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Fluorescence Properties and Reactions of a Novel Fluorescence Thiol Reagent, N-(7-Dimethylamino-4-methyl-3-coumarinyl)maleimide(DACM)[1]

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N-(7-Dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) was applied to egg albumin and it was found that approximately 0.6 mol of DACM was incorporated into the protein molecule as revealed by chemical and spectroscopic analyses. DACS as the model compound of the reaction product of DACM with thiols was used for dependency of pH and solvent.

Keywords——maleimide-type; egg albumin; spectroscopic measurement; pH dependency; imide hydrolysis; Boyer's PCMB method; amino acid analysis

In a previous paper[1] we have described that N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM) **1** is a preferable fluorescent thiol reagent of maleimide-type which has fluorescent maxima in a significantly longer wavelength region than those of the "intrinsic" fluorescence of protein. In the present paper we report the reaction of DACM with thiol substrates including small to macro molecular compounds, and detailed fluorescence characteristics of these addition products as well as those of the related model compounds.

Experimental

Materials——Preparation of N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) **1**, N-(7-dimethylamino-4-methyl-3-coumarinyl)succinimide (DACS) **2** and N-(7-dimethylamino-4-methyl-3-coumarinyl)succinamic acid (DACSA) **3** was described in a previous paper.[1a] Five times crystallized egg albumin was a commercial product of Nutritional Biochemical Co. lot No. 9336. The concentration of egg albumin was determined spectrophotometrically using $E^{1\%}_{1cm}$ 7.35 at 280 nm.

Preparation of DACM-Treated Egg Albumin (4b or 5b)——Egg albumin (45 mg) was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0) and treated with 1.6 ml (8 fold molar excess for protein) of monoglyme solution of DACM (5 mM) under cooling. After the reaction mixture was stood in a cold room for 3 days, the reaction mixture was applied to a Sephadex G-25 column (10 g) and eluted with water. Fractions containing material which absorbs at 280 nm and 350 nm were combined and lyophilized.

Spectroscopic Measurements——Fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer MPF-2A. Absorbances of the solution were kept below 0.2 at the excitation wavelength. Absorption spectra were recorded on a Shimadzu double beam UV-200 spectrophotometer.

Titration of the Thiol Groups of Egg Albumin with *p*-Chloromercuribenzoic Acid (PCMB)——The content of the thiol groups of egg albumin was estimated spectrophotometrically by the PCMB method of Boyer.[3,4] A solution of PCMB (9 mg) in 0.1 ml of 1 N NaOH and 0.9 ml of 0.15 M sodium pyrophosphate was diluted

1) *a*) Fluorescent Thiol Reagents. XIII. Part XII: M. Machida, N. Ushijima, T. Takahashi, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **25**, 1289 (1977); *b*) Preliminary communication: M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *ibid.*, **23**, 1385 (1975); *c*) The abbreviations used are: DACM=**1**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)maleimide; DACS=**2**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)succinimide; DACSA=**3**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)succinamic acid; PCMB=*p*-Chloromercuribenzoic acid.

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3) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

4) M. Machida, T. Sekine, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **22**, 2642 (1974).

to 10 ml with deionized water. To 4 ml of this solution was added 0.5 ml of 0.15 M sodium pyrophosphate and the total volume was made to 10 ml with 0.33 M acetate buffer (pH 4.6). For spectrophotometric titrations, aliquots of a PCMB solution were transferred into two 1 cm cells. One was used for the reference, the other for the sample, and each of cells contained buffer solution and protein in acetate buffer. The absorbancy of sample containing a protein in 0.33 M acetate buffer was read periodically at 250 nm against a blank (reference) containing same volume of PCMB solution added, and the absorption values were corrected for the volume change of the reagent added.

pH Dependency of the Absorption and Fluorescence Spectra of DACS (2)——Absorbances and fluorescence intensities were monitored at 396 nm and 478 nm, respectively. A stock solution of 6×10^{-4} M was prepared by dissolving 2 in monoglyme. For measurement of absorption and fluorescence spectra 0.1 ml of the stock solution was diluted to 10 ml with various buffer solutions. Excitation wavelength was 396 nm.

Reaction Rates of the Imide Hydrolysis——Kinetic measurements of the imide hydrolysis were carried out in 1 cm cell using a Hitachi 356 two-wavelength double beam spectrophotometer, equipped with a water jacketed cell holder connecter to a circulating constant temperature bath (Komatsu-yamato Coolnics Thermo-Bath CTE-2). Operational mode of the instrument was set at different wavelengths of $\lambda_1=383$ nm and $\lambda_2=415$ nm (nonscanning). A stock solution (0.07 ml) of 5 mM DACM was made to 10 ml with various buffer solutions which were warmed prior to each kinetic run. A mixture was vigorously shaken in a flask for several seconds, transferring into 1 cm cell and immediately measured at 30°.

In experiments to see the effect of pH on the absorption and fluorescence spectra of DACS (2) and on the hydrolysis of DACM (1) the following buffers were used; 0.1 M H_3PO_4–KH_2PO_4 for the range of pH 1.5—4.0, 0.1 M citric acid–Na_2HPO_4 for pH 4.8—6.0, 0.1 M KH_2PO_4–Na_2HPO_4 for pH 6.5—8.0 and 0.1 M sodium borate–KH_2PO_4 for pH 9.0—9.7.

Reaction Rates of DACM (1) with N-Acetyl-L-cysteine——A solution of 1 mM N-acetyl-L-cysteine (0.05—0.5 ml; 5—50 eq. mol) was transferred into 1 cm cell and the total volume was made to 3 ml with 0.1 M phosphate buffer (pH 7.0). To this solution was added 0.01 ml of 1 mM DACM in monoglyme and simultaneously the fluorescence enhancement was automatically recorded as a function of time at 25°.

Amino Acid Analysis——Amino acid analysis was performed by the method and on the same instrument as described in the previous paper.[4]

Results

Absorption Spectra of the DACM-Treated Protein

When maleimide-type reagents are covalently introduced in proteins, a question is raised whether the initially formed succinimide ring **4** is retained or further hydrolyzed to the succinamic acid form **5** (Chart 1). We have already proposed the certain succinimide and succinamic acid derivatives as simple model compounds of the thiol adduct,[5] and in this spectroscopic studies DACS (**2**) and DACSA (**3**) were selected as the model compounds for the imide type and the ring-opening type, respectively. The molar absorptivity used for calculation

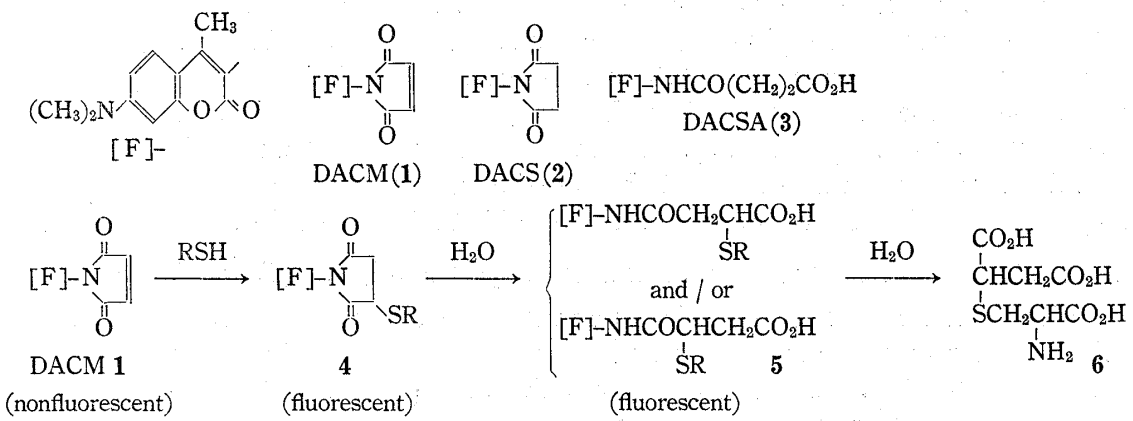

Chart 1

5) Y. Kanaoka, M. Machida, H. Kokubun, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **16**, 1747 (1968).

of the content of the thiol groups in proteins were ε=20800 for DACSA (3) and ε=20900 for DACS (2) at 380 nm. For determining the concentration of DACM-treated proteins the absorption at 280 nm was corrected for turbidity and contribution of absorption of the DACM chromophore at this wavelength. Figure 1 shows the absorption spectra of the modified egg albumin, DACS (2) and DACSA (3). The absorption spectrum of protein is almost superimposable on that of DACS (2). On the basis of molar absorptivity of DACS (2) and DACSA (3) the extent of labeling was 0.5 mole per mole of the DACM-treated egg albumin.

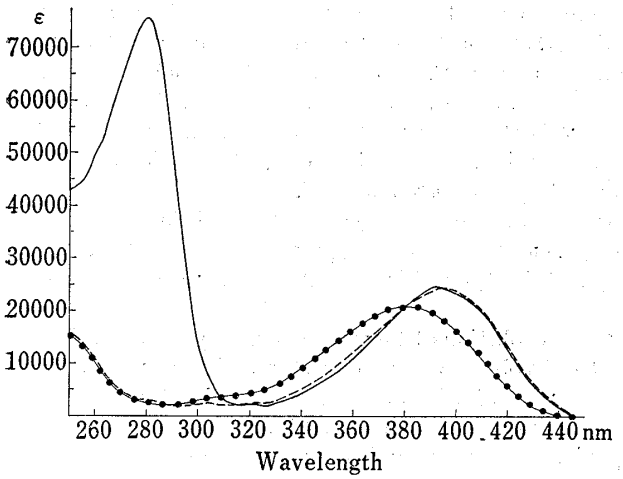

Fig. 1. Absorption Spectra of DACM-Treated Egg Albumin, DACSA and DACS in 0.1 M Phosphate Buffer (pH 7.0)

———: DACM-treated egg albumin.
– – – – –: DACS (2).
—●—●—: DACSA (3).

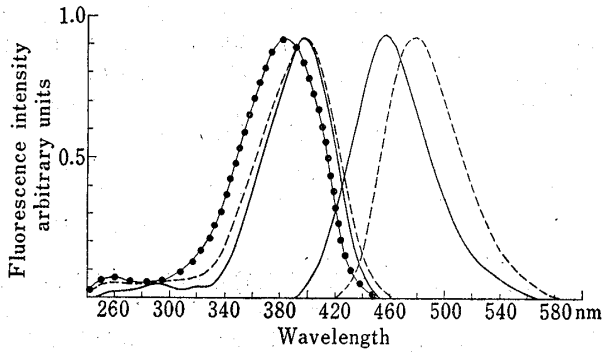

Fig. 2. Fluorescence Excitation and Emission Spectra of DACM-Treated Egg Albumin, DACSA and DACS in 0.1 M Phosphate Buffer (pH 7.0)

———: DACM-treated egg albumin.
– – – – –: DACS (2).
—●—●—: DACSA (3).

Fluorescence Spectrum of the DACM-Treated Protein (4b or 5b)

Since the fluorescence spectrum of DACS (2) is superimposable on that of the addition product of DACM with N-acetyl-L-cysteine, as expected, DACS and DACSA can serve as the model compounds of fluorescence. Although fluorescence emission spectra of DACS and DACSA are superposed from each other, the excitation spectrum of DACSA is blue shifted compared with that of DACS (Fig. 2). While the excitation spectrum of the DACM-treated egg albumin is similar to that of DACS, the emission maximum of the modified protein considerably shifted to the blue compared with those of DACS and DACSA.

Solvent Effects on the Absorption and Fluorescence Spectra of DACS (2) and DACSA (3)

In order to examine whether DACS (2) and its derivatives can be used as the models of indication of microenvironmental polarity, their absorption and fluorescence spectra were measured in various solvents. Table I shows the absorption and fluorescence maxima of DACS and DACSA in various solvent systems. As a percentage of nonpolar solvent in water is increased, absorption maxima of DACS and DACSA are first red shifted then blue shifted, while fluorescence maxima of those are blue shifted. The variation observed in the absorption maxima of DACSA in different solvents is smaller than those observed in those of DACS. This phenomenon is observed also in their fluorescence maxima. It must be noted, however, that the polarity dependency of the fluorescence maxima of dimethylaminocoumarin derivatives is less significant than those of N-arylaminonaphthalene derivatives.[6]

6) D.C Turner and L. Brand, *Biochemistry*, 7, 3381 (1968).

Table I. Absorption and Fluorescence Maxima of DACS (2) and DACSA (3) in Various Solvent Systems[a]

Solvent system	DACS		DACSA	
	λ_{abs}^{max} (nm)[b]	λ_{em}^{max} (nm)[c]	λ_{abs}^{max} (nm)[d]	λ_{em}^{max} (nm)[e]
Water	395	477	382	476
EtOH–water (10%)	396	475	381	473
EtOH–water (20%)	396	473	383	472
EtOH–water (30%)	394	471	383	469
EtOH–water (40%)	393	468	381	468
EtOH–water (50%)	391	467	380	466
EtOH–water (60%)	390	465	380	465
EtOH–water (70%)	388	464	379	464
EtOH–water (80%)	386	462	378	461
EtOH–water (90%)	384	459	376	459
EtOH	380	456	369	456
MeOH	381	460	372	459
2-Propanol	378	450	368	452
CH_3CN	375	446	368	449
Acetone	373	441	365	446
Benzene	364	414	363	436
Monoglyme	368	432	362	441

a) The wavelength of absorption band in the longest wavelength.
b) 0.07 ml of 5×10^{-3}M DACS in monoglyme was diluted to 10 ml with various solvents.
c) Further, 0.5 ml of solution used to measuring the absorption spectra was mixed with 2 ml of various solvents. Excitation wavelength was 380 nm. In a EtOH–water solvent system 0.1 ml of 6×10^{-4}M DACS in monoglyme was diluted to 10 ml with ethanol containing water of various concentration (6×10^{-6}M).
d) A stock solution of 2×10^{-3}M DACSA was prepared by dissolving the succinamic acid derivative in diglyme containing ethanol (10% v/v) and 0.2 ml of this solution was diluted to 10 ml with various solvents.
e) A stock solution (2 ml) of DACSA was diluted to 5 ml with monoglyme and 0.1 ml of this solution was diluted to 10 ml with various solvents (8×10^{-6}M).

pH Dependency of the Absorption and Fluorescence Spectra of DACS (2)

Since DACM has a dimethylamino group as a basic functional group, the fluorescence spectra of DACM derivatives change as the pH of the solution is altered. Figure 3 shows the pH dependency of the absorbance at 396 nm and the fluorescence intensity at 478 nm of DACS. Two curves thus obtained run almost parallel with each other, and apparently the dimethylamino group of DACS is not protonated above pH 4.

Stoichiometry of the Reaction of DACM with Thiols of the Protein

In order to verify that DACM reacts with the thiol groups in protein, the amount of unreacted thiol groups were titrated by the Boyer's PCMB method.[4,5] As shown in Fig. 4 and Fig. 5 the amount of the thiols per mole of native egg albumin and the DACM-treated egg albumin were 3.8 and 2.9 moles, respectively, indicating the extent of labeling of cysteine in the protein to be 0.7 mole. Table II shows the comparison of the amino acid composition of native egg albumin and the DACM-treated egg albumin. There is no significant change in the quantity of any other amino acids before and after the treatment of the protein with DACM. On the basis of the amino acid analysis of S-succinylcysteine (**6**), the extent of labeling for the DACM-treated egg albumin was 0.66 in good agreement with the value obtained by the Boyer's method.

Rate Constants of Hydrolysis of DACM (1)

Gregory[7] has studied the stability of N-ethylmaleimide and its reaction with thiol groups, reporting that N-ethylmaleimide reacts not only with thiol compounds but also with water

7) J.D. Gregory, *J. Am. Chem. Soc.*, 77, 3922 (1955).

rapidly at above pH 8, but the reaction with thiol groups is much faster than the hydrolytic decomposition of the reagent. Thus, the reactivity of a maleimide for thiol and decomposition of the imide moiety were both accelerated at higher pH. In order to examine the effect of pH for decomposition of DACM (**1**), values of log k_{obs} of the hydrolysis were plotted against various pH values in Fig. 6. The rate for hydrolysis increases as a function of pH values. The k_{obs} of DACM at pH 7.0 (30±0.2 °) was 10^{-4} sec^{-1} and its half-lifetime was 115 min. The

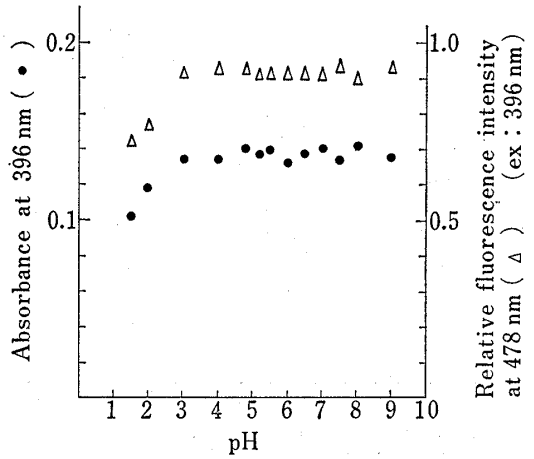

Fig. 3. pH Dependency of the Absorption and Fluorescence Spectra of DACS (**2**)

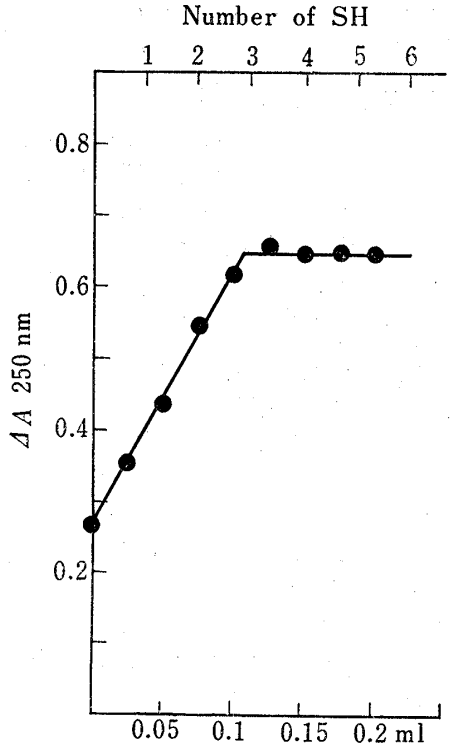

Fig. 5. Titration of Thiol Groups in the DACM-Treated Egg Albumin (0.82×10^{-5} M) with PCMB

The concentration of the added PCMB solution was 6.86×10^{-4} M.

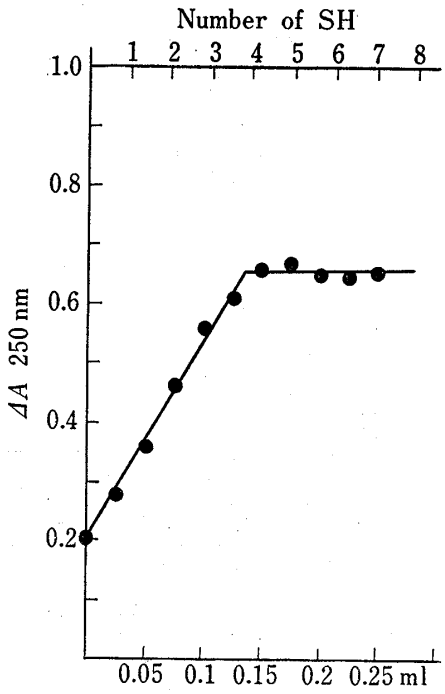

Fig. 4. Titration of Thiol Groups in Native Egg Albumin (1.6×10^{-5} M) with PCMB

The concentration of the added PCMB solution was 1.42×10^{-3} M.

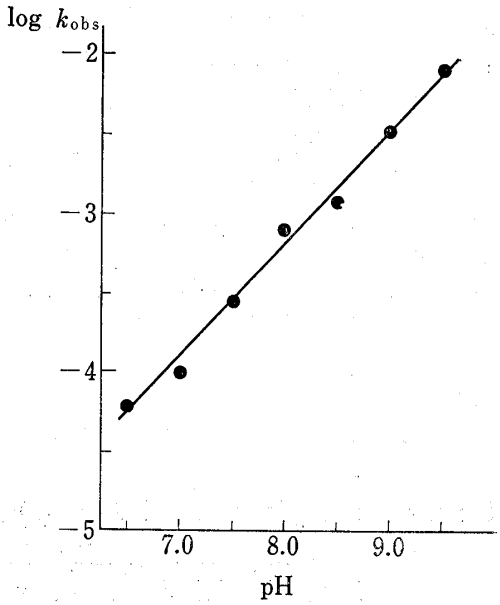

Fig. 6. The pH Rate Profile for Hydrolysis of DACM

TABLE II. Amino Acid Composition of DACM-Treated Egg Albumin

Amino acid	Reported values[a] egg albumin	Native egg albumin	DACM-treated egg albumin
Lys	20	20.5	19.6
His	7	7.1	6.9
Arg	15	15.1	14.1
S-Succinylcysteine			0.66
Asp	32	32.8	32.4
Thr	16	14.1	13.9
Ser	36	29.0	28.9
Glu	52	53.3	52.9
Pro	14	16.5	15.3
Gly	19	19.7	20.4
Ala	35	35.9	36.3
Val	28	30.7	29.2
Met	16	15.3	15.0
Ileu	25	24.3	24.2
Leu	32	32	32
Tyr	9	9.9	9.5
Phe	21	20.5	20.0

The amino acid compositions are expressed in terms of the molar ratios of the constituent amino acids. Each values based on 32 leucine per mole of protein are the result of a single analysis hydrolyzed for 72 hr and not corrected for decomposition during hydrolysis.
a) G.R. Tristram, *Adv. in Protein Chem.*, **5**, 83 (1949).

pH rate profile for hydrolysis of DACM is similar to that for hydrolysis of N-phenylmaleimide[8] under the same conditions. General hydrolytic behavior of *p*-substituted N-phenylmaleimides will be reported elsewhere.

Discussion

In a previous paper[4] egg albumin was selected as a standard thiol protein in evaluating a fluorescent thiol reagent for protein, and it was found that approximately 0.6—0.7 mole of BIPM is incorporated into one mole of egg albumin. Likewise, when egg albumin was treated with DACM the extent of labeling calculated from the amino acid analysis was 0.6 mole in good agreement with the value obtained from PCMB titration. The value agreed also with the data obtained with BIPM. On the basis of the absorption spectrum of the DACM-treated egg albumin, however, the incorporation of DACM was approximately 0.5 mole per mole of protein. This somewhat low value probably results from molar absorptivity at slightly blue shifted maximum (λ_{max} 392 nm) of the DACM moiety, which exists in a form of a succinimid (DACS; λ_{max} 395 nm). The existence of a succinimide form is suggested based on the fluorescence excitation spectrum of DACM-treated egg albumin, which is close to that of DACS but not to that of DACSA. Thus, DACM moiety exists in a form of the succinimide and DACM behaves in principle as a fluorescent reagent with a high degree of specificity for thiol groups in protein.

As seen in Table I fluorescence and absorption properties of DACS are affected in various solvents, but the variation observed in the emission maxima of DACS is smaller than that of ANM,[9] which is proposed as a typical hydrophobic probe directed to a thiol in protein. In general, fluorescence spectra of hydrophobic probes which are sensitive to the microenvironmental polarity indicate a large increase in the quantum yield of fluorescence and a blue shift

8) M.I. Machida, Thesis, Hokkaido University, (1973).
9) Y. Kanaoka, M. Machida, M.I. Machida, and T. Sekine, *Biochim. Biophys. Acta*, **317**, 563 (1973).

in the emission maximum in going from a polar medium to nonpolar one. As described in the previous paper[1] the quantum yields of DACS (Q=0.67) and DACSA (Q=0.70) in ethanol solution are enhanced compared with those (Q=0.11 for DACS, Q=0.01 for DACSA) in aqueous solution, respectively. Thus, since the fluorescence of DACS is relatively sensitive to the polarity of the solvent, it could be used, though not typical, as a hydrophobic probe. From the emission data of ANM, the Z value of thiol residue in egg albumin was estimated as 79—82.[8,9] Likewise, from the emission data of DACS the Z value of the environment of the reactive thiol(s) in egg albumin is estimated as approximately 79 in agreement with the value obtained from the ANM-treated egg albumin.

Fluorescence spectra of most aromatic compounds containing acidic or basic functional groups are very sensitive to the pH and hydrogen-bonding ability of the solvent. Since the pH dependency of the absorbance of DACS is parallel with that of the fluorescence intensity, it seems that the acidity of dimethylaminocoumarin derivatives in the ground state is roughly equal to that in the excited state. Similar result has been reported for diethylaminocoumarin derivative,[10] whose protonation takes place with pK_a=3.4 causing a spectral change. Since dimethylamino group of DACS is not protonated above pH 4, the fluorescence of DACS is rather invariable in the pH range from 4 to 9. From a practical view point it is important that the fluorogenic group maintains high fluorescence intensity in such a wide pH range.

Because the chemical modification of protein is carried out in aqueous solution and the reaction of the maleimide-type reagents with thiol compound competes with the hydrolytic reaction of maleimide ring, it is desirable to examine stability of reagents in aqueous solution. The half-lifetime of DACM is 115 min (pH 7.0, 30±0.2°), longer than that of BIPM (60 min, 30±0.2°) and nearly same as that of N-phenylmaleimide (107 min, 30±0.2°) in aqueous solution.[8] In small molecules the second-order rate constant for the reaction of DACM with N-acetyl-L-cysteine as measured by the fluorometry was 1.5×10^3 mol^{-1} sec^{-1} (0.1 M phosphate buffer, pH 7.0, 25°). When compared with the rate constants of other reagents,[8,11] this value for DACM indicates that the reactivity of maleimide-type reagents was not significantly affected by the substituent group on a nitrogen atom of the maleimide moiety. Since it seems that the reaction of DACM with thiol compounds is faster than the decomposition of DACM, incubation time of DACM with protein may be sufficiently shorter than 3 days at cold room. DACM is relatively stable in aqueous solution under the conditions described in the text.

In conclusion, the dimethylaminocoumarin ring is one of the candidate fluorogenic groups of choice. DACM has favorable water solubility in comparison with other fluorescent thiol reagents as well as the longer excitation range beyond of the emission of tryptophan in protein to avoid spectral overlap. Since DACM shows favorable and group-specific reactivity with thiol compounds it may be used in study of the kinetic analysis of the reactivity of various organic thiols as BIPM.[12] In view of that DACM derivatives have large molar absorptivity (ε=24500 in 0.1 M phosphate buffer, pH 7.0 for DACS) and high quantum yield (Q=0.67 in ethanol for DACS), DACM may be advantageously employed as a tracer or marker of any components containing thiol groups and a microfluorometry of thiol compounds. Systematic studies of the application of DACM to a variety of biological problems are currently under way.

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10) O. Takenaka, Y. Nishimura, A. Takenaka, and K. Shibata, *Biochim. Biophys. Acta*, **207**, 1 (1970).
11) *a*) J.R. Heitz, C.D. Anderson, and B.M. Anderson, *Arch. Biochem. Biophys.*, **127**, 627 (1968); *b*) C.C. Lee and E.R. Samuels, *Can. J. Chem.*, **42**, 168 (1964).
12) T. Sekine, K.A. Kato, K. Takamori, M. Machida, and Y. Kanaoka, *Biochim. Biophys. Acta*, **354**, 139 (1974).