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Fluorescence and Structure of Proteins as measured by Incorporation
of Fluorophore. III.¹⁾ Fluorescence Characteristics
of N-[*p*-(2-Benzoxazolyl)phenyl]maleimide
and the Derivatives²⁾

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Among fluorescent-labeled sulfhydryl reagents hitherto synthesized in this laboratory, N-[*p*-(2-benzoxazolyl)phenyl]maleimide (I) was selected as a typical example and its fluorescent characteristics were determined. In contrast to that I shows no substantial fluorescence, its cysteine ester-adduct (II) exhibits strong fluorescence with emission maxima at 352 and 365 mμ. This spectroscopic behavior of this type of reagent may be favorably utilized in the application to proteins. Since N-[*p*-(2-benzoxazolyl)phenyl]succinimide (III) is regarded as the simplest model compound of the adducts of I with sulfhydryl substrates, fluorescent spectra of III in various media were examined. The absorption spectra of III were also systematically examined by comparing spectra of IV, VII, VIII and III. General procedures of fluorescence determination were described and interpretation of the spectra was discussed.

In an attempt to find a novel approach to studies of structure and function of proteins, several maleimide derivatives containing benzazole moieties have been synthesized as fluorescence-labeled sulfhydryl reagents.^{1,4)} Some of the reagents were shown to react readily with cysteine derivatives, simple model system, and further, with certain protein and enzyme system.^{1,5)} The present paper describes fluorescence characteristics of N-[*p*-(2-benzoxazolyl)phenyl]maleimide (I), one of the above reagents as a typical example.

Experimental

Materials

Preparation of N-[*p*-(2-benzoxazolyl)phenyl]maleimide (I), S-[N-[*p*-(2-benzoxazolyl)phenyl]succinimido]cysteine ethyl ester hydrochloride (II), N-[*p*-(2-benzoxazolyl)phenyl]succinimide (III), N-[*p*-(2-benzoxazolyl)phenyl]succinamic acid (IV), 6-[[*p*-(2-benzoxazolyl)phenyl]carbamoylmethyl]-5-oxo-3-thiomorpholinecarboxylic acid ethyl ester (V), and 2-(*p*-aminophenyl)benzoxazole (VIII) was described in a previous paper.¹⁾ Samples used for fluorescence measurement were analytically pure.

Quinine sulfate (VI) was purchased from Iwaki Co. (Pharmacopoeia Japonica grade).

Urea (Koso Chemical Co. (guaranteed reagent)), guanidine hydrochloride (Nakarai Chemicals Co. (guaranteed reagent)) and guanidine sulfate (Kanto Chemical Co. (extra pure reagent)) were used without purification.

- 1) Part II: Y. Kanaoka, M. Machida, Y. Ban, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **15**, 1738 (1967).
- 2) Presented before the 87th Meeting of Japan Pharmaceutical Association, Abstr. p. 431, April, 1967, Kyoto.
- 3) Location: a) *Kita-12, Nishi-6, Sapporo*; b) *Kita-12, Nishi-7, Sapporo*; c) Present address: *Department of Chemistry, Faculty of Science, Tohoku University, Sendai*; d) *Hongo, Tokyo*.
- 4) Y. Kanaoka, T. Sekine, M. Machida, Y. Soma, K. Tanizawa, and Y. Ban, *Chem. Pharm. Bull.* (Tokyo), **12**, 127 (1964).
- 5) a) T. Sekine, M. Machida, and Y. Kanaoka, in preparation; b) T. Sekine, K. Kuroda, Y. Kanaoka, and M. Machida, "The 17th Symposium on Protein Structures," Abs. p. 41, Nov., 1966, Kyoto; c) T. Sekine, Y. Kanaoka, T. Kameyama, M. Machida, and A. Takada, "The 7th International Congress of Biochemistry," Abstr. p. 773, Aug., 1967, Tokyo.

2-Phenylbenzoxazole (VII) was prepared from *o*-aminophenol and benzoic acid to form colorless prisms of mp 101° (lit.,⁹) mp 102°).

Methods

Measurement of Fluorescence—Fluorescence spectra were measured with a spectrofluorometer consisting of a Hitachi EPU-2 quartz monochromator and a Hamamatsu TV R-106 photomultiplier. Excitation was provided by the combination of an Ushio 150 watt xenon lamp and a Hitachi G-3 grating monochromator. The fluorescence was detected at right angles to the excitation beam.

To obtain the "true fluorescence spectra," the monochromator-photomultiplier combination was calibrated by measuring the fluorescence spectra of standard substances⁷⁾ of known spectral distribution in relative quanta per unit wave number. The correction factor thus obtained was applied to apparent spectra. The detail of the calibration procedure was explained elsewhere.⁸⁾

Absorption spectra were recorded on a Hitachi EPS-3 spectrophotometer.

Determination of Fluorescence Quantum Yield—Fluorescence quantum yield was determined by the method of Parker and Rees,⁹⁾ in which it is assumed that the integrated area (*A*) under the fluorescence emission curve is proportional to the total intensity of fluorescent light (*F*) emitted by the solution. *F* is proportional to the product, $I_0 \cdot \phi \cdot \epsilon \cdot c \cdot d$, where I_0 = intensity of exciting light, ϕ = quantum yield of fluorescence, ϵ = molar extinction coefficient, c = concentration, and d = optical path length. With the same apparatus and at the same intensity of excitation light, *I*, the ratio of the two fluorescence intensities is given by the equation:

$$\frac{F_1}{F_2} = \frac{A_1}{A_2} = \frac{\phi_1 \epsilon_1 c_1 d_1}{\phi_2 \epsilon_2 c_2 d_2} = \frac{\phi_1}{\phi_2} \cdot \frac{OD_1}{OD_2} \quad (1)$$

Hence,

$$\frac{\phi_1}{\phi_2} = \frac{A_1/OD_1}{A_2/OD_2} \quad (2)$$

This equation can be applied only to sufficiently dilute solution. If one of the value of quantum yield, say ϕ_1 , is already known, the other yield is easily determined. The fluorescence quantum yield of the reference quinine solution is known to be 0.55 at room temperature.¹⁰⁾

Values for optical density (OD) at the excitation wave length and of the area under the observed emission spectral curve were determined for each compound based on the corrected spectra as described in the preceding paragraph. The excitation wave length was maintained constant at 310 μ with a constant slit width.

Since the intensity of the xenon lamp varied because of fluctuation in line voltage and increasing age of the lamp during the period of measurement, a correction was made using a reference solution. The fluorescence of a reference solution (quinine bisulfate solution in 0.1 N sulfuric acid) was recorded at the same time as the measurement of a sample.

- 6) F.F. Stephens and J.D. Bower, *J. Chem. Soc.*, **1949**, 2971.
- 7) E. Lippert, W. Nagele, I. Seibold-Blankenstein, U. Staiger, and W. Voss, *Z. Anal. Chem.*, **170**, 1 (1959).
- 8) H. Kokubun and M. Kobayashi, *Ohyodenki Kenkyusho Iho*, **18**, 117 (1965).
- 9) C.A. Parker and W.T. Rees, *Analyst*, **85**, 587 (1960).
- 10) W.H. Melhuish, *New Zealand J. Sci. Technol.*, **37.2B**, 142 (1955).

Results and Discussion

Fig. 1 shows fluorescence spectra of the addition product of the reagent to cysteine (II) and related compounds (III—V). Table I contains quantum yields of these compounds

TABLE I. Fluorescence Quantum Yields of Benzoxazole Derivatives in Ethanol

Compound	Relative fluorescence intensity	Quantum yield (ϕ)
II	1.59	0.87
III	1.62	0.89
IV	1.55	0.85
V	1.62	0.89
VI ^{a)}	1.00	0.55

a) in 0.1 N sulfuric acid

calculated as described in the experimental section.

Comparison of fluorescence spectra of a series of compounds shown in Fig. 1 and Table I reveals the significant aspects of the fluorescent characteristics of the reagent (I) and the derivatives. In contrast to that I shows no substantial emission, its cysteine ester-adduct, II exhibits strong fluorescence of a high quantum yield in ethanol solution with emission maxima at 352 and 365 m μ and a shoulder at 330 m μ . If the reagent (I) is applied to a solution of protein or enzyme (P), therefore, only the incorporated fraction of I (F), which forms a new covalent bond with sulfhydryl group in protein, should exhibit fluorescence as outlined in the Chart 1. This relationship in spectroscopic behavior between I and its protein-conjugate (F) may have definite advantage in that one can conveniently discriminate the reagent-incorporated protein (F) from intact portion (P) by fluorometric technique.

Chart 1

Since high quantum yield values were obtained for all derivatives (II—V) in Table I except the reagent itself (I), it may be reasonably postulated from comparison of chemical structures of I—V that the maleimide moiety of I involving the olefinic *endo* double bond is responsible

Fig. 1. Fluorescence Spectra of Benzoxazole Derivatives in Ethanol Solution

OD values at the wave length of excitation (310 m μ) of sample solutions were 0.202, 0.315, 0.406, 0.359 and 0.365 for II —, III ·····, IV — — —, V - - - - and VI - · - ·, respectively.

for the observed quenching of emission of I. It has been well accepted that N-substituted maleimide derivatives generally react rapidly and specifically with sulfhydryl groups in proteins by an electrophilic addition mechanism to give an addition product¹¹⁾ as illustrated in Chart 1. These adducts may be looked upon as N-substituted succinimide derivatives in nature. Based on the above results and discussion, N-[*p*-(2-benzoxazolyl)phenyl]succinimide (III) was selected as a key compound in investigating spectroscopic properties of these derivatives because III is apparently the simplest parent compound of N-substituted succinimides of this series.

First, comparison of the absorption and emission spectra of a series of derivatives related to III was made in order to analyze the chromophore system of III. At least in a formal sense, III is regarded as a derivative of 2-phenylbenzoxazole (VII). Namely, the parent compound (VII) is assumed to be aminated to give VIII, which is then monoacylated to form IV, followed by cyclization or diacylation leading ultimately to III.

The ultraviolet absorption spectra of above series of compounds (VII, VIII, IV, and III) are shown in Fig. 2. In going from VII to VIII, an enhanced shift to longer wave length with increased intensity was observed in agreement with the well known substitution effect of amino group on aromatic nucleus.¹²⁾ Conversely, monoacylation of the above-introduced amino group resulted in blue shift as seen in the absorption of IV, which further showed progressive blue shift accompanied by hypsochromic effect by the diacylation in going to III.

Fig. 2. Ultraviolet Absorption Spectra of 2-Phenylbenzoxazole (Parent Compound) and Its *p*-Substituted Derivatives

III ————— IV ·········
VII ————— VIII - - - - -

Fig. 3. Fluorescence Spectra of Benzoxazole Derivatives at 77°K

Intensities are in arbitrary units, solvent, ethanol
III ————— VII ·········
VIII - - - - -

The fluorescence spectra of them are shown in Fig. 3. In consistent with the data in the absorption, the emission spectrum of VII showed a red shift in going to VIII, then showed blue shift in going from VIII to III. The spectra in Fig. 3 were measured at low temperature under cooling with liquid nitrogen and show distinct vibrational structures, which become blurred at a room temperature. The fluorescence spectra thus obtained satisfy the mirror image relation to the longest absorption band suggesting that the absorbing and emitting chromophores are essentially of the same origin.

11) R. Cecil, "The Proteins," Vol. 1, ed. by H. Neurath, Academic Press, N.Y., 1963, p. 402.

12) H.H. Jaffe and M. Orchin, "Theory and Applications of Ultraviolet Spectroscopy," J. Wiley, N.Y., 1962.

This parallel behavior of absorption and fluorescence spectra of the series of compounds indicates that the spectra of III can be largely interpreted as consisting of the parent chromophore (VII) substituted with diacylated amino group.

In his spectral studies, Matsuo concluded that the longest wave length absorption band of N-substituted maleimides are due to the $n \rightarrow \pi^*$ transition of the carbonyl group.¹³⁾ This transition is of course perturbed by the $\pi \rightarrow \pi^*$ transition of the cyclic imide system in various way as a nature of a substituent at the nitrogen atom is varied. The maximum of this band is located at around 300 m μ as Fig. 4 shows an example. On the other hand, VII, the parent chromophore system of the reagent (I) as discussed above, also has an absorption maximum at around 300 m μ . By formation of compound I, thus, the $^1(n \rightarrow \pi^*)$ state will become the lowest excited state or that lying close to the $^1(\pi \rightarrow \pi^*)$ state. Radiationless transition to the $^3(\pi \rightarrow \pi^*)$ state through the $^1(n \rightarrow \pi^*)$ state might be responsible for the non fluorescent character of I. This kind of interpretation has been applied to many cases.¹⁴⁻¹⁶⁾

An absorption maximum of succinimide was observed at about 240 m μ with $\log \epsilon \approx 2$ in aqueous solution.¹⁷⁾ This band may be assigned as due to $n \rightarrow \pi^*$ transition judging from its relatively low absorption intensity. Consequently, the $^1(n \rightarrow \pi^*)$ state of succinimide must be much higher in

Fig. 4. Ultraviolet Absorption Spectra of Maleimide and Succinimide Derivatives

I ——— NEM
III - - - - -

Fig. 5. Comparison of the Fluorescence Spectra of III in Various Solvents

III (11.415 mg) was dissolved in 25 ml of ethanol to make a stock solution. 0.25 ml of the stock solution was diluted to 25 ml with each solvent shortly before measurement. All spectra were calibrated with respect to the same OD value (0.42) at 310 m μ . Intensities are in arbitrary units.

ethanol ——— tetrahydrofuran
glycerine - - - - -

Fig. 6. Change of the Fluorescence Spectra of III by the Addition of Urea and Guanidine Salts

0.25 ml of the stock solution was diluted to 25 ml of various aqueous solution shortly before measurement. Spectra were obtained as in the case of Fig. 5. OD value was 0.385. 25 ml of aqueous solution of guanidine sulfate contains 0.1 ml of conc. sulfuric acid.
water ———, 8 M urea and 4 M urea
1 M guanidine hydrochloride - - - - -, 4 M guanidine
(lowest), 1 M guanidine sulfate - - - - -

13) T. Matsuo, *Bull. Chem. Soc. Japan*, **38**, 557 (1965).

14) N. Mataga and S. Tsunoi, *Bull. Chem. Soc. Japan*, **30**, 368 (1957).

15) K. Brederick, Th. Förster, and H.-G. Oesterlin, "Luminescence of Organic and Inorganic Materials," ed. H.P. Kellmann, G. Marmor Spruch, J. Wiley, N.Y., 1962, p. 161.

16) H. Kokubun and M. Kobayashi, *Z. Phys. Chem. (Frankfurt)*, **41**, 245 (1964).

17) J. Schurz, A. Ullrich, and H. Bayzer, *Monatsh. Chem.*, **90**, 29 (1959).

energy than that of maleimide. This fact may indicate that the $^1(n \rightarrow \pi^*)$ state in III lies much higher in energy than that in I. As a result, the probability of intersystem crossing through $^1(n \rightarrow \pi^*)$ as discussed in the case of I may decrease to a great extent in III. This interpretation may tentatively provide a general explanation for the marked difference in fluorescence characteristics of the maleimide system (I) and the adduct (F).

Effects of various solvents on the fluorescence of the key compound (III) were next examined. Fig. 5 and 6 show fluorescence spectra of III in tetrahydrofuran, ethanol, glycerine and water. The spectrum in tetrahydrofuran solution had a vibrational structure, whereas those measured in protic solvents such as water or alcohol had more broad shapes. As a whole, however, change in dielectric constant or viscosity of solvents seemed to have no significant influence on the emission of III.

Dependence of the fluorescence of III in aqueous solution upon both urea and guanidine salt was tested since aqueous solutions containing certain concentrations of urea or guanidine salt are the most commonly used media in studies of denaturation of proteins. As seen in Fig. 6, the presence of 4 M or 8 M urea did not appear to affect largely the intensity of the fluorescence of III. Although the fluorescence in 4 M urea solution decreased to about 71% in quantum yield to that in aqueous solution, no more quenching was observed in 8 M urea solution, both fluorescence spectra being practically identical. Addition of guanidine hydrochloride, however, produced more pronounced decrease in intensity as shown in Fig. 6. The presence of guanidine hydrochloride in 4 M concentration, a typical condition for protein denaturation, showed quenching effect up to 37% in terms of quantum yield. Therefore, these results obtained with this simple model system concerning the influence of medium upon the fluorescence of the incorporated fluorophore must be considered when the reagent is applied to protein system. Quantum yield values for these solvent perturbation are listed in Table II.

TABLE II. Relative Fluorescence Intensity of III in Various Solvents

Solvent	Relative fluorescence intensity	Solvent	Relative fluorescence intensity
Ethanol	1	8 M Urea	0.94
Dioxane	0.97	4 M Urea	0.93
Tetrahydrofuran	0.87	1 M Guanidine hydrochloride	0.73
Glycerine	0.79	4 M Guanidine hydrochloride	0.49
Water	1.32	1 M Guanidine sulfate	0.76
Water	0.67 ^{a)}	4 M Guanidine sulfate	0.69

a) compound IV

At present it is uncertain whether the observed quenching is due to chloride ion, guanidinium ion, or other reasons. Though measurement with guanidine bisulfate was made in an attempt to find a role of anion in the quenching, results were ambiguous since the bisulfate had poor solubility to give solutions of necessary concentration. The curve in Fig. 6 was obtained in 0.144 N sulfuric acid solution. Other factors such as pH dependence or effect of oxygen, which might substantially influence fluorescent properties, are still remained for further study.

Succinimide ring can be easily hydrolyzed particularly in alkaline medium. After incorporation of the reagent to protein sulfhydryl group, a newly formed succinimide ring (F) would be further opened hydrolytically either owing to pH of medium or some interaction of neighboring group participation in protein molecule as shown in the Chart 2. Fig. 7 gives fluorescence spectra of III and IV in aqueous solution, which are regarded as model systems