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Supplemental Studies on Relationship between Structure and Spectrum of Fluorescein[1]

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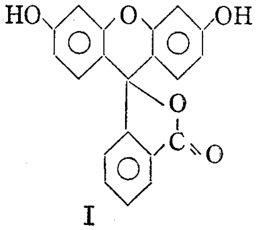
The relationship between the structure and the spectrum of fluorescein in aqueous solution was investigated by mainly comparing the absorption and fluorescence properties of fluorescein with those of monomethylfluorescein, dimethylfluorescein, and fluorescein methyl ester. Fluorescein was proved to be predominantly present in its univalent cation (V), neutral lactonoid (I), univalent anion (III) and bivalent anion (IV) forms at pH$<$1, pH 3, pH 5—6 and pH$>$8, respectively. All forms except I were fluorescent and IV fluoresced most intensely. The neutral quinonoid form (II) was not detected in aqueous solution against to the results of previous authors.

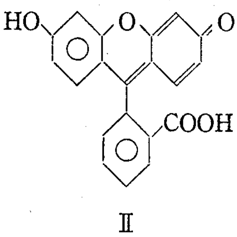
Keywords——fluorescein; monomethylfluorescein; dimethylfluorescein; fluorescein methyl ester; fluorescence; absorption; structure-fluorescence relationship; structure-absorption relationship

Fluorescein was first prepared[3] and assigned[4] to have a lactonoid structure (I) by Baeyer. On the other hand, a *p*-quinonoid structure (II) was also presented by Bernthsen,[5] Meyer,[6] Kropp and Decker,[7] and Pope and Howard,[8] when fluorescein was in its salts and esters.

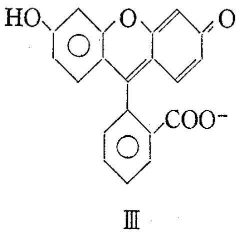
Ramart-Lucas[9] reported that the colorless lactonoid form (I) and the colored quinonoid form (II) were in equilibrium in aqueous solution of fluorescein. Based on the pH-dependent absorption spectra of fluorescein solution, Zanker and Peter[10] concluded that univalent anion



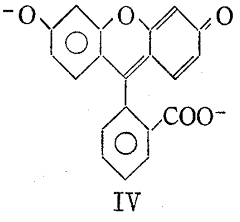
I



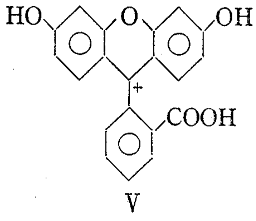
II



III



IV



V

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1) This work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978.
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
3) A. Baeyer, *Ber.*, **4**, 555 (1871).
4) A. Baeyer, *Ann.*, **212**, 351 (1882).
5) Bernthsen, *Chem.-Ztg.*, **16**, 1956 (1892).
6) R. Meyer, *Ber.*, **28**, 428 (1895).
7) W. Kropp and H. Decker, *Ber.*, **42**, 578 (1909).
8) F.G. Pope and H. Howard, *J. Chem. Soc.*, **97**, 1023 (1910); *ibid.*, **99**, 545 (1911).
9) P. Ramart-Lucas, *Compt. rend.*, **205**, 1409 (1937).
10) V. Zanker and W. Peter, *Chem. Ber.*, **91**, 572 (1958).

(III), bivalent anion (IV) and univalent cation (V) were additionally present in dioxane-water or water depending on pH. However, the absorption properties of these various forms remain obscure. Although the fluorescence properties of fluorescein have been much investigated[11-16] owing to its intense fluorescence, there are many deviations among these papers. Thus, the relationship between structure and fluorescence of fluorescein has not been fully elucidated.

In the present study, the authors attempted to clarify the above ambiguity by comparing the absorption spectra and fluorescence spectra of dimethylfluorescein (model of neutral lactonoid form), monomethylfluorescein (model of univalent anion), and fluorescein methyl ester (model of bivalent anion and neutral quinonoid form) with those fluorescein.

Experimental

Apparatus——In this study, a Hitachi MPF-2A Fluorescence Spectrophotometer and a Shimadzu UV-202 Spectrophotometer were used. The wavelengths indicated were uncorrected. The pH of solutions was checked with a Toa HM-5 pH Meter.

Materials——Fluorescein was obtained from Daiichi Pure Chemicals Co., Tokyo, and purified as reported by Orndorff, *et al.*[17] Monomethylfluorescein, dimethylfluorescein, and fluorescein methyl ester were prepared by Ullmann method,[18] Decker-Koch method[19] and conc. sulfuric acid-methanol method,[20] respectively, and purified by silica gel column chromatography and recrystallizations. All these compounds gave single spots on silica gel thin-layers. Their elemental analysis data and the yields obtained are shown in Table I. The structures of these fluorescein derivatives synthesized are shown in Chart 1.

Table I. Elemental Analysis Data of Purified Materials

	Calcd. (%)		Found (%)		Yields (%)[a]
	C	H	C	H	
Fluorescein (F)	72.28	3.64	72.01	3.76	—
Monomethyl F	72.83	4.07	73.10	4.15	24
Dimethyl F	73.33	4.48	73.56	4.55	32
F methyl ester	72.83	4.07	72.93	4.16	40

a) Yields of crude products of a single synthesis.

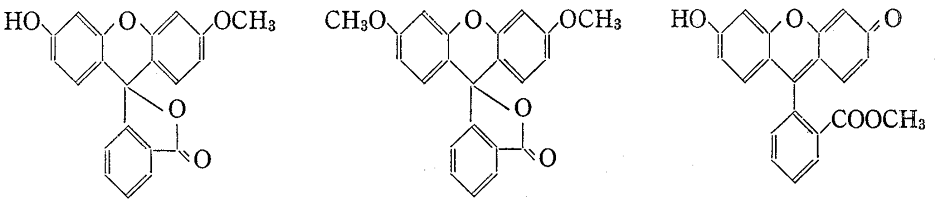
monomethyl fluorescein dimethyl fluorescein fluorescein methyl ester

Chart 1. Structures of Model Compounds

One molar perchloric acid, 0.1 M perchloric acid, McIlvain's solution, 0.2 M dibasic sodium phosphate, Ringer's solution, 0.1 M sodium hydroxide and 1 M sodium hydroxide were used as media for spectrophotometry.

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11) A. Boutaric and M. Roy, *Compt. rend.*, **209**, 162 (1939).
12) E. Shotton and A.F.S.A. Habeeb, *J. Pharm. and Pharmacol.*, **7**, 456 (1955).
13) M. Rozwadowski, *Acta Phys. Polon.*, **20**, 1005 (1961).
14) J. Glowacki and U. Kamińska, *Acta Phys. Polon.*, **23**, 43 (1963).
15) D. Težak and B. Težak, *Croat. Chem. Acta*, **36**, 59 (1964).
16) M.M. Martin and L. Lindqvist, *J. Lumin.*, **10**, 381 (1975).
17) W.R. Orndorff and A.J. Hemmer, *J. Am. Chem. Soc.*, **49**, 1272 (1927).
18) F. Ullmann, *Ann.*, **327**, 104 (1903).
19) H. Decker and O. Koch, *Ber.*, **40**, 4794 (1907).
20) W. Feuerstein and J. Wallach, *Ber.*, **34**, 2641 (1901).

Results and Discussion

Determination of Chemical Species of Fluorescein at Various pH's

In strong acid aqueous solutions, the absorption spectrum of fluorescein having a maximum at 437 nm was common to those of all of its methyl derivatives in Chart 1 (Fig. 1). Therefore, this spectrum should be attributed to the form V as reported by Zanker and Peter.$^{10)}$ In alkaline solutions, on the other hand, the spectrum of fluorescein became to have a maximum at 491 nm and was common to only that of its methyl ester (Fig. 1, a and d) and not to that of mono- and di-methyl fluorescein (Fig. 1, b and c). So, this spectrum should be attributed to the form IV.$^{8-10)}$ In a solution of pH 5, the spectrum of fluorescein had two peaks at 454 and 474 nm. The spectrum was common to that of monomethylfluorescein at pH 5—13, to that of fluorescein methyl ester at pH 4—5, and not to that of dimethylfluorescein which showed no absorption at this pH range. The results demonstrate that both the form III and form II of fluorescein should have the quite similar spectrum of peaks at 454 and 474 nm. Hence, if the form II were present in aqueous solution of fluorescein, it would have two absorption maxima between 454 and 477 nm at pH 4. The spectrum of fluorescein had an isosbestic point at 463 nm between pH 6 and 8, while it showed no isosbestic point in acidic media. All these data described above indicate that the spectrum of fluorescein at pH 5 is practically attributed to the form III, and that the neutral form of fluorescein is practically lactonoid. This was confirmed by the change of the absorbance at fixed wavelength with pH. Unlike fluorescein methyl ester (Fig. 4), fluorescein (Fig. 2) and monomethylfluorescein (Fig. 3) showed the formation of neutral species which had no absorption between 380 and 520 nm around pH 2—5. These findings differ from the supposi-

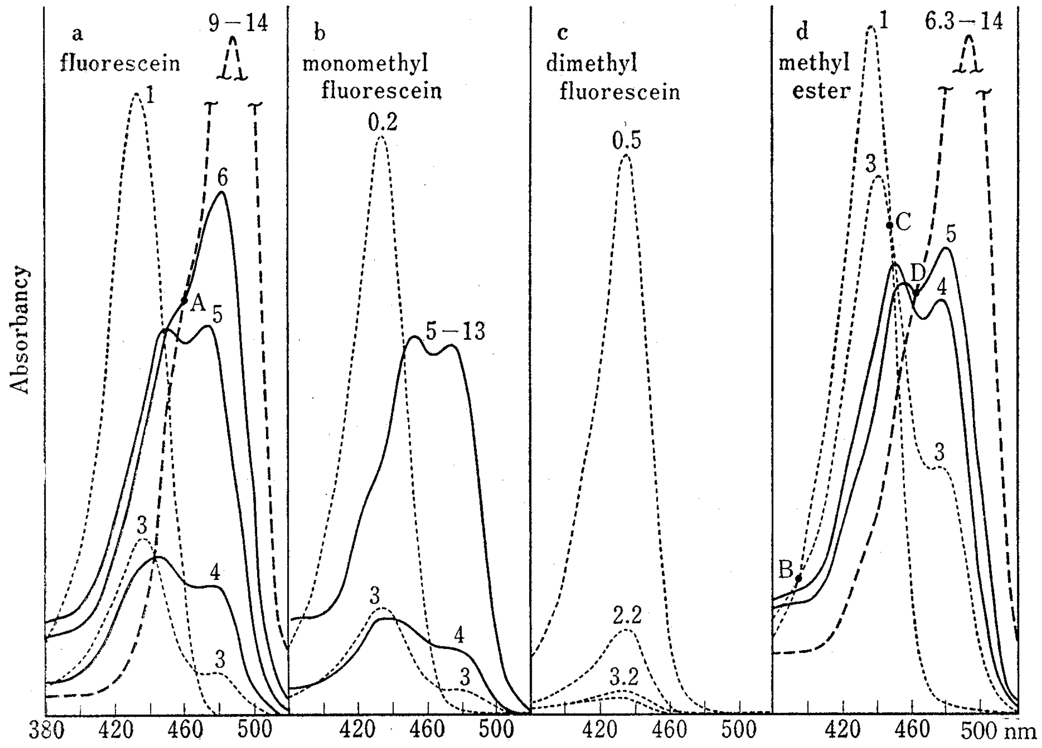

Fig. 1. Absorption Spectra of Fluorescein and Its Derivatives

The pH values of solutions are given on the peaks. The final concentration of dimethylfluorescein was 9.5×10^{-6} M (10% ethyl alcohol) and those of others are indicated in Fig. 2, 3, and 4. The isosbestic points are indicated by A, B, C, and D.

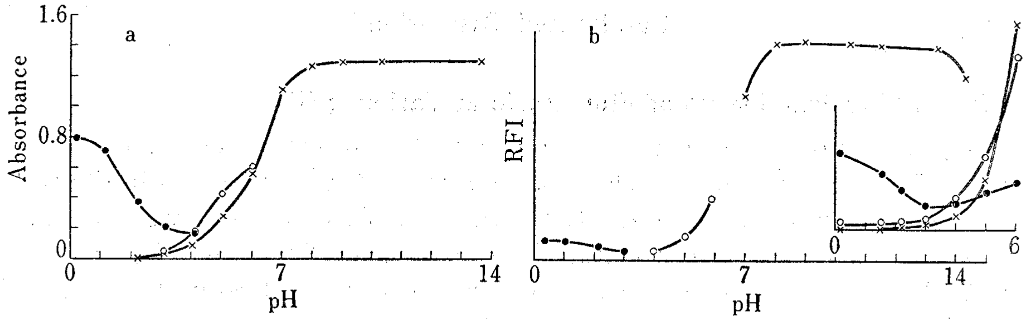

Fig. 2. Effect of pH on the Absorbance (a) and Fluorescence Intensity (b) of Fluorescein of Concentration 1.6×10^{-5} M and 3.1×10^{-7} M, Respectively

The absorbance was measured at 437 nm (●), 474 nm (○), and 491 nm (×). The fluorescence intensity was measured with excitation at 439 nm (●), 469 nm (○), and 490 nm (×), and emission at 512 nm.

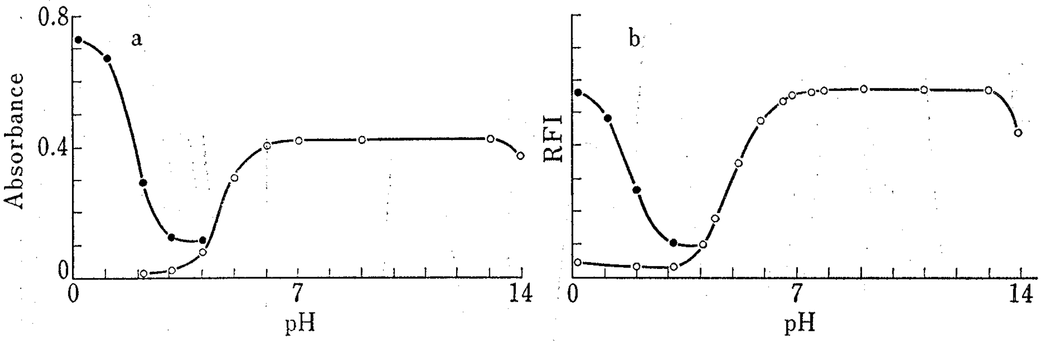

Fig. 3. Effect of pH on the Absorbance (a) and Fluorescence Intensity (b) of Monomethylfluorescein of Concentration 1.3×10^{-5} M (5% Ethyl Alcohol) and 1.1×10^{-6} M, Respectively

The absorbance was measured at 437 nm (●) and 475 nm (○). The fluorescence intensity was measured with excitation at 438 nm (●) and 469 nm (○) and emission at 512 nm.

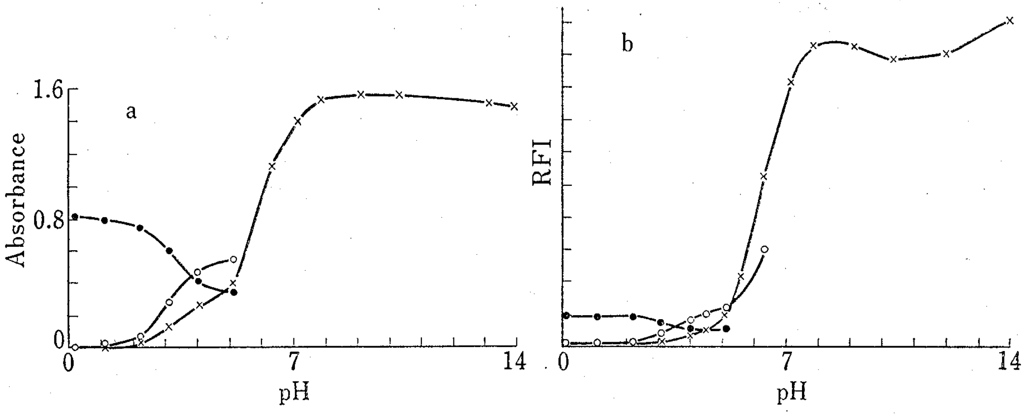

Fig. 4. Effect of pH on the Absorbance (a) and Fluorescence Intensity (b) of Fluorescein Methyl Ester of Concentration 1.6×10^{-5} M (5% Ethyl Alcohol) and 3.3×10^{-7} M, Respectively

The absorqance was measured at 438 nm (●) 475 nm, (○) and 493 nm (×). The fluorescence intensity was measured with excitation at 442 nm (●), 470 nm (○), and 494 nm (×), and emission at 516 nm.

tion of Orndorff *et al.*,22) Ramart-Lucas,9) Zanker *et al.*,10) Martin,16) Lindqvist,21) and Nagase *et al.*23) offering that neutral quinonoid form might be present in aqueous solution.

From above elucidation, we propose Chart 2 for the protolytic reactions of fluorescein in aqueous solution.

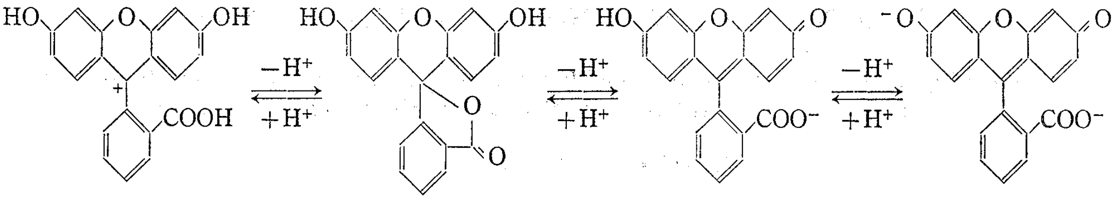

Chart 2. Proposed Protolytic Reactions of Fluorescein

Correlation between Chemical Species and Fluorescence Intensity of Fluorescein

As shown in Fig. 2b, except the form I, the forms III, IV and V emitted green-yellow fluorescence at 512 nm when they were excited at 439 nm, 469 nm and 490 nm, respectively, and fluorescein fluoresced most intensely in the form IV. In the solution of pH 9, the fluorescence intensity of fluorescein, present thoroughly in the form IV, was similar to that of fluorescein methyl ester, and about eight times stronger than that of monomethylfluorescein on the molar basis. The results indicated that the fluorescence intensity of the form IV is about eight times stronger than that of the form III, although the latter form of fluorescein is not able to exist alone in an aqueous solution.

Conclusion

Based on the above findings, it is concluded that

1) the neutral quinonoid form (II) is not detected in the aqueous solution of fluorescein against to the results of previous authors.

2) except neutral lactonoid form (I), all other forms are fluorescent.

3) fluorescein fluoresces most intensely in its bivalent anion form (IV).

4) the univalent cation (V), neutral lactonoid form (I), univalent anion (III), and bivalent anion (IV) of fluorescein are predominantly present in aqueous solutions of $pH<1$, pH 3, pH 5—6 and $pH>8$, respectively.

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21) L. Lindqvist, *Arkiv Kemi*, **16**, 79 (1960).
22) W.R. Orndorff, R.C. Gibbs, and C.V. Shapiro, *J. Am. Chem. Soc.*, **50**, 819 (1928).
23) Y. Nagase, T. Ohno, and T. Goto, *Yakugaku Zasshi*, **73**, 1033 (1953).