[Chem. Pharm. Bull. 34(11)4479—4485(1986)]

Estimation of Critical Micelle Concentrations of Lysolecithins with Fluorescent Probes

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(Received May 12, 1986)

The estimation of the critical micelle concentration (cmc) of various lysolecithins with 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) and 1-anilinonaphthalene-8-sulfonate (ANS) as fluorescent probes was investigated. The fluorescence yield of TNS in palmitoyl lysolecithin micellar phase was found to be somewhat larger than that of ANS. Further, the fluorescence yield of TNS in the aqueous phase is known to be much smaller than that of ANS. Thus, TNS should be useful for estimating very small cmc values of surfactants such as lysolecithins. The cmc values of lysolecithins estimated by using TNS were 0.5 mM for lauroyl lysolecithin, 63 μM for myristoyl lysolecithin, 8.3 μM for palmitoyl lysolecithin and 6.6 μM for oleoyl lysolecithin, in 20 mM Tris–HCl buffer solution (pH 7.0) at 25 °C. It was also found that stearoyl lysolecithin does not form micelles at room temperature, and the Krafft point is about 60 °C.

Keywords——lysolecithin; lauroyl lysolecithin; myristoyl lysolecithin; palmitoyl lysolecithin; stearoyl lysolecithin; oleoyl lysolecithin; critical micelle concentration; fluorescent probe; 2-*p*-toluidinylnaphthalene-6-sulfonate; 1-anilinonaphthalene-8-sulfonate

Lysolecithins (*sn*-1-*O*-acyl-glycerol-3-phosphorylcholine) have properties quite different from diacyl phosphatides. The relatively high solubility in water, and the ability to form micelles in aqueous solutions have been rationalized on the basis of the relatively large hydrophilic head group in relation to the hydrocarbon tail as compared with other phospholipids. Lysolecithins have important effects in biological systems. These include membrane lysis,[1] membrane fusion,[2] increased membrane permeability[3] and enhanced immune reactions.[4] Recently, it has been recognized that lysolecithin in the presence of cholesterol forms a stable lamellar phase[5] and that lysolecithin and cholesterol form a stable equimolar complex[5]; the the solubilization of drugs such as steroids[6] has also been investigated. Many of the effects of lysolecithins on biological systems are related to the amphiphilic character of the molecules. The surface activities of lysolecithins as well as other physical properties in solution have been studied by a number of workers. However, the values of critical micelle concentration (cmc) of lysolecithins are known only for egg yolk lysolecithin[7] and palmitoyl lysolecithin,[8] so far as we are aware.

In early investigations, the possibility of the use of fluorescent probes as a tool to determine the cmc of surfactants has been suggested. Two examples of fluorescent probes are 1-anilinonaphthalene-8-sulfonate (ANS) and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). These compounds have been used to investigate protein conformations and hydrophobic regions on the protein surface.[9] Vendittis *et al.* suggested that ANS is useful for estimation of the cmc values of anionic, zwitterionic and nonionic surfactants.[8b] The purpose of this work was to examine the usefulness of TNS as a fluorescent probe for estimation of the cmc and to determine the cmc of lysolecithins with a variety of acyl chains by the use of TNS as a fluorescent probe.

Experimental

Materials——L-α-Lauroyl lysolecithin (LlysoPC), L-α-myristoyl lysolecithin (MlysoPC), L-α-palmitoyl lysolecithin (PlysoPC), L-α-stearoyl lysolecithin (SlysoPC) and L-α-oleoyl lysolecithin (OlysoPC) were purchased from Sigma Chemical Co., Ltd. The purity of the lysolecithins was monitored by thin layer chromatography on Silica Gel 70 with chloroform–methanol–water (65 : 35 : 4) as the eluting solvent. A single spot was obtained in each case by using chemical detection procedures.[8a)] TNS potassium salt and ANS ammonium salt were supplied by Aldrich Chemical Company Inc. and Wako Pure Chemical Industries Ltd., respectively. Tris(hydroxymethyl)aminomethane (Tris) was used as received. Water was doubly distilled from a quartz still. The lysolecithins were stocked as chloroform–ethanol (1 : 1) solutions below 4 °C.

Measurements——Fluorescence spectra were obtained at 25 °C using a Jasco FB-550 spectrofluorometer with a Hitachi 056 recorder. The excitation wavelengths were 366 and 356 nm for TNS and ANS, respectively. Quinine sulfate and β-naphthol were used to correct the fluorescence intensities. The absorbance of the fluorescent probes at the excitation wavelength was less than 0.1. The absorption spectra were obtained at 25 °C using a Hitachi 220 spectrophotometer.

Fluorescence titrations were performed by stepwise additions of small aliquots of dense lysolecithin solution to the fluorescent probe solution, buffered with 20 mM Tris–HCl at pH 7.0. Corrections were made for dilution of the probes which, however, did not exceed 10%.

Results

Figure 1 shows the absorption and emission spectra of TNS and ANS in 120 μM PlysoPC micellar solution. The emission maxima were at about 460 nm for TNS and about 500 nm for ANS. Figure 2 shows the effect of PlysoPC on the emission spectra of TNS. No significant

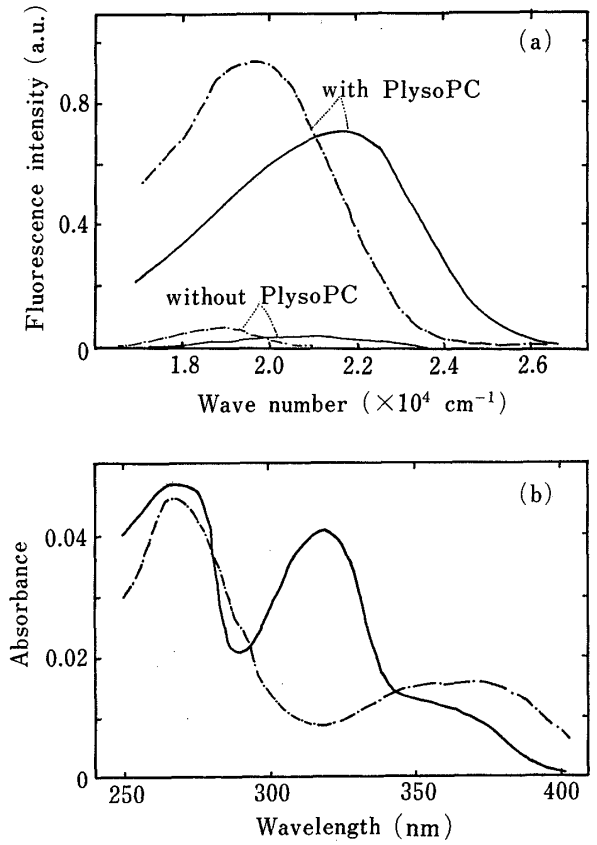


Fig. 1. Emission (a) and Absorption (b) Spectra of 2 μM TNS (——) and 2 μM ANS (- - - -) in 120 μM PlysoPC Micellar Solution (20 mM Tris–HCl, pH 7.0) at 25 °C

Fluorescence was excited at 366 and 356 nm for TNS and ANS, respectively.

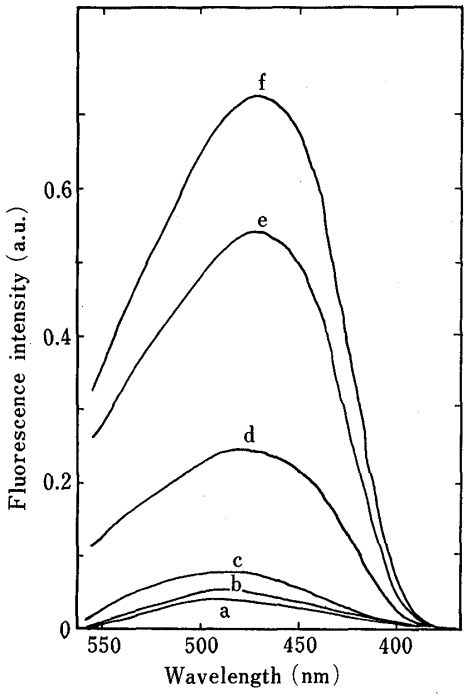


Fig. 2. Fluorescence Spectrum of TNS at Various Concentrations of PlysoPC

Increasing amounts of a 3 mM stock solution of PlysoPC were added to 10 ml of 20 μM TNS in 20 mM Tris–HCl, pH 7.0, at 25 °C. Fluorescence was excited at 366 nm. PlysoPC concentrations: (a), 0; (b), 4; (c), 12; (d), 40; (e), 80; (f), 110 μM.

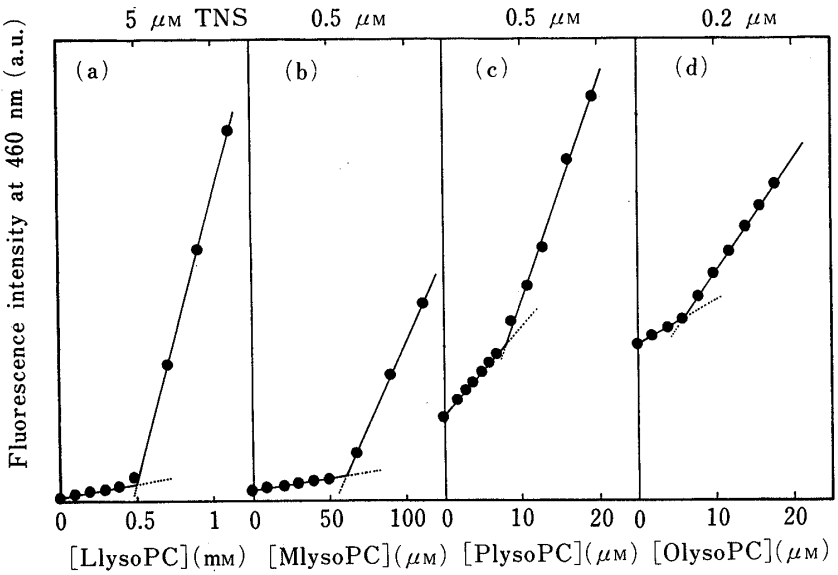


Fig. 3. Fluorescence Titrations of TNS with LlysoPC (a), MlysoPC (b), PlysoPC (c), and OlysoPC (d) at 25 °C

The concentration of TNS was 0.5 μM for PlysoPC and MlysoPC, 5 μM for LlysoPC and 0.2 μM for OlysoPC.

TABLE I. Critical Micelle Concentrations (cmc) of Various Lysolecithins at 25 °C

Lysolecithins		cmc[a] (M)
Lauroyl	(12:0) lysolecithin	5.0×10^{-4}
Myristoyl	(14:0) lysolecithin	6.3×10^{-5}
Palmitoyl	(16:0) lysolecithin	8.3×10^{-6}
Stearoyl	(18:0) lysolecithin	—[b]
Oleoyl	(18:1) lysolecithin	6.6×10^{-6}

a) In 20 mM Tris–HCl buffer solution at pH 7.0. *b*) The Krafft point is about 60 °C.

spectral changes were observed at concentrations of PlysoPC below the cmc (8.3 μM). When PlysoPC was present at a concentration above the cmc, there was a large increase in the emission intensity of TNS and a blue shift of about 40 nm in the peak wavelength. It is generally observed that surfactant solutions show sharp changes in physical properties such as density, refractive index, surface tension, equivalent conductance, solubility, *etc.* when the cmc is reached.[10]

Figure 3c shows the enhancement of fluorescence intensity of TNS at 630 nm as a function of the concentration of PlysoPC. Two straight lines can be drawn through the experimental points; their intersection (8.3 μM) is taken as the concentration above which PlysoPC aggregates to form micelles. This is supported by the fact that the cmc determined in this study agreed with the values measured by other methods; fluorometric methods with ANS (8.4 μM, 10 mM Na-phosphate pH 7.2, 0.1 M KCl)[8a] or rhodamine 6G (7.5 μM, 10 mM Na-phosphate pH 7.2, 0.1 M KCl)[8a] as a probe, and the equilibrium dialysis method (7.0 μM, multicomponent buffer with ionic strength 0.2 M).[8b] The results of fluorescence titration of TNS with LlysoPC, MlysoPC and OlysoPC are shown in Figs. 3a, 3b and 3d. The intersections of the lines in these figures led to cmc values at 25 °C of 0.50 mM for LlysoPC, 63 μM for MlysoPC and 6.6 μM for OlysoPC, as shown in Table I. In the titration of TNS with SlysoPC at 25 °C, large changes in fluorescence intensity were not observed. However, increase in the incubation temperature at a constant concentration (20 μM) of SlysoPC led to

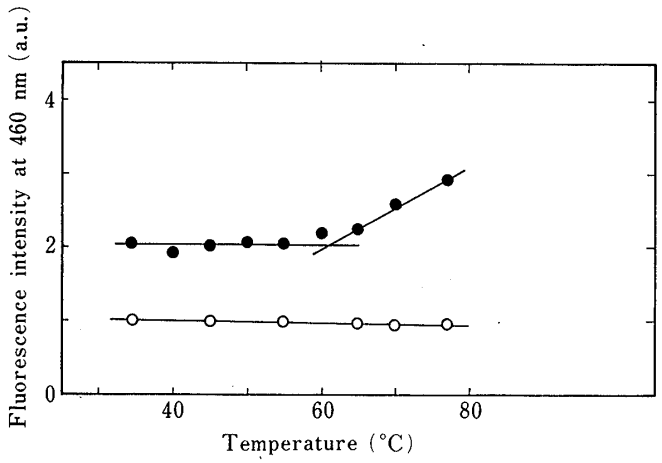


Fig. 4. Enhancement of TNS Fluorescence as a Function of Incubation Temperature at a Constant Concentration (20 μM) of SlysoPC

○, without SlysoPC; ●, with SlysoPC.

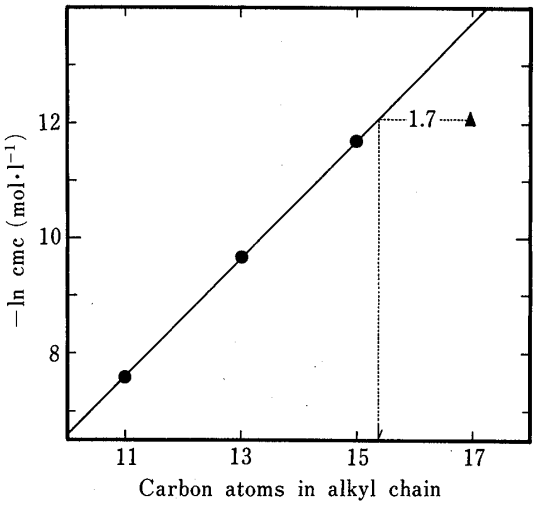


Fig. 5. Logarithm of cmc as a Function of Carbon Numbers of the Alkyl Chain in Lysolecithins

(●), lysolecithins with a saturated alkyl chain; (▲), OlysoPC, which has a double bond near the middle of the hydrocarbon chain.

enhancement of the fluorescence of TNS at 460 nm above about 60 °C, as shown in Fig. 4, indicating the micellar formation at above that temperature. This result suggests that the solubility of SlysoPC at room temperature is small, and the Krafft point is about 60 °C.

Figure 5 shows the relation between the cmc and the carbon number of the alkyl chain in lysolecithin molecules. These plots give a straight line for saturated lysolecithins, and its slope leads to a value for the energy of micellar formation of −0.61 kcal/mol per methylene group of the alkyl chain.

The binding of the fluorescent probes to the PlysoPC micelle was estimated from the partition coefficients of the fluorescent probes from the aqueous phase to the micellar pseudophase. The partition coefficient (P) may be described by[11]

$$P=\frac{x/V_m}{(a-x)/V_w} \tag{1}$$

where a and x are the total amount (mol) of the probe in the solution and that of the probe in the micellar pseudophase, and V_m and V_w are the volumes of the micellar pseudophase and the aqueous phase, respectively. Assuming that the following relation holds between fluorescence intensities (I_f) and x/V_m,

$$I_f=c\left(\frac{x}{V_m}\right) \tag{2}$$

and that the fluorescence intensity of the probes in the aqueous phase may be ignored,[9a] we have

$$\frac{V_m/V_w}{I_f}=\frac{1}{I_f^\infty}(V_m/V_w)+\frac{1}{I_f^\infty}\frac{1}{P} \tag{3}$$

where c is a constant, I_f is the fluorescence intensity at 460 and 498 nm for TNS and ANS, respectively and $I_f^\infty=c(a/V_m)$; I_f^∞ stands for the fluorescence intensity when the probe is completely solubilized in the micellar pseudophase. Here, the volume of the micellar pseudophase is evaluated with the use of 1.0 g/cm^3[10] as the density of the micellar phase in the aqueous solution. Figure 6 shows $(V_m/V_w)/I_f$ as a function of V_m/V_w according to Eq. 3.

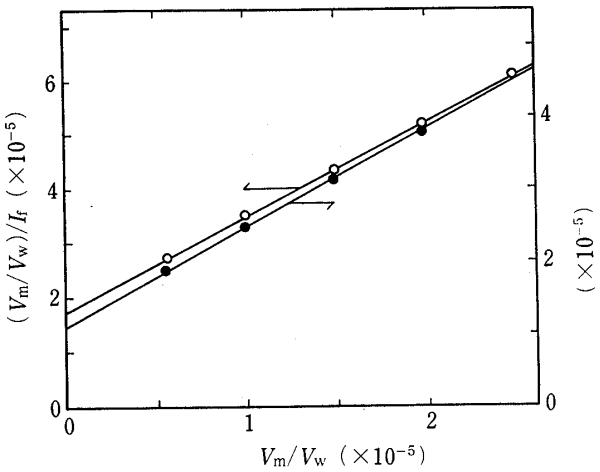


Fig. 6. Plots of $(V_m/V_w)/I_f$ as a Function of V_m/V_w for Partitions of TNS and ANS to PlysoPC Micelles

TABLE II. Comparison of Partition Coefficients and Fluorescence Yields

	ANS	TNS
P	1.1×10^4	1.2×10^4
I_f^∞	7.47	10.3
Abs.	0.0109	0.0164
ϕ_f^{Rm}	1	1.1
ϕ_f^{Rw}	1	0.25

P, partition coefficient of the probes from the aqueous phase to the PlysoPC micellar pseudophase; I_f^∞, fluorescence intensity at 460 nm and 498 nm for TNS and ANS, respectively, when the probes are completely solubilized in the micellar pseudophase; Abs., absorbance at the excited wavelength (at 366 nm and 356 nm for TNS and ANS); ϕ_f^{Rm} and ϕ_f^{Rw}, relative fluorescence yields of the probes in the micellar pseudophase and aqueous phase, respectively.

These plots yielded straight lines, and the slopes and intercepts led to values for P of 1.2×10^4 and 1.1×10^4, and for I_f^∞ of 7.47 and 10.3 for TNS and ANS, respectively.

The absorbance values of the probes at the excitation wavelength were 0.0109 for TNS and 0.0164 for ANS. The absorbance of each probe at the excitation wavelength is proportional to the number of photons absorbed by the probe. Therefore, if the fluorescence intensity at the monitoring wavelength is in proportion to the area of the fluorescence peak, the absolute fluorescence yield (ϕ_f^m) of the probes in the micellar phase may be estimated from the value obtained by dividing I_f^∞ (7.47 for TNS and 10.3 for ANS) by the absorbance at the excitation wavelength (0.0109 for TNS and 0.0164 for ANS). According to the above procedures, the relative value of ϕ_f^m for TNS with respect to that of ANS (ϕ_f^{Rm}) was estimated to be 1.1 (Table II). On the other hand, McClure and Edelman[9a] and Turner and Brand[9b] reported the values of absolute fluorescence yield (ϕ_f^w) of these probes in water to be 0.0008 and 0.0032 for TNS and ANS, respectively. The relative value of ϕ_f^w for TNS with respect to that of ANS (ϕ_f^{Rw}) was estimated to be 0.25 (Table II).

Discussion

Comparison of the Fluorescence Yields and Micellar Bindings of TNS and ANS

A fluorescent probe for determining the cmc of surfactants should possess the following properties; (i) strong binding of probes to micelles, and (ii) high fluorescence yield of probes in the micellar phase and low fluorescence yield in the aqueous phase. The bindings of TNS to cyclodextrins are about one order of magnitude larger than those of ANS.[12] The hy-

drophobic interaction between the tolyl group of the TNS molecule and the cavity of the cyclodextrins was suggested to contribute to the relatively strong binding of the probes to the cyclodextrins.[12] In this study, the difference in the bindings (the P values) of TNS and ANS to the micelles was found to be small. Though this result indicates that the binding of the probes to the PlysoPC micellar surface might be affected by various specific structural interactions other than the hydrophobic ones, a more detailed examination of the bindings is in progress in our laboratory.

The binding of TNS as estimated from the partition coefficient (P) from the aqueous phase to the PlysoPC micellar pseudophase is slightly stronger than that of ANS. The difference between the fluorescence yields in the micellar phase and in the aqueous phase was larger for TNS than for ANS (Table II). From these results, it is suggested that TNS is a suitable fluorescent probe for precisely estimating very small cmc values of surfactants such as lysolecithins. Therefore, the cmc values of a variety of lysolecithins were determined by using TNS as a fluorescent probe.

The cmc Values of Various Lysolecithins

In the case of lysolecithins having a saturated acyl chain, the plot of the logarithm of the cmc values *versus* carbon numbers of the alkyl chain in lysolecithin molecules gave a good linear relationship and followed Traube's rule,[13] as shown in Fig. 5. The value for the energy of micellar formation per methylene group of the alkyl chain ($\mu_m^0 - \mu_w^0$) is -0.61 kcal/mol, as estimated from the slope of the line.

Klevens suggested that the introduction of a double bond (in these instances near the middle of the hydrocarbon chain) has an effect equivalent to removal of 1 to 1.5 carbon atoms from a saturated fatty acid chain on the basis of cmc values for potassium salts of saturated and unsaturated fatty acids.[14] The cmc value of OlysoPC corresponds to that of lysolecithin having a saturated hydrocarbon chain of 15.3 carbon atoms, as shown by the arrow in Fig. 5. This indicates that the introduction of a double bond into the alkyl chain corresponds to removal of 1.7 carbon atoms from a saturated alkyl chain, and this result is consistent with that for unsaturated fatty acid salts.

Figure 7 compares the cmc values of lysolecithins and two types of analogs of lysolecithins, ether-deoxy lysolecithins (1-alkyl-propanediol-3-phosphorylcholine),[15] in which the ester link to the phosphoglyceride moiety is replaced by an ether link, and phosphorylcholines (alkyl-phosphorylcholine),[16] which have the alkyl chain attached directly to phosphate. The cmc values for lysolecithins are close to those for ether-deoxy lysolecithins. This indicates that the contribution of hydroxy and carbonyl groups in lysolecithin molecules

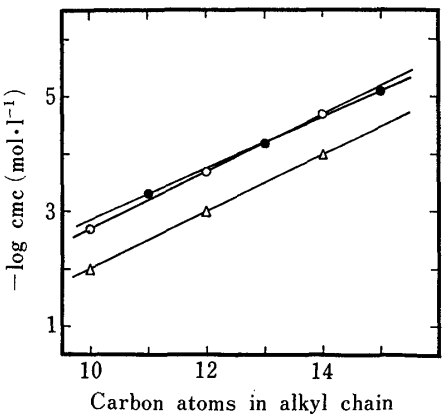
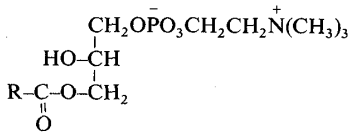


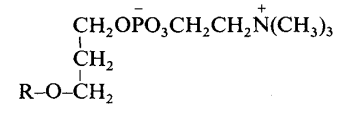
Fig. 7. Comparison of cmc Values of Lysolecithins and Lysolecithin Analogs

(●), lysolecithins; (○), ether-deoxy lysolecithins;[15] (△), phosphorylcholines.[16]

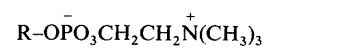
●: lysolecithins,



○: ether-deoxy lysolecithins,



△: phosphorylcholines,



to the cmc values is small. The cmc values for lysolecithins are one order of magnitude lower than those for phosphorylcholines, and close to those for ether-deoxy lysolecithins. The reason for this result has been suggested by Tanford[17]; the zwitterionic groups in lysolecithins and ether-deoxy lysolecithins are separated considerably from the alkyl chain by the intervening glyceryl moiety. Between lysolecithins or ether-deoxy lysolecithins and phosphorylcholines, there is a difference of about 2 carbon atoms in the carbon atom number of the alkyl chain between lysolecithins and their analogs which have the same cmc values. This indicates that the glyceryl moiety in lysolecithins and ether-deoxy lysolecithins is effectively equivalent to an ethylene group.

In conclusion, it appears that TNS is a useful fluorescent probe, as is ANS, for estimating the cmc values of lysolecithins, and TNS may also be useful for determining the cmc values of other surfactants. The cmc values of various lysolecithins were accurately determined by using TNS as a fluorescent probe.

Acknowledgement The present work was supported in part by a Grant-in-Aid for Scientific Research (No. 59370048) from the Ministry of Education, Science and Culture.

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