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A Chemometric Approach to the Estimation of the Absorption Spectra of Dye Probe Merocyanine 540 in Aqueous and Phospholipid Environments

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Merocyanine 540 (MC540) is a widely used dye probe for membranous environments. However, fundamental knowledge of the spectral features of this dye in aqueous and hydrophobic environments is still lacking. Such knowledge is important because biomembranes involve a hydrophobic environment surrounded by a hydrophilic environment. Because many investigations so far have been performed based on indistinct spectral estimations, the interpretation of the data obtained using this dye as a fluorescent transmembrane probe remains controversial. In order to determine the exact spectra in both aqueous and hydrophobic environments, we adopted principal factor analysis (PFA), a method of multivariate analysis. The PFA method can also determine the number of molecular species present in the reaction mixture, which is three in pure water and two in phospholipid suspension. Two of the species in both water and phospholipid suspension were the monomer and dimer. The third species in water was the trimer, but its amount was so small at 10 μM MC540 solution that the spectral data in water can be approximated neglecting this molecular species. The monomer spectrum changed its form markedly with a bathochromic shift when transferred from the water to phospholipid environment, whereas the dimer remained similar in its shape except for a remarkable red shift. In water, the dissociation constants, K_1 and K_2, for the assumed stacking-model reactions, $M+M \rightleftharpoons M_2$ and $M+M_2 \rightleftharpoons M_3$, were 3.1×10^{-4} M and 5.7×10^{-4} M, respectively. In the phospholipid environment, the dissociation constant K^* for the assumed stacking-model reaction, $M^*+M^* \rightleftharpoons M_2^*$, was 1.9×10^{-5} M. The fluorescent intensities of MC540 were also measured in both water and phospholipid environments. A comparison based on the absorption and fluorescence spectra suggested that the temporal increase in the amount of the monomer on the excitable membrane contributes to the fluorescent intensity change observed in the transmembrane potential change.

Key words: absorption spectrum, factor analysis, fluorescence spectrum, merocyanine 540, potential probe.

In biological fields many dyes have been used as probes to monitor various kinds of physiological phenomena. The fluorescent dye probe merocyanine 540 (MC540) is a typical example. Originally, MC540 was developed as a sensitizing agent for photographic suspensions (*1*). However, this dye was later found to be a useful probe for monitoring changes in transmembrane potential in nerve cells (*2*), and, based on this observation, MC540 has been used to monitor such changes in several systems including nerves (*3–6*), muscle (*7*), mitochondria (*8*, *9*), and liposomes (*10*). However, MC540 has since been largely replaced by other more sensitive and less phototoxic dyes.

Instead, MC540 is now used extensively to obtain information about the environment of phospholipid micelles or bilayers or vesicles (*11–30*). However, there remains a lack of fundamental knowledge concerning this dye. For example, the precise number of molecular species of MC540, even in aqueous solution, is not clear. For this reason, the spectral features of MC540 molecular species remain controversial. Exact knowledge of the spectral features of the molecular species of this dye present in aqueous media or hydrophobic environments is undoubtedly the basis for understanding the mechanism of this dye as a probe.

Fortunately, recent developments in computer-assisted principal factor analysis (PFA), a method of multivariate analysis, may help to resolve this problem (*31*). To establish a basic methodology using PFA, we developed suitable programs for evaluating the spectra of the ligand associated with a protein molecule, employing a simple solution system including bovine serum albumin (BSA) and bromocresol purple (BCP) (*32*, *33*). Using the PFA method, we can obtain information about the number of molecular species involved in the reaction system, and about the spectral figures with physiological meaning for those molecular species. The purpose of the present study is to apply the PFA method to the determination of the number of molecular species present in an aqueous solution of MC540 and in phospholipid suspensions containing MC540, and to obtain

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exact spectra for those molecular species by searching for a suitable reaction model to fit the observed spectral data. Finally, the absorption spectra are discussed in relation to the observed fluorescence spectra of the dye.

MATERIALS AND METHODS

Computer Calculation and Programming—Principal factor analysis (PFA) can provide information about the number of molecular species present in a dye solution, the association constants in the equilibrium state, the amounts and exact spectra of those species, and the reaction mechanism. Since we have already applied the PFA method to the determination of those parameters in a BSA-BCP system, the theoretical background of the computer calculations using PFA has been described previously (*32*, *33*).

An absorbance matrix (**D**) can be expressed as the product of (**E**), a molecular absorption coefficient matrix, and matrix (**C**), a concentration matrix, that is,

$$\mathbf{D} = \mathbf{EC}.$$

Estimation of the number of molecular species is based on the eigenanalysis. Because inevitable experimental errors yield an excess number of eigenvalues, we estimated the valid number of the eigenvalues statistically. For this purpose the IND parameter proposed empirically by Malinowski (*31*, *34*, *35*) was convenient for deducing the number of reliable eigenvalues. In the actual calculations, a matrix prepared directly from the measured absorbance was used instead of matrix **E**, and matrix **C** was multiplied by 10^5 to keep the observed absorbance unit in matrix **D**. As a result, the value of K became large enough for PFA calculations.

After the conversion of matrix **D** into a covariance matrix (**Z**), it was further decomposed into abstract row (**R**) and column (**C**) matrices. Since both the **R** and **C** matrices have neither physical nor chemical meaning, the axes of both matrices were rotated to yield matrices having physiological meaning. To obtain a least-squares transformation matrix (**T**) for the rotation, a suitable reaction model was constructed and equilibrium constants were empirically assumed, and the true K values were searched by minimizing the difference between the empirical concentration matrix ($\mathbf{C}^*$) and matrix ($\mathbf{C}^*$) rotated by **T**.

All programs used in the present paper were developed in N_{88}BASIC, and all calculations were performed with a PC-9821Nr233 (NEC), using double precision of this language for the numerical calculations.

Spectral Data—The spectrum of MC540 was measured using the double-beam mode of a Hitachi 557 spectrophotometer. The data were sent to the host computer through an RS232C interface in the form of digital data pairs, wavelength at 1 nm intervals and the corresponding absorption intensity.

To raise the MC540 concentration, a cuvette with a 0.5 cm light-path length was used for both the aqueous solution and phosphatidylcholine (PC) suspension. The cuvette containing 2 ml of MC540 solution was preincubated for 5 min in a cell-holder at 25°C, and spectral measurement was started at the same temperature. In a typical series of data, the final concentrations of MC540 were 20, 30, 40, 50, 60, and 70 μM in water and 8, 12, 16, 20, 24, 28, μM in PC solution. To avoid the effects of light scattering and turbidity, we added the same amount of PC to the solutions in both the sample and reference cuvettes.

Fluorescence spectra were obtained using a Hitachi 204 fluorescence spectrophotometer in a 1-cm light-path cuvette containing MC540 solution at 25°C. The light scatterings in both the emission and excitation spectra were examined using cuvette containing PC suspension without MC540 dye, and take those spectra into consideration when discussing the results.

Reagents—Merocyanine 540 (MC540) was purchased from Sigma Co., and used by dissolving it in water just before each experiment, because MC540 is unstable in aqueous solution. Phosphatidylcholine (PC) was purchased from Sigma Co., and a PC stock solution of 2 mg/ml was prepared by sonication (20 kHz, 25 W, 10–30 A, 3 min) in water. The water used in the reaction mixtures was twice distilled.

RESULTS

Effects of Dye Concentration on the Spectra in Water and PC Solution—In water: Several investigators have observed that an MC540 dye solution shows monomer-dimer-like conversions in its spectrum as its concentration is changed (*11*, *24*, *36*). As shown in Fig. 1A, we also observed the same kind of spectral change. To clarify the spectral tendency, we express the spectral data per 10 μM and 1 cm light path length in Fig. 1B. This figure clearly shows that the absorbances near 500 nm increase, whereas those near

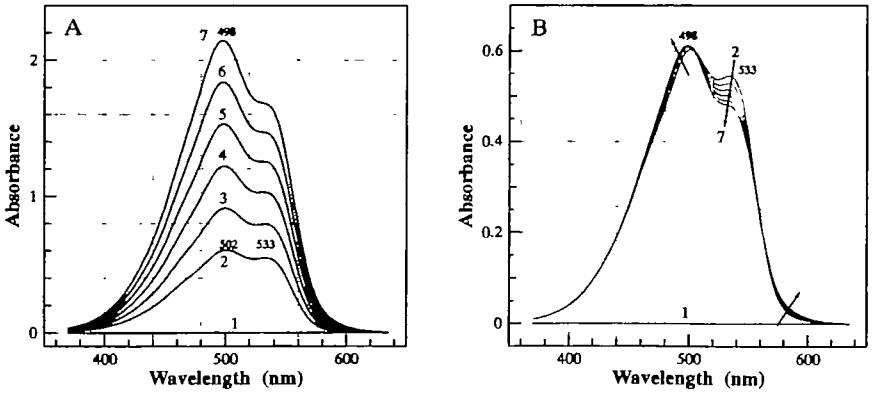

Fig. 1. **(A) Effects of MC540 concentration on the absorption spectrum in an aqueous solution.** MC540 concentrations: spectrum 1, 0; 2, 20; 3, 30; 4, 40; 5, 50; 6, 60; 7, 70 μM. Other conditions; total 2 ml of aqueous solution in cells with a 0.5 cm light path length, at 25°C. The absorbances shown are the values measured in a 0.5 cm cell. **(B) Absorption spectra per unit concentration (10 μM) converted from each spectrum in Fig. 1A.** The absorbances shown are the values per 1 cm cell. Arrows show the direction as the MC540 concentration is increased.

533 nm decrease, and that two isosbestic points are apparent at 502 and 561 nm. At first glance, the presence of the isosbestic points seems to show that the spectral system is composed of only two molecular species.

In phospholipid solution: To examine the effect of a phospholipid (PC) environment on the absorption spectrum of MC540, we added various amounts of PC to the MC540 solution. As shown in Fig. 2, the increase in the amount of PC in the MC540 solution caused a red-shift of the peak wavelength from 502 nm to 532 and 566 nm, with a decrease in the absorbance at 502 nm. Instead, the absorbances at 532 and 566 nm increased; this increase was especially remarkable at 566 nm. This seems to indicate that the spectra of MC540 bound to PC comprise at least two components, one with a peak wavelength at 532 nm, and the other with a peak wavelength at 566 nm.

To clarify this point, the spectra of MC540 were measured at sufficient amounts of PC to bind almost all MC540 molecules. Figure 3A clearly shows that the spectra of MC540 are composed of two peaks at high concentrations of PC. It also appears that the higher the concentration of MC540, the larger the increase in the absorbance at 532 nm compared with that at 566 nm. We displayed all spectra per 10 μM in order to define this tendency. As shown in Fig. 3B, two isosbestic points were apparent at 542 and 596 nm. The presence of the isosbestic points gives the impression that the spectrum of MC540 comprises the spectra of two molecular species.

Calculation of the Number of Components in Aqueous Medium and Phosphatidylcholine Solution—Apart from the above conjectures based on the spectral features, we discuss the exact number of molecular species of MC540 present in aqueous solution and phosphatidylcholine solution, using factor analysis (FA) on the data matrix (**D**). The statistical parameters used to estimate the number of valid eigenvalues were residual standard deviation (RSD), imbedded error (IE), root-mean-square error (RMS), and factor indicator function (IND) (*34*, *35*). Table I and Table II show the results of the calculation. All of the parameters in these tables indicate that the rank number, *i. e.*, the number of molecular species with independent spectra, is three in water and two in PC solution. Two of the species in water are undoubtedly monomeric and dimeric forms of MC540, but the third component is not easily identified. According to a stacking reaction model, however, the third component appears to be a trimeric form. It is natural, in

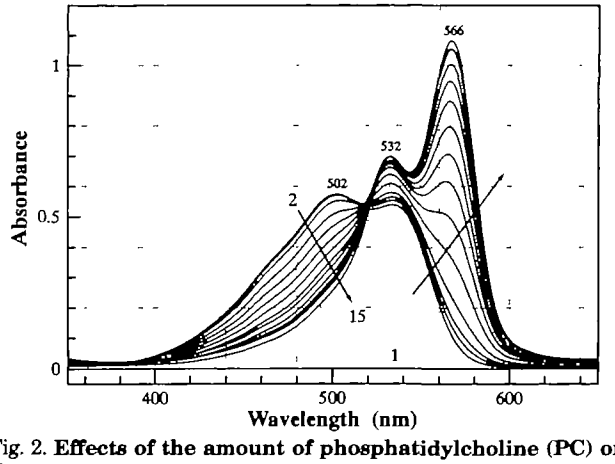

Fig. 2. **Effects of the amount of phosphatidylcholine (PC) on the absorption spectrum in 10 μM MC540 solution.** PC amounts: spectrum 1, base line (only water); 2, 0; 3, 0.05; 4, 0.1; 5, 0.2; 6, 0.4; 7, 0.6; 8, 0.8; 9, 1.0; 10, 1.2; 11, 1.4; 12, 1.6; 13, 1.8; 14, 2.0; 15, 2.2 mg. Other conditions; total 3 ml of solution in a cell with a 1 cm light path length, at 25°C. Arrows show the direction as the amount of PC is increased. Numbers above the spectra indicate peak wavelengths.

TABLE I. **A typical example of the results of rank analysis using the spectral data in water.** The data matrix was constructed from six spectra (curves 2–7) in Fig. 1A.

n	Eigenvalue	RSD	IE	RMS	IND
1	92.67488789	0.017489	0.007140	0.015966	0.000700
2	0.03205527	0.000857	0.000496	0.000702	0.000054
3	0.00005723	0.000276	0.000195	0.000195	0.000031
4	0.00000369	0.000163	0.000133	0.000094	0.000041
5	0.00000064	0.000149	0.000136	0.000061	0.000149
6	0.00000047	—	—	—	—

TABLE II. **A typical example of the results of rank analysis using the spectral data in phospholipid suspension.** The data matrix was constructed from six spectra (curves 1–6) in Fig. 3A.

n	Eigenvalue	RSD	IE	RMS	IND
1	49.06158428	0.037628	0.015362	0.034350	0.001505
2	0.11294664	0.002248	0.001298	0.001836	0.000141
3	0.00024236	0.001300	0.000919	0.000919	0.000144
4	0.00004030	0.001129	0.000922	0.000652	0.000282
5	0.00003755	0.000449	0.000410	0.000183	0.000449
6	0.00000323	—	—	—	—

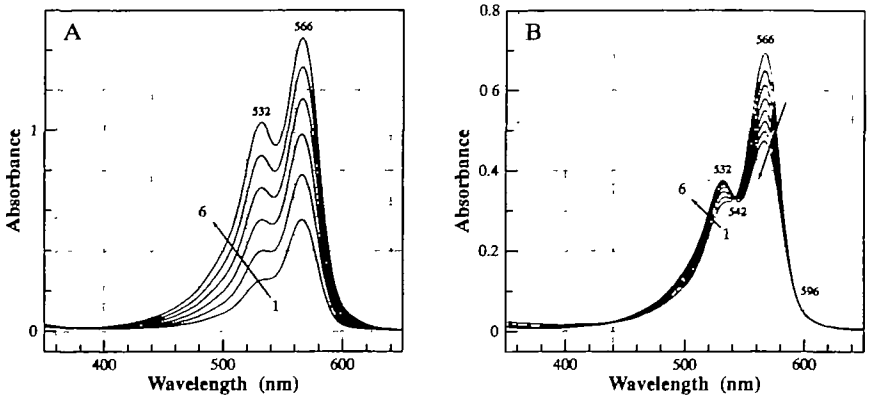

Fig. 3. **(A) Effects of MC540 concentration on the absorption spectrum in PC suspension.** MC540 concentrations: spectrum 1, 8; 2, 12; 3, 16; 4, 20; 5, 24; 6, 28 μM. Other conditions; total 2 ml of solution in a cell with a 0.5 cm light path length, 1.6 mg PC/2 ml, at 25°C. **(B) Absorption spectra per unit concentration (10 μM) converted from each spectrum in A.** Arrows show the direction as the MC540 concentration is increased.

solutions with high concentration of PC, to consider that the two molecular species are a bound monomer and a bound dimer of MC540.

Spectral Separation by a Monomer-Dimer Equilibrium Model in Water—Since Fig. 1B support the view, described above, that the MC540 solution system in water is an approximate equilibrium between monomeric and dimeric forms, we first analyzed the data matrix according to the following one-step reaction model:

$$M + M \underset{K}{\rightleftharpoons} M_2,$$

where M, M_2, and K represent an MC540 monomer, an MC540 dimer and a dissociation constant, respectively. When a dissociation constant K and the resultant concentration matrix $\mathbf{C}^\circ$ are given tentatively, a transformation matrix $\mathbf{T}$ can be calculated from the equation,

$$\mathbf{T} = \mathbf{C}^\circ\mathbf{C}'(\mathbf{C}\mathbf{C}')^{-1},$$

and a matrix $\mathbf{C}^*$ with physiological meaning can also be calculated from $\mathbf{T}$. The two matrices, $\mathbf{C}^\circ$ and $\mathbf{C}^*$, coincide with each other when K has a true value. We searched for the true value of K by finding the minimal value of a residual sum of squares (RSS) by changing the K value, and calculating the RSS by the equation

$$RSS = (\Sigma(d^\circ - d^*)^2)^{1/2},$$

where d° and d^* are an element of $\mathbf{C}^\circ$ and the corresponding element of $\mathbf{C}^*$, respectively.

Figure 4A shows the results of the calculation performed by the above principle, clearly showing the minimal RSS at $K = 31$. Using this value for K, a set of $\mathbf{R}^*$ and $\mathbf{C}^*$ matrices with physiological meaning were calculated by the following relationships:

$$\mathbf{D} = \mathbf{RC} = (\mathbf{RT}^{-1})(\mathbf{TC}) = \mathbf{R}^*\mathbf{C}^*,$$

where the row matrix ($\mathbf{R}^*$) includes the predicted spectra of the assumed one-step reaction model. Figure 4B shows such spectra which are the predicted monomer (curve 1, solid line) and the predicted dimer (curve 2, broken line), expressed as absorbances per 10 μM of MC540 monomer or dimer solution. The dimer spectrum drawn in this figure (curve 2) is for one MC540 molecule involved in one dimeric unit. It is interesting that the monomer has two intrinsic peaks in its spectrum. The dimer of MC540 also has two peaks, but the absorbance is far greater at the shorter wavelength (488 nm) than at the longer (562 nm). This spectral difference can explain the behavior of the spectral changes seen in Fig. 1, that is, decreases in absorbance near 533 nm and increases in absorbance near 500 nm when the concentration of MC540 is raised.

In a reaction model with two components, a system involving a trimer or tetramer instead of a dimer is also possible. We used the PFA to calculate for such reaction models, but the basic spectral shapes of the trimer and tetramer were the same as that of the dimer.

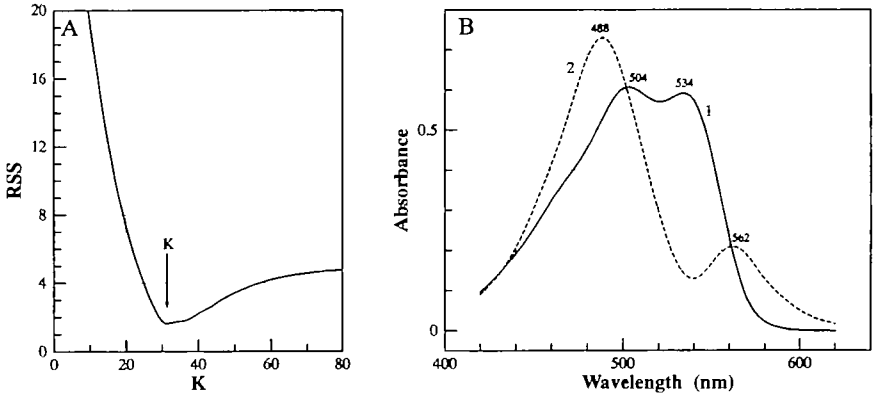

Fig. 4. **Search for the spectra of two components in water using the reaction model M + M = M_2 (K as a dissociation constant).** (A) Result of the search for K giving the smallest residual sum of squares (RSS), as indicated by the arrow, in the PFA calculation using the spectral data of 2 to 7 in Fig. 1A. (B) Predicted spectra using K (= 31.0 in a relative value) found in (A); curve 1 (solid line), monomer (M); curve 2 (broken line), dimer (M_2). The absorbance of the dimer spectrum (curve 2) is expressed as the spectrum for one molecule of MC540 composed in one dimer.

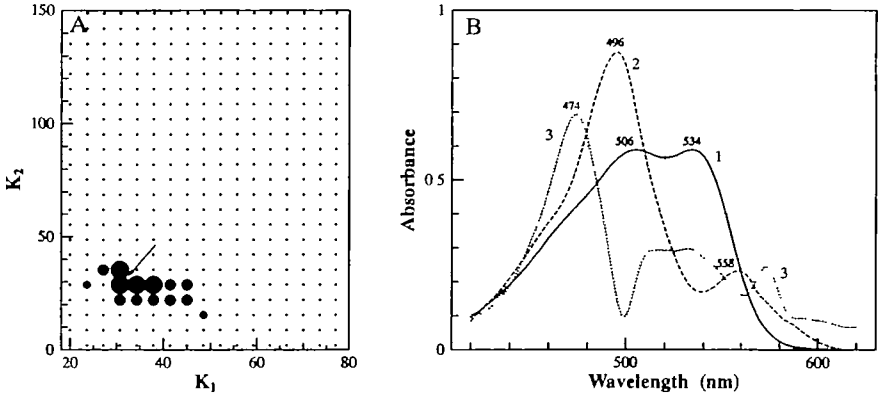

Fig. 5. **Search for the spectra of three components in water using the reaction model M + M = M_2, M_2 + M = M_3 (K_1 and K_2 as dissociation constants at each step).** (A) Two-dimensional display of the search for K_1 and K_2 giving the smallest RSS. The inverse of the RSS (=1/RSS) is expressed by the size of the circles. (B) Result of the separation into three spectra using K_1 (=30.7) and K_2 (=28.6) found in (A) as indicated by the arrow: curve 1 (solid line), monomer (M); curve 2 (broken line), dimer (M_2); curve 3 (dotted line), trimer (M_3). Absorbances of the dimer and the trimer are expressed as the absorption spectra of one molecule of MC540 composed of those molecular aggregates. Numbers above the peaks indicate the peak wavelengths.

Spectral Separation by a Two-Step Model in Water—According to the number of molecular species in Table I (*i.e.*, three), we tried to isolate a trimer spectrum by assuming the following reaction:

$$M + M \underset{K_1}{\rightleftharpoons} M_2$$

$$M_2 + M \underset{K_2}{\rightleftharpoons} M_3.$$

Expression on a two-dimensional plane is convenient to search for a K_1 and K_2 pair that gives the minimal RSS for this reaction model. In Fig. 5A, the magnitude of the RSS is expressed as an inverse of the RSS, and, therefore, the larger the circle, the smaller the RSS value is. As indicated by the arrow in Fig. 5A, we adopted K_1 = 30.7 and K_2 = 28.6 as the minimal RSS in this reaction model. Figure 5B shows three separated spectra obtained by PFA calculation. As in Fig. 5B, the spectra of the dimer (curve 2, broken line) and the trimer (curve 3, dotted line) were for one MC540 molecule involved in one dimer or one trimer molecule, respectively.

The isolated spectra for the monomer and the dimer thus obtained resemble those calculated by the one-step reaction model, as shown in Fig. 4B. The spectrum of the trimer has three peaks (curve 3, dotted line in Fig. 5B). Since a reaction model involving a tetramer instead of a trimer is also possible, we calculated using the PFA for such a model, but could not obtain a reasonable figure for a tetramer. The following reaction also seems to be possible and should be further analyzed: $M + M \rightleftharpoons M_2$, $M_2 + M_2 \rightleftharpoons M_4$; $M + M \rightleftharpoons M_2$, $M_2 + 2M \rightleftharpoons M_4$.

The dissociation constants, K_1 and K_2, are important parameters not only in the PFA calculation, but also in the general interaction between ligand and protein molecules. We used those parameters as relative values in the present study, but it is easy to convert them into the actual absolute values, that is, $K_1 = 3.1 \times 10^{-4}$ M and $K_2 = 5.7 \times 10^{-4}$ M. Using these dissociation constants and the reaction model, the amounts of the three components can easily be calculated at any given MC540 concentration. According to such a calculation, the amount of the trimer is small enough to regard the one-step reaction model as a practical reaction system.

Spectral Separation by a Monomer-Dimer Equilibrium Model in PC Solution—As shown in Fig. 3B and Table II, the reaction system containing high concentrations of phosphatidylcholine (PC) seems to be at an equilibrium between the monomeric and dimeric forms of MC540. The obvious differences between the spectra in PC suspension and those in water indicate that both of the molecular species are present in the hydrophobic environment of PC. Thus, we analyzed the data matrix according to the following one-step reaction model,

$$M^* + M^* \underset{K^*}{\rightleftharpoons} M^*_2,$$

where M^*, M^*_2, and K^* represent a monomer of MC540 bound to PC, a dimer of MC540 bound to PC, and a dissociation constant, respectively. After determining the K^* value (=1.9) for these spectral data (Fig. 6A), we performed the PFA calculation using this K^* value and obtained two component spectra for MC540 molecular species in PC solution (Fig. 6B), one a bound monomer (curve 1, solid line), and the other a bound dimer (curve 2, broken line), expressed as absorbances per 10 μM solution. The dimer spectrum drawn in this figure (curve 2, broken line) was for one MC540 molecule involved in one dimeric molecule. It is noticeable that the bound monomer no longer has two peaks as in water, but one peak with a slight shoulder at the shorter wavelength. The spectrum of the dimer bound to PC has a large peak at 530 nm and a small peak at 576 nm, resembling the spectrum of the dimer in water in shape, but showing an overall bathochromic shift.

Converting the relative value (K = 1.9) into the absolute value, we obtained 1.9×10^{-5} M as a dissociation constant in PC solution. This value was about one-sixteenth that in water.

Relationship between Absorbance and Fluorescence—Since MC540 has been used as a fluorescent probe, we measured its fluorescence excitation and emission spectra in water and in PC solution, and compared the results with the absorption spectra obtained by the PFA calculation.

Fluorescence in Water—In water, as shown in Fig. 7, maximal fluorescence in the emission spectrum was observed at 578 nm (curve 1, solid line) at an excitation wavelength of 465 nm. Thus, the excitation wavelength was

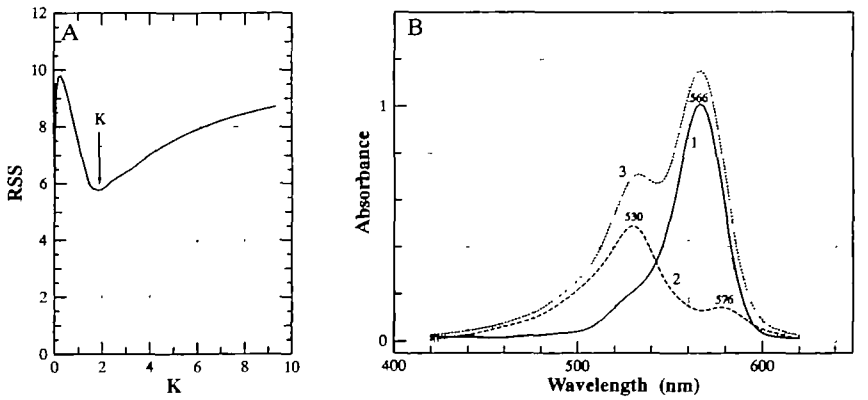

Fig. 6. **Search for spectra of two components in PC suspension using the reaction model $M^* + M^* \rightleftharpoons M^*_2$ (K as a dissociation constant).** The PFA calculation in the Type-II data, the data type obtained by changing the dye concentrations such as the present case of MC540, has been shown to make it difficult to obtain a clear inflection point of the residual sum of squares (RSS) (*33*). For this reason we examined the PFA calculation in many data series, and obtained an average image of the result. Therefore, the results shown in 3A and 3B are typical examples of the calculations obtained using different series of data. (A) At typical case of the search for K that yields the smallest residual sum of squares (RSS), a position indicated by the arrow, in the PFA calculation using spectral data for 4, 6, 8, 10, 12, and 14 μM MC540; 1.3 mg PC/2 ml. (B) Typical predicted spectra using K (=1.9 in a relative value) found in (A). The data series used for the calculation was the same as shown in Fig. 3; curve 1 (solid line), monomer (M^*); curve 2 (broken line), dimer (M^*_2). Absorbance of the dimer spectrum (curve 2) is expressed as the spectrum for one molecule of MC540 composed of one dimer.

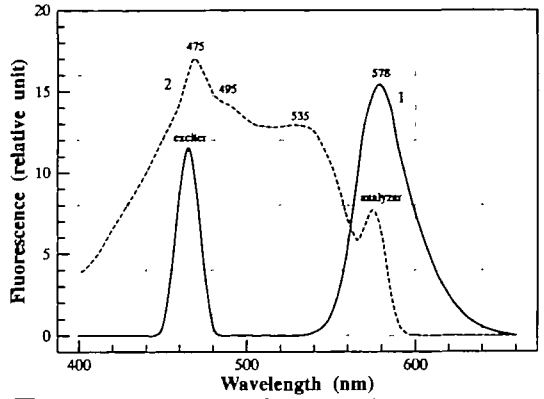

Fig. 7. **Fluorescence spectra of 60 μM MC540 in water at 25°C.** Curve 1 (solid line), fluorescence emission spectrum (exciter, excitation at 465 nm); curve 2 (broken line), fluorescence excitation spectrum (analyzer, emission at 578 nm). Numbers above peaks indicate the peak wavelengths.

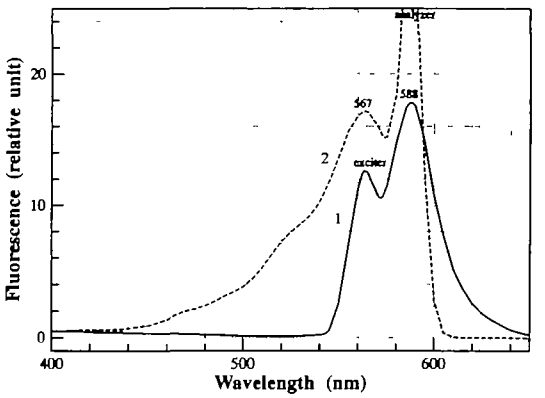

Fig. 8. **Fluorescence spectra of 14 μM MC540 in a 4 mg/3 ml PC suspension at 25°C.** Curve 1 (solid line), fluorescence emission spectrum (exciter, excitation at 560 nm); curve 2 (broken line), fluorescence excitation spectrum (analyzer, emission at 588 nm). Since the fluorescence intensity in the PC suspension was so high, the recording was performed at one-fiftieth the sensitivity of that used for water. Numbers above peaks indicate the peak wavelengths. To estimate the effect of light scattering, we obtained excitation spectra in the cuvette without MC540 using an analyzer at several wavelengths, and confirmed the consistency of 567 nm as a peak wavelength in the MC540 spectrum.

scanned at an emission wavelength of 578 nm (curve 2, broken line). Three peaks were observed in this excitation spectrum. Interestingly, these three peaks correspond approximately to the peak-wavelengths in the absorption spectra of the trimer, the dimer, and the monomer, respectively, as isolated by PFA calculation in water. Therefore, it is likely that although the fluorescence intensity is very small, these three molecular forms participate in the fluorescent excitation of MC540 to a certain extent in water.

Fluorescence in Phospholipid Solution—In the PC solution, as shown in Fig. 8, maximal fluorescence in the emission spectrum was observed at 588 nm (curve 1, solid line) at an excitation wavelength of 560 nm. Thus, we obtained the excitation spectrum by scanning at 588 nm as an analyzer (curve 2, broken line). It is also interesting that the peak wavelength of the excitation spectrum is near that for the absorption spectrum of the monomer bound to PC (Fig. 6B). However, no clear peak in the excitation spectrum was observed near the peak wavelength of the absorption spectrum of the dimer, except for a small shoulder near 530 nm. This result suggests that the molecular species involved in the fluorescence excitation is not the dimer, but the monomer.

DISCUSSION

According to the principle of principal factor analysis (PFA), all of the spectra calculated by PFA are independent and do not correlate with one another; this means that all spectra observed can be synthesized using the isolated element spectra. In water, the number of element spectra for MC540 is three. It is reasonable to assume a monomer, a dimer, and a trimer if a stacking reaction model is adopted. However, since the amount of the trimer is small, the monomer and dimer alone are sufficient to approximate the observed spectral data. According to a calculation, the proportion of the MC540 monomer in water is greater than 95% at 5 μM MC540 solution. A spectrum obtained under these conditions is nearly monomeric in shape, as also suggested by Lelkes *et al.* (*11*) and Ehrenberg *et al.* (*24*) from the spectra at low concentrations of MC540. However, a dimer spectral shape is never obtained in spectrophotometric measurement, even at 100 μM MC540, because an increase in higher molecular aggregates, such as the trimer or, possibly, the tetramer, in addition to the remaining monomer.

The present data clearly show that the two peaks of the spectrum of the MC540 monomer at 504 and 534 nm reflect the intrinsic nature of this species. Onganer *et al.* (*37*) stated that in a protic solution such as pure water, a zwitterionic structure is more stable than an uncharged structure due to hydrogen-bonding interactions with the negatively charged oxygen atom of MC540. They also describe *trans-cis* conformations in MC540. Although the reason for the presence of two peaks for an MC540 monomer remains unclear, the wavelength width of 30 nm between these two peaks, corresponding to the wave number 1,115 cm^{-1} or 3.2 kcal/mol, is so small that a single hydrogen bond in a protic solvent would be sufficient to yield such an energy difference.

With an increase in the amount of phosphatidylcholine, the spectrum of the MC540 solution shows a remarkable bathochromic shift through a complicated spectral pattern (Fig. 2). At sufficient amounts of PC, the number of element spectra is two; undoubtedly, one is a monomer bound to PC, and the other is a dimer bound to PC. The calculated spectrum for the monomer bound to PC no longer has two peaks as in water, with the missing peak at the shorter wavelength, suggesting that the hydrogen bond disappears in an aprotic environment.

A dimer spectrum with two peaks in the presence of phospholipid has been reported by Waggoner *et al.* (*38*), based on the existence of isosbestic points that suggest the presence of two molecular species. The dimer spectrum determined by PFA calculation in the presence of PC also has two peaks that resemble those of the dimer spectrum in water, that is, with the large peak at the shorter wavelength and the small peak at the longer wavelength. However, while the overall figures of the two dimer spectra resemble one another, there is a remarkable red shift of

about 40–50 nm in the dimer spectrum in the presence of PC. This strongly indicates that the dimer is restricted to the hydrophobic environment created by the PC. It should also be emphasized that, according to the exciton model proposed by Kasha *et al.* (*39*), a wavelength width of 50–70 nm, 1,600–2,700 cm^{-1}, between two peaks is typical of a dimer. The resemblance of spectral figures between the dimers, in water and PC also suggests that the orientation of the MC540 molecules in hydrophobic environment is similar to that in the hydrophilic environment. The dissociation constant between a monomer and dimer in water, 3.1×10^{-4} M, is ten times larger than that in PC solution, 1.9×10^{-5} M, indicating that the dimer is formed more easily in PC solution than in water.

The fluorescence intensity of the monomer in the phospholipid environment is far higher than that of the dimer in phospholipid or any MC540 species in water. Thus, it seems very likely that a certain transmembrane potential change causes a temporal transformation of the dimer into the monomer in the hydrophobic environment, with a resultant increase in fluorescence emission, as suggested by Ross *et al.* (*3*) and Waggoner *et al.* (*38*).

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