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Fluorescence Thiol Reagents: Syntheses and Properties of N-Acridinylmaleimides and Their Derivatives[1]

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N-(3-Acridinyl)maleimide (3-AM) and N-(9-acridinyl)maleimide (9-AM) were prepared and their reactions with thiol compounds including egg albumin were examined. Both 3-AM (**7**) and 9-AM (**9**) react readily with thiol compounds to form strongly fluorescent addition products. Their absorption and fluorescence spectra were measured, and it was found that they are useful fluorescence thiol reagents. Comparative use of the both reagents will afford information on the states of thiols and they serve as a special kind of hydrophobic probe for microenvironment of thiols in macromolecules.

Keywords——fluorogenic group; egg albumin; fluorescence spectra; pH dependence; fluorescence quantum yield; N-acetyl-L-cysteine; fluorometry; solvent effect; empirical rule; hydrophobic probe

In general, as the degree of conjugation in hydrocarbon compounds increases, the intensity of fluorescence often increases and a bathochromic shift is observed. Most poly-condensed aromatic compounds are known to exhibit an intense fluorescence in the visible region, and are a profilic source of fluorescent derivatives. Therefore, the poly-condensed aromatic compounds with increased conjugation may be good candidates to serve as fluorescent probes which meet various spectroscopic requirements. In fact, in a series of studies on thiol reagents,[3] substituted naphthalene,[4] fluoranthene[5] and other aromatic hydrocarbon compounds were selected as fluorogenic groups. For example, it was found that N-(3-fluoranthyl)maleimide (FAM) is a fluorescent probe for thiols with a medium life time.[5] Among heterocyclic aromatic compounds, acridine and its derivatives have biological and pharmacological properties, and are particulary of interest as a fluorescence probe for drug-protein binding studies.[6] In addition, acridine has special fluorescence characteristics: *e.g.* it fluoresces weakly in hydro-

1) *a*) Fluorescence Thiol Reagents. XVI. Part XV: K. Yamamoto, Y. Kanaoka, and T. Sekine, *Anal. Biochem.*, **79**, 83 (1977); *b*) Preliminary reports of portion of this work have been presented: Y. Kanaoka, M. Machida, and K. Itoh, Abstracts of Papers, The 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, Apr. 1969, p. 190 and M. Machida, T. Takahashi, Y. Takahashi, and Y. Kanaoka, Abstracts of Papers, The 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, Apr. 1975, IV, p. 21; *c*) *cf.* T. Takahashi, Master Thesis, p. 3, Hokkaido University, 1976; *d*) The abbreviation used are: 3-AM=N-(3-acridinyl)maleimide (**7**); 9-AM=N-(9-acridinyl)maleimide (**9**); 3-AM–NAC=adduct of N-(3-acridinyl)maleimide with N-acetyl-L-cysteine (**12a**); 9-AM–NAC=adduct of N-(9-acridinyl)maleimide with N-acetyl-L-cysteine (**13a**); PCMB=*p*-chloromercuribenzoic acid.

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3) Y. Kanaoka, *Angew, Chem.*, **89**, 142 (1977); *Angew. Chem. Intern. Ed. Engl.*, **16**, 137 (1977).

4) M. Machida, M. Bando, Y. Migita, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **24**, 3045 (1976).

5) Y. Kanaoka, T. Takahashi, M. Machida, K. Yamamoto, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **24**, 1417 (1976).

6) J.K.H. Ma, P.-L. Hsu, and L.A. Luzzi, *J. Pharm. Sci.*, **63**, 32 (1974).

carbon solvents and more intensely in polar solvents.[7-9] In the present paper we report the syntheses and the application of acridinylmaleimides, a maleimide system with a three-fused ring containing nitrogen, as a part of our systematic search for fluorescence thiol reagents.[3] Independently of our work, Tuzimura, *et al.* have reported synthesis and application of N-(9-acridinyl)maleimide.[10]

Syntheses

The key intermediates for the syntheses of the desired maleimides are the suitable substituted fluorogenic groups including an amino group, which is, by way of the maleamic acid, to be converted to the corresponding maleimide. 3-Aminoacridine (**1**) was synthesized from 3-aminodiphenylamine according to the procedure of Albert.[11] N-(3-Acridinyl)maleamic acid (**3**) was obtained from **1** and maleic anhydride, and cyclized to the corresponding maleimide (**7**) by the usual method[4] (Chart 1). As a model compound of the reaction product of **7** with

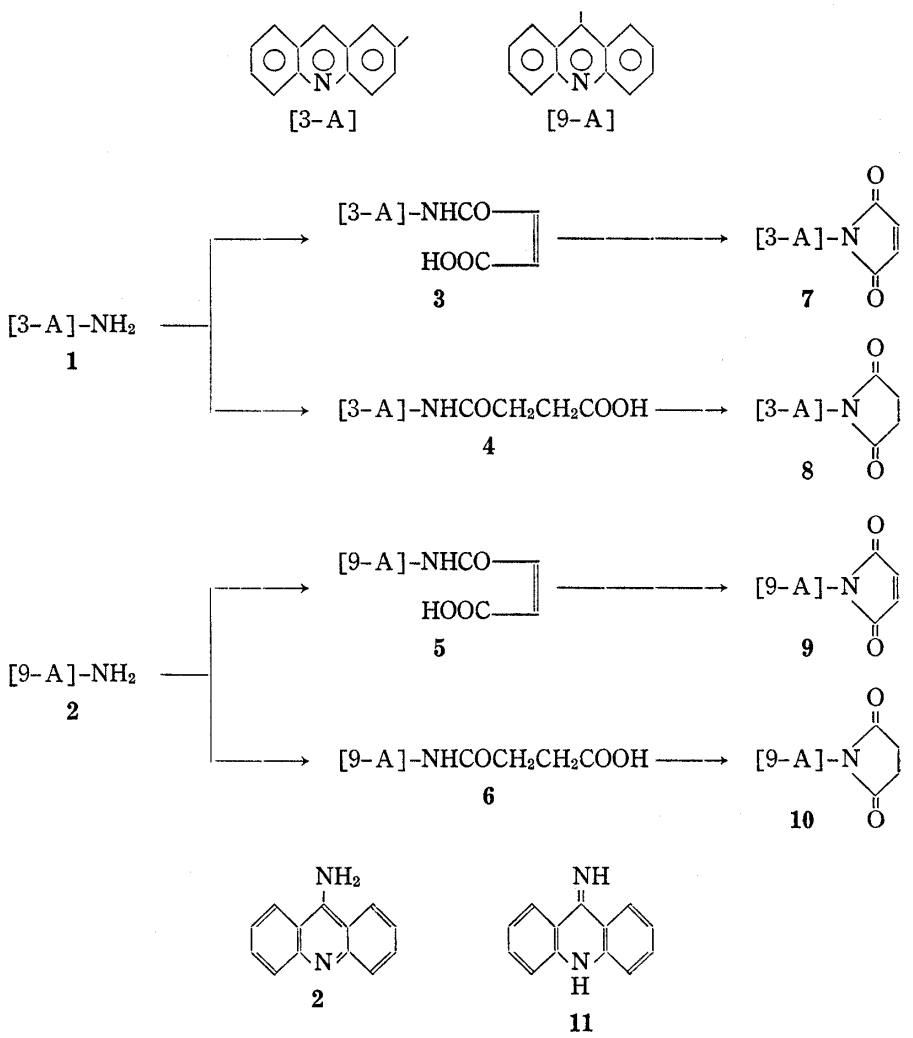


Chart 1

7) B.L. Van Duuren, *Anal. Chem.*, **32**, 1436 (1960).
8) N. Mataga, Y. Kaifu, and M. Koizumi, *Bull. Chem. Soc. Jpn.*, **29**, 373 (1956).
9) E.J. Bowen and J. Sahu, *J. Chem. Soc.*, **1958**, 3716.
10) *a*) Y. Nara and K. Tuzimura, *Bunseki Kagaku*, **22**, 451 (1973); *b*) H. Takahashi, Y. Nara, and K. Tuzimura, *Agr. Biol. Chem.*, **40**, 2493 (1976).
11) A. Albert, *J. Chem. Soc.*, **1948**, 1225.

a thiol, **8** was also synthesized. Synthesis of the 9-derivatives was examined in a similar manner. When the cyclization of crude **5** was attempted with polyphosphoric acid (PPA),[12] or sodium acetate and acetic anhydride,[4] **9** was isolated only in a very poor yield. The corresponding succinimide (**10**) was not obtained. This is probably due to that the formation of **5** or **6** was incomplete under the conditions because 9-aminoacridine (**2**) takes mainly the tautomeric acridoimine form (**11**)[13] consisting of two basic imino sites, one with a pK_a value of 10 (ring) and the other with a very low pK_a value, <-2 (9-amino).[14] The resulting **9** has a little higher melting point than the reported one,[10a] but shows the reasonable data in the elemental analysis and infrared (IR) spectrum in support of the assigned structure.

Results

Absorption Spectra of the N-Acridinylmaleimide Derivatives

The adducts of the acridinylmaleimides with N-acetyl-L-cysteine (3-AM-NAC=**12a** or 9-AM-NAC=**13a**) were used in the spectroscopic measurements as a simple model compound of the reaction products of the reagents with thiols. Fig. 1 and 2 show the absorption spectra of these 3-AM and 9-AM derivatives in 0.1 M phosphate buffer (pH 7.0). The maximum of 9-AM-NAC (**13a**) is slightly red shifted and intensified compared with that of 3-AM-NAC (**12a**). In going from **12a** to **14a** by hydrolysis of an imide moiety, the absorption is intensified in the wide range of wavelength. In the spectrum of 3-AM-treated egg albumin, in which a long wavelength maximum of **12b** and/or **14b** is slightly red shifted compared with that of 3-AM-NAC (**12a**), the peak at about 280 nm due to aromatic amino acids of protein is masked by the chromophores derived from 3-AM existing as a ring-opened form (**14b**). The spectrum of 9-AM-treated egg albumin (**13b** and/or **15b**), however, has the well-defined peak at 280 nm

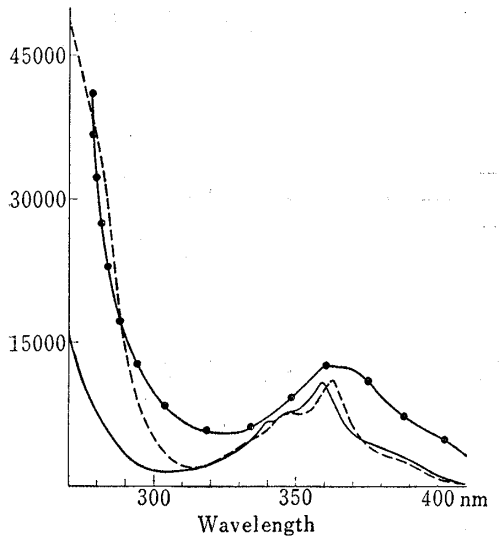


Fig. 1. Absorption Spectra of the 3-Acridinylmaleimide Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

——: 3-AM-NAC (**12a**).
------: 3-AM-treated egg albumin (**12b** and/or **14b**).
—●—: hydrolyzate of 3-AM-NAC (**14a**).

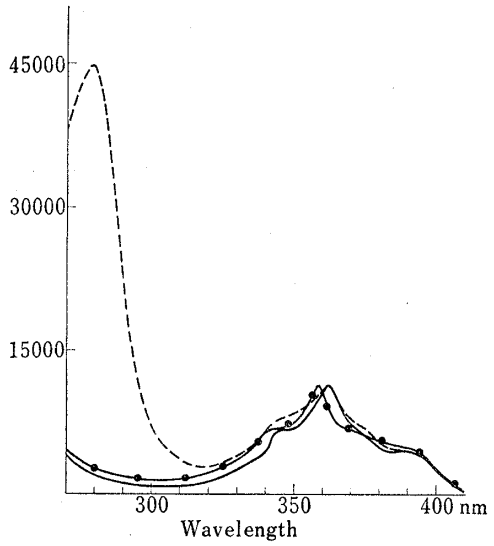


Fig. 2. Absorption Spectra of the 9-Acridinylmaleimide Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

——: 9-AM-NAC (**13a**).
------: 9-AM-treated egg albumin (**13b** and/or **15b**).
—●—: hydrolyzate of 9-AM-NAC (**15a**).

12) Y. Kanaoka, T. Sekine, M. Machida, Y. Soma, K. Tanizawa, and Y. Ban, *Chem. Pharm. Bull.* (Tokyo), **12**, 127 (1964).
13) A. Albert, S.D. Rubbo, and R. Goldacre, *Nature* (London), **147**, 332 (1941).
14) S. Schuldiner, H. Rottenberg, and M. Avron, *Eur. J. Biochem.*, **25**, 64 (1972).

which essentially originates from aromatic amino acid residues, and the longest wavelength band is close to that of 9-AM-NAC (**13a**).

Fluorescence Spectra of N-Acridinylmaleimide Derivatives

The fluorescence emission spectrum of the hydrolyzate (**14a**) of 3-AM-NAC (**12a**) is red shifted compared with that of 3-AM-NAC (**12a**) (Fig. 3). When **12a** was hydrolyzed to **14a**, maximum of **14a** in the excitation spectrum shifted slightly toward the red and the longer wavelength range than 380 nm is intensified, and a peak at 290 nm appears. The fluorescence spectrum of 3-AM-treated egg albumin shows an approximately additive spectrum of **12a** and **14a**, indicating that the succinimide ring produced by the reaction with 3-AM has been partially hydrolyzed forming a mixture of **12a** and **14a**. By contrast, as shown in Fig. 4, the emission spectrum of 9-AM-treated egg albumin (**13b**) is almost similar to that of 9-AM-NAC (**13a**).

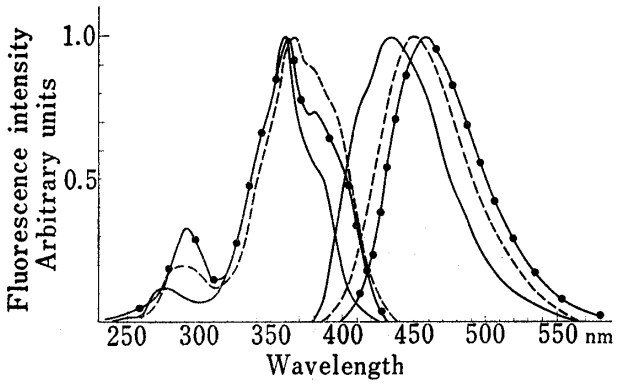


Fig. 3. Fluorescence Spectra of the 3-Acridinylmaleimide Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

——: 3-AM–NAC (**12a**).
------: 3-AM-treated egg albumin (**12b** and/or **14b**).
—●—: hydrolyzate of 3-AM–NAC (**14a**).

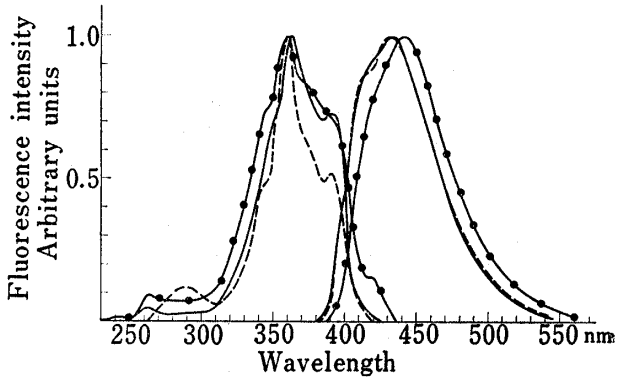


Fig. 4. Fluorescence Spectra of the 9-Acridinylmaleimide Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

——: 9-AM–NAC (**13a**).
------: 9-AM-treated egg albumin (**13b** and/or **15b**).
—●—: hydrolyzate of 3-AM–NAC (**15a**).

Fluorescence Quantum Yields of the Acridinylmaleimide Derivatives

As listed in Table I the introduction of the fluorogenic group in protein results in decrease of fluorescence quantum yields. Similarly, the fluorescence spectrum of acridine derivative is strongly quenched upon binding to the enzyme and it is found that the marked change in fluorescence intensity between the free and bound forms of the probe is compatible with the observation that the fluorescence intensity is extremely sensitive to solvent environment in the absence of enzyme.[15] The quantum yields of **12a** and **13a** are nearly of same values.

Table I. Fluorescence Quantum Yields of the Acridinylmaleimide Derivatives (Excitation: 360 nm, in 0.1 M phosphate buffer (pH 7.0))

	3-AM-NAC (**12a**)	3-AM-treated egg albumin	9-AM-NAC (13a)	9-AM-treated egg albumin
Quantum yield	0.18	0.02	0.17	0.06

Effects of Solvent on the Fluorescence Spectra

As shown in Fig. 5 and 6, as a percentage of alcohol in water increases the fluorescence intensities of **12a** and **13a** decrease with a slightly blue shift of emission maxima. Since the

15) G. Mooser, H. Shulman, and D.S. Sigman, *Biochemistry*, **11**, 1595 (1972).

spectral behavior of **12a** is similar to that of **13a**, the fluorescence properties of these acridine derivatives are not significantly affected by the position of the substituents on the acridine ring.

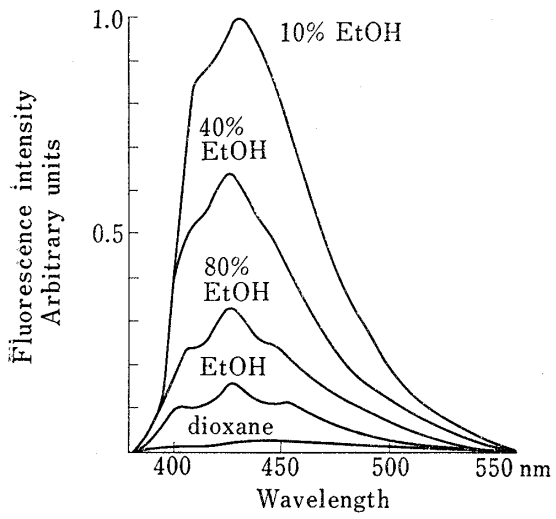


Fig. 5. Solvent Effects on the Fluorescence Spectra of 3-AM-NAC (**12a**)

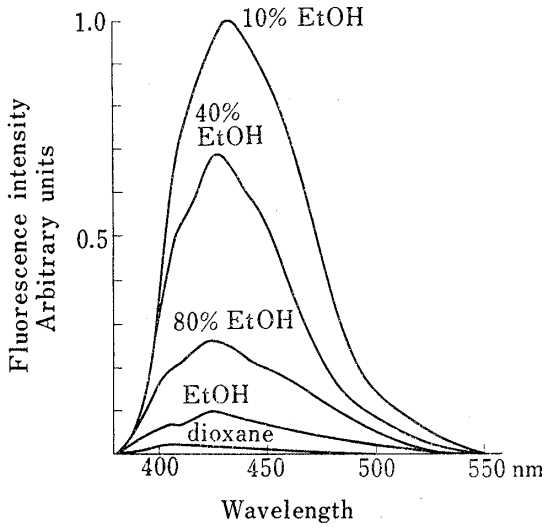


Fig. 6. Solvent Effects on the Fluorescence Spectra of 9-AM-NAC (**13a**)

pH Dependence of Absorbance and Fluorescence Intensity of N-Acridinylmaleimide Derivatives

Compounds containing a nitrogen atom usually exhibit shifts in maxima of electronic spectra when the pH of the solutions is changed. Since acridine ring has one nitrogen atom which may act as a proton acceptor, the fluorescence spectra of its derivatives are very sensitive to the pH.[8] Fig. 7 and 8 show the pH dependence of the absorbance at 358—360 nm and the fluorescence intensity at 432—433 nm of **12a** and **13a**, respectively. In **13a** the plot of the absorbances at the monitored wavelength shows roughly a straight line, while that in

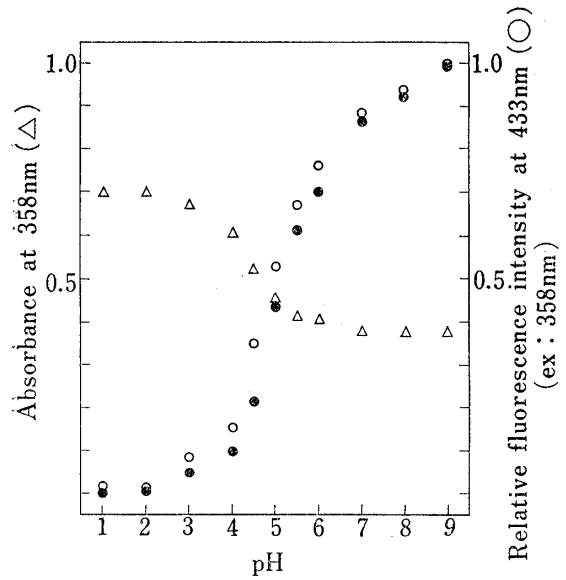


Fig. 7. pH Dependence of Absorbance and Fluorescence Intensity of 3-AM-NAC (**12a**)

The values (●) obtained by dividing the fluorescence intensities by absorbances; the value at pH 9 is taken as unity.

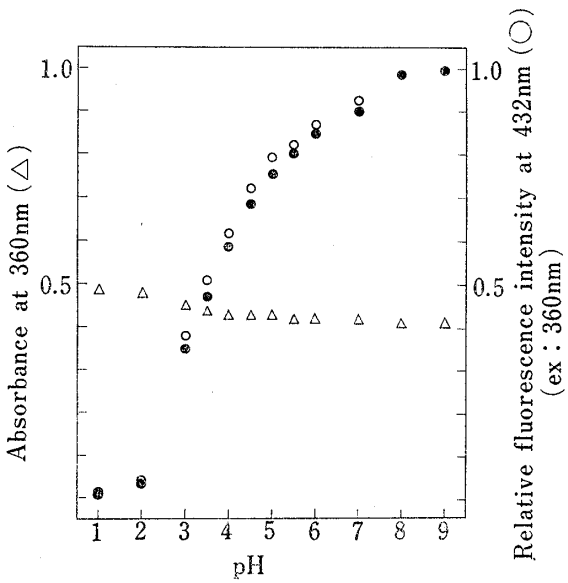


Fig. 8. pH Dependence of Absorbance and Fluorescence Intensity of 9-AM-NAC (**13a**)

The values (●); obtained by dividing the fluorescence intensities by absorbances the value at pH 9 is taken as unity.

12a forms rather a sigmoid curve. Plots of the values obtained by deviding the fluorescence intensities by absorbance at the monitored wavelength, run almost parallel with the corresponding fluorescence intensities both in **12a** and **13a**. The fluorescence spectrum of **12a** changes by the proton addition and shifts toward the red pH 2.0 (Fig. 9). Similar behavior is also seen in the fluorescence spectrum of **13a** (Fig. 10).

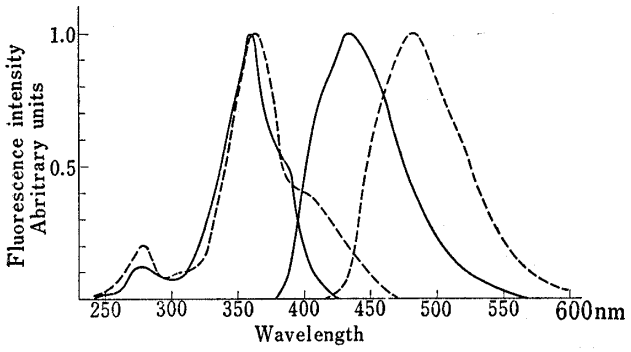


Fig. 9. Fluorescence Spectra of 3-AM-NAC (**12a**) at pH 2.0 and 7.0

------: pH 2.0, 0.05 M HCl-AcONa solution.
——: pH 7.0, 0.1 M phosphate buffer.

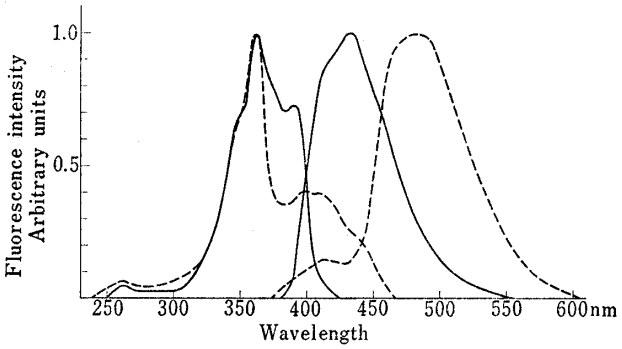
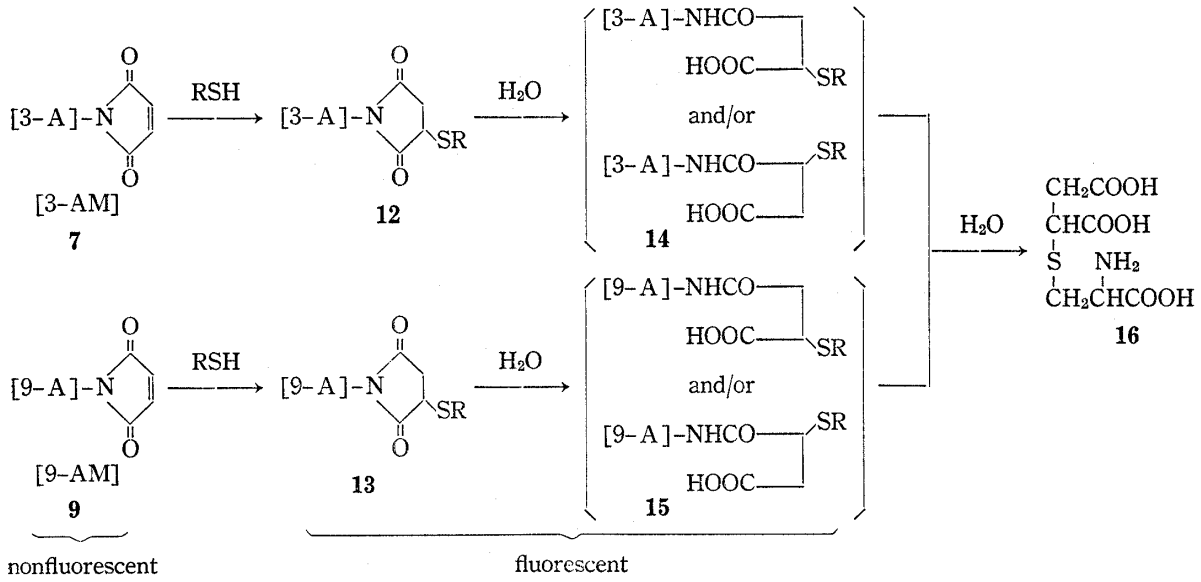


Fig. 10. Fluorescence Spectra of 9-AM-NAC (**13a**) at pH 1.0 and 7.0

------: pH 1.0, 0.05 M HCl-AcONa solution.
——: pH 7.0, 0.1 M phosphate buffer.

Reactivity of N-Acridinylmaleimides with Thiol Compounds

Since the maleimide-type fluorescent reagents are fluorescent only when reacted with thiols obeying the empirical rule we had proposed[3,16] (Chart 2), the reaction rate of the N-acridinylmaleimides with thiol compound can be also readily followed from the fluorescence enhancement as a function of time.[17] The second order rate constants for the reactions of 3-AM and 9-AM with N-acetyl-L-cysteine as measured by fluorometry were 1.2×10^3 and 1.7×10^3 $1\,mol^{-1}\,sec^{-1}$ in 0.1 M phosphate buffer (pH 7.0) at 25°, respectively. The second



a : RSH=N-acetyl-L-cysteine
b : RSH=egg albumin

Chart 2

16) Y. Kanaoka, M. Machida, K. Ando, and T. Sekine, *Biochim. Biophys. Acta*, **207**, 269 (1970).
17) M. Machida, M.I. Machida, T. Sekine, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **25**, 1678 (1977).

order rate constants for the reactions of 3-AM and 9-AM with egg albumin were 1×10^2 and 0.9×10^2 1 mol^{-1} sec^{-1} respectively, under similar conditions.

Stoichiometry of the Reaction of N-Acridinylmaleimides with Thiols of Egg Albumin

In order to confirm that the N-acridinylmaleimides react with thiol groups in protein, the amount of unreacted thiol groups in egg albumin were titrated after the reaction by the PCMB method.[17] When the protein was incubated with 3-AM or 9-AM for one day in a cold room, the contents of labeling of thiols in protein with 3-AM or 9-AM were 1.3 or 0.8 mol, respectively, on the basis of the PCMB titration (Table II). On the basis of the amino acid analysis of S-succinylcysteine (**16**),[18] the extents of labeling for the 3-AM-treated egg albumin and 9-AM-treated egg albumin were 1.1 and 0.6 mol, respectively, in approximate agreement with the above values.

Table II. The Contents of Labeling of Thiols in Protein by the PCMB Titration (mol per one mol of protein)

	Native egg albumin	3-AM-treated egg albumin	9-AM-treated egg albumin
Amount of titrable thiols	3.2	1.9	2.4
Amount of modified thiols	0	1.3	0.8

Discussion

N-Acridinylmaleimides react readily with thiol compounds such as N-acetylcysteine and strongly fluoresce in aqueous solutions. In comparison with the other reagents of this class (BIPM,[16] DACM[17] and ANM[19]), the rate constants of N-acridinylmaleimides with N-acetyl-L-cysteine are on the same order, indicating that the reaction is not significantly affected by the molecular size of the fluorogenic groups which are three fused ring system.

In this series of work egg albumin has been used as a standard thiol protein in evaluating the fluorescent thiol reagents, *e.g.* BIPM, ANM, FAM and DACM. Incorporation of these reagents were 0.3—0.7 mol per one mol of the protein. In the present study, egg albumin was likewise treated with the N-acridinylmaleimide (3-AM or 9-AM). For the 9-AM-treated egg albumin the extents of labeling were 0.6—0.8 mol, in good agreement with the values obtained with the related reagents as mentioned above. Although the rate of reaction of 3-AM with a small thiol compound is of the same order as that of 9-AM, the incorporation of 3-AM into the protein was in the range of 1.1—1.3 mol. In contrast, the reaction rate of 3-AM or 9-AM with a macromolecule is slower by a factor of ten compared with that for a small molecule. In addition, it was found that the extents of labeling vary with a position of the imide group on the acridine ring. It seems that a second thiol group of the protein was modified by 3-AM but not by 9-AM. These results are, at least in part, explained by stereochemical factors involved in N-acridinylmaleimides. While the slower rate for a macromolecule than for a smaller molecule may be due to bulkiness of the tricyclic acridine ring interacting with the microenvironments of thiols, the greater extent of the reaction of 3-AM with thiols is ascribed to the less crowded environment of the maleimide in 3-AM than in 9-AM. In fact, approximately 2 mol of thiols were modified with less hindered maleimide reagents such as NEM and N-(dimethylaminonaphthyl-4)maleimide.[20] Thus dual employment of the set of the reagents, 3-AM and 9-AM, will be applied to probe relative accessibility and therefore the states of thiols in protein.

18) M. Machida, T. Sekine, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **22**, 2642 (1974).
19) Y. Kanaoka, M. Machida, M.I. Machida, and T. Sekine, *Biochim. Biophys. Acta*, **317**, 563 (1973).
20) M.I. Machida, Thesis, Hokkaido University, (1973).

In general, the fluorescence of reagents that are called as hydrophobic probes varies in intensity and other spectral characteristics with the polarity of the solvent,[19] that is, the emission maximum is shifted toward the blue, and the fluorescence intensity increases as the polarity of solvent decreases. Although acridine is known to fluoresce weakly in hydrocarbon solvents and more intensely in ethyl alcohol solution, the fluorescence of acridine derivatives is varied by different substitutions on the acridine ring.[6] In various solvent systems the fluorescence of the acridinylmaleimide derivatives (**12a, 13a**) decreases with the blue shift of the emission maximum as the polarity of solvent increases. These fluorescence changes of **12a** and **13a** by the solvent are very similar to those of a parent compound, acridine. Therefore, these solvent effects are interpreted to indicate that the unshared electrons on the nitrogen atom in acridine ring become involved in hydrogen bonding with proton-donating solvents. As a result, intersystem crossing to the triplet state through n, π^* transition become less likely and, therefore, enhanced fluorescence is observed. This solvent dependency of the fluorescence of **12a** and **13a** is worth noting because they exhibit inversely proportional effects to polarity shown by conventional hydrophobic probes.[19]

In conclusion, N-acridinylmaleimides 3-AM and 9-AM readily form, upon reaction with thiols, addition products with fluorescence maxima in a longer wavelength region distinct from backbone of protein. They may be employed as an analytical tool for microdetermination of small molecular thiol compounds, as well as a tracer or marker of thiols in biological studies. Comparative use of both of them will afford information on the states of thiols. Finally, they would serve as a special kind of hydrophobic probe for microenvironment of thiols in macromolecules.

Experimental

Materials

3-Nitrodiphenylamine——3-Nitrodiphenylamine was prepared from 3-nitroacetanilide and bromobenzene in nitrobenzene.[21] Recrystallization from ethanol gave 3-nitrodiphenylamine as reddish brown prism, mp 109—111° (lit.[21] 112°).

3-Aminodiphenylamine——3-Nitrodiphenylamine (10.7 g, 50 mmol) was hydrogenated in 200 ml of ethanol with 10% Pd–C (4 g) as catalyst. After the catalyst was filtered off, solvent was removed *in vacuo*. Distillation under reduced pressure gave 3-aminodiphenylamine as yellow viscous oil, bp 196—207°/3 mmHg, 93% yield (lit.[11] mp 75°). 3-Aminodiphenylamine was used for cyclization without further purification.

3-Aminoacridine (**1**)——Compound **1** was prepared according to the procedure of Albert. Recrystallization from ethanol afforded **1** as yellow plates, mp 220.5—222° (lit.[11] mp 216°).

N-(3-Acridinyl)maleamic Acid (**3**)——To a solution of maleic anhydride (490 mg/10 ml in tetrahydrofuran (THF), 5 mmol) was gradually added **1** (970 mg, 5 mmol) in 20 ml of THF with stirring. After standing over night the precipitate was collected, washed with THF and dried. 1.3 g (91%), reddish orange powder of mp 189—192° (dec.). IR ν_{max}^{Nujol} cm^{-1}: 1680 (–COOH), 1635 (amide).

N-(9-Acridinyl)maleamic Acid (**5**)——To a solution of maleic anhydride (490 mg/10 ml in THF, 5 mmol) was added 9-aminoacridine (**2**) (970 mg, 5 mmol) in THF with stirring. After standing over night the precipitate was collected, washed with THF and dried. 1.3 g, yellow powder, mp 232—234° (dec.). IR ν_{max}^{Nujol} cm^{-1}: 1690 (–COOH), 1640 (amide).

N-(3-Acridinyl)succinamic Acid (**4**)——This compound was prepared from succinic anhydride and 3-aminoacridine (**1**) in THF, 82%. Reddish orange powder, mp 192—195° (dec.).

N-(3-Acridinyl)maleimide (**7**)——A mixture of **3** (292 mg, 1 mmol), anhydrous sodium acetate (25 mg) and acetic anhydride (2 ml) was heated at 100° for 20 min. After cooling the mixture was poured into ice-cold water, neutralized with $NaHCO_3$ powder and extracted with CH_2Cl_2. The extract was washed with sat. aq. $NaHCO_3$ and sat. aq. NaCl solution, and dried over anhydrous Na_2SO_4. Solvent was removed *in vacuo*. Recrystallization from AcOEt–*n*-hexane gave **7** as pale yellow prisms in 52% yield, mp 219—220.5°. UV λ_{max}^{EtOH} nm (ε): 254 (83000), 258 (89000), 360 (12000); IR ν_{max}^{Nujol} cm^{-1}: 1715 (imide). *Anal.* Calcd. for $C_{17}H_{10}N_2O_2$: C, 74.44; H, 3.68; N, 10.21. Found: C, 74.44; H, 3.68; N, 9.99.

N-(9-Acridinyl)maleimide (**9**)——This compound was prepared from N-(9-acridinyl)maleamic acid (**5**) (876 mg), anhydrous sodium acetate (252 mg) and acetic anhydride (5 ml) according to the usual procedure.[4] The temperature was maintained at 100° for 2 hr. Purification of **9** was carried out by column chromato-

21) I. Goldberg, *Chem. Ber.*, **40**, 4541 (1907).

graphy of silica gel using CH_2Cl_2 as eluent. Recrystallization from acetone afforded **9** as pale yellow prisms in 8% yield, mp 255—258° (lit.[14] mp 248°). UV λ_{max}^{EtOH} nm (ε): 251 (159500), 343 (sh, 7700), 360 (12400), 382 (sh, 4700). IR ν_{max}^{Nujol} cm^{-1}: 1710 (imide). *Anal.* Calcd. for $C_{17}H_{10}N_2O_2$: C, 74.44; H, 3.68; N, 10.21. Found: C, 74.58; H, 3.62; N, 10.11.

N-(3-Acridinyl)succinimide (8)——A mixture of **4** (294 mg, 1 mmol), anhydrous sodium acetate (25 mg) and acetic anhydride (2 ml) was heated at 100° for 20 min. After cooling the reactant was poured into ice-cold water, neutralizing with $NaHCO_3$ powder, and extracted with CH_2Cl_2. The extract was washed with sat. aq. $NaHCO_3$ solution and sat. aq. NaCl solution, and dried over anhydrous Na_2SO_4. Solvent was removed *in vacuo*. Recrystallization from EtOH gave **8** as pale yellow prisms in 43% yield, mp 250—252.5°. UV λ_{max}^{EtOH} nm (ε): 254 (87000), 342 (sh, 7700), 360 (10000). IR ν_{max}^{Nujol} cm^{-1}: 1780, 1715 (imide). *Anal.* Calcd. for $C_{17}H_{12}N_2O_2$: C, 73.90; H, 4.38; N, 10.14. Found: C, 73.89; H, 4.48; N, 10.43.

Methods

Spectroscopic Measurements——Fluorescence and absorption spectra were measured with a Hitachi MPF-2A fluorescence spectrophotometer and a Shimadzu double beam UV-200 spectrophotometer, respectively. IR spectra were measured with a Jasco IR-S spectrophotometer. The samples used for fluorescence studies have absorbances below 0.2 at the exciting wavelength. Quantum yields of the fluorescence spectra were obtained by the method described in the previous paper.[22] For determining the concentration of the modified egg albumin, the absorption at 280 nm was corrected for turbidity and contribution of the absorption of AM-derivatives at this wavelength. All stock solutions of 3-AM and 9-AM were prepared in monoglyme ($1-5\times10^{-3}$ M).

Reaction of Acridinylmaleimides with N-Acetyl-L-cysteine (Preparation of AM-NAC, 12a or 13a)——A mixture of 0.5 ml of 5 mM AM and 10 ml of 5 mM N-acetyl-L-cysteine in 0.1 M phosphate buffer was incubated at room temperature for 1 hr and 5 ml of the mixture was diluted to 25 ml with 0.1 M phosphate buffer (pH 7.0) for the measurement of the absorption and the fluorescence spectra.

Hydrolysis of AM-NAC (Preparation of 14a and 15a)——A mixture of 3-(or 9-) AM monoglyme solution (5 ml, 2×10^{-3} M) and 5 ml of N-acetyl-L-cysteine (8.16 mg/5 ml, 0.1 M phosphate buffer, pH 7.0) was incubated at 25° for 30 min. To a reaction mixture (1 ml) was added 5 ml of 0.1 N NaOH and stood for 1 hr. For measurement of spectra the reaction mixture was neutralized with 2 N HCl and the total volume was made to 25 ml with 0.1 M phosphate buffer (pH 7.0).

Solvent Effects on the Fluorescence of AM-NAC (12a and 13a)——A mixture of 1 ml of 5 mM AM and 9 ml of 3 mM N-acetyl-L-cysteine in ethanol was stood at room temperature for 1 hr and 0.1 ml of the mixture was diluted to 10 ml with various solvent systems. Excitation was 360 nm.

pH Dependence of the Fluorescence of AM-NAC (12a and 13a)——A mixture of 1 ml of 5 mM AM and 9 ml of 3 mM N-acetyl-L-cysteine in monoglyme was stood at room temperature for 1 hr and 0.1 ml of the mixture was diluted to 10 ml with various buffer solutions. Excitation was 360 nm. In experiments to see the effect of pH the following buffers were used; 0.05 M HCl–AcONa for pH 1.0—5.0, 0.05 M citric acid–Na_2HPO_4 for pH 5.0—7.0 and 0.05 M KH_2PO_4–sodium borate for pH 7.0—9.0.

Reaction Rate of Acridinylmaleimides with Thiol Compounds——To a solution of 1 mM N-acetyl-L-cysteine (0.05—0.5 ml, 5—50 eq. mol for the thiol compound) was added 0.01 ml of 1 mM AM and instantly stirred, and immediately the enhancement of the fluorescence intensity at 433 nm was automatically recorded by exciting at 360 nm at 25°. The total volume was made to 3 ml with 0.1 M phosphate buffer in 1 cm cell. Egg albumin was similarly treated with 1 mM AM.

Preparation of Modified Egg Albumin——A mixture of egg albumin (45 mg/40 ml, 0.1 M phosphate buffer, pH 7.0) and 1.6 ml of 5 mM AM was stood in cold room for one day. The reaction mixture was applied to a Sephadex G-25 column (7 g) and eluted with water. Fraction containing materials which absorb at 362 nm were combined and lyophilized.

Titration of the Thiol Groups of Egg Albumin with PCMB——Titrations of the thiol groups of egg albumin were carried out by the Boyer's method.[17,18] The PCMB solution was prepared as described in the previous paper.[17]

Amino Acid Analysis——Amino acid analysis was performed by the method and on the same instrument as described in the previous paper.[18]

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