uncorrected.

5-Acetylaminophthalaz-1, 4-dione (II). Luminol (I) in DMF reacted with acetyl chloride in the presence of pyridine to afford faint yellow needle crystals, mp 315°C (decomp.).

5-Acetylamino-1-acetoxyphthalaz-4-one (III). I, reacted with an excess of acetyl chloride, gave light yellow crystals, mp 251°C (decomp.). UV: λ_{max}^{DMF} nm (ε) 329 (7700), 343 (7400). IR (KBr) 3420 (NH), 1760 (C=O), 1690 (C=N), 1530 (amide II), 1200 cm^{-1} (C–O–C).

5-Acetylamino-2-acetyl-phthalaz-1, 4-dione (IV). I (1 g), acetyl chloride (1 m*l*), and pyridine (5 m*l*) were mixed at 0°C; then the mixture was refluxed for 2 hr. The crystals which separated were recrystallized from pyridine to give colorless crystals, mp 260°C (decomp.).

Found: C, 55.12; H, 4.10; N, 15.94%. Calcd for $C_{12}H_{11}O_4N_3$: C, 55.17; H, 4.24; H, 16.09%. UV: λ_{max}^{DMF} nm (ε) 333 (9100), 347 (7700). IR (KBr): 3420 (NH), 1720 (C=O), 1520 cm^{-1} (amide II).

Reaction of Luminol with Acetic Anhydride (V). I (1 g), was refluxed with an excess of acetic anhydride (40 m*l*) at 120°C for 2 hr, then the mixture was cooled to afford faint yellow needles, mp 195°C.

Found: C, 58.65; H, 3.86; N, 17.06%. Calcd for $C_{12}H_9O_3N_3$; C, 59.26; H, 3.73; N, 17.28%. UV: λ_{max}^{DMF} nm (ε) 344 (6400). IR (KBr): 1730 (C=O), 1690 (C=N), 1200 cm^{-1} (C–O–C).

Acetyl Derivatives of Phthalaz-1, 4-dione. Mono-acetyl and diacetyl derivatives were synthesized by the method of Drew *et al.*,[2] and their IR spectra were measured: 4-acetoxyphthalaz-1-one, mp 178°C, IR (KBr): 1705 cm^{-1} (C=O). 2-Acetylphthalaz-1, 4-dione, mp 160°C, IR (KBr): 1760 (C=O), 1700(C=N), 1200 cm^{-1} (C–O–C). 4-Acetoxy-2-acetyl-phthalaz-1-one, mp 140°C, IR (KBr): 1765 (C=O), 1730 (C=O), 1695 (C=N), 1200 cm^{-1} (C–O–C).

3-Aminophtalic Acid. 3-Nitrophthalic acid (10 g), when reacted with stannous chloride (40 g) and hydrochloric acid (100 m*l*), gave 3-aminophthalic acid hydrochloride, which was then dissolved in a small amount of cold water to separate 3-aminophthalic acid, mp 187°C.[5]

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[4] T. Miyake, *Vacuum Chem. Japan*, **13**, 198 (1965).

[5] J. R. Scott and J. B. Cohen, *J. Chem. Soc.*, **1921,** 664.

3-Acetylaminophthalic Acid. Into 3-aminophthalic acid hydrochloride (1.5 g), dissolved in a 2 N sodium hydroxide solution (15 m*l*), a 2 N sodium hydroxide solution (30 m*l*) and acetic anhydride (3 m*l*) were alternately stirred over a 20 min period. After the solution had then been allowed to stand for 20 min, it was acidified with 5 N sulfuric acid and evaporated under reduced pressure at room temperature. From the residue 3-acetylaminophthalic acid was extracted with ethanol. The ethanol solution was concentrated at room temperature to separate 3-acetylaminophthalic acid, mp 206°C.

Found: C, 53.58; H, 4.34; N, 6.05%. Calcd for $C_{10}H_9O_5N$: C, 53.81; H, 4.06; N, 6.28%. UV: λ_{max}^{DMF} nm (ε) 313 (2600). IR (KBr): 3400 (NH), 1700 (C=O), 1625 (amide I), 1535 cm^{-1} (amide II).

Measurement of Chemiluminescence. *The Chemiluminescence in Water.* To an alkaline solution (2 m*l*) containing I or II (10^{-4} mol/*l* in a 0.1 N sodium hydroxide solution), hydrogen peroxide (1 m*l*) and then 1 m*l* of a 0.6% potassium ferricyanide solution were added. The chemiluminescence intensity curve was then measured at room temperature (20—25°C) by means of a Hitachi photomultiplier-amplifier equipped with an automatic recorder.

The Chemiluminescence in DMF-Water. An alkaline solution (1 m*l*) containing potassium ferricyanide (0.3% in a 1 N sodium hydroxide solution) was added to 3 m*l* of a DMF solution of I or II (10^{-4} mol/*l*). The temperature of the reaction mixture was 38—40°C. The chemiluminescence spectra were measured by a Hitachi recording spectrophotometer, EPS 2, equipped with a fluorescence attachment.