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COLLOIDS AND SURFACES A

Cholic acid behavior in water and organic solvent: study of normal and inverted aggregates

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Abstract

Water-soluble fluorescent probes, including 1-pyrene butyric acid, rhodamine B, sodium salt 1,5-diaminoanthraquinone-2,6-disulfonic acid, quinine hemisulfate and monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid were used to determine the critical micelle concentration (cmc) of inverted micelles formed by cholic acid in tetrahydrofuran. Another water-insoluble fluorescent probe, namely pyrene, was used to determine the cmc of normal micelles formed by sodium cholate in water. Relative scattered light intensity measurements give direct evidence of transition concentrations similar to cmc in surfactants. Our results indicate that in the inverted micelles, hydrophilic sites placed in the core are present in cholic acid aggregates above the cmc region at 5–7 mM. In normal micelles, hydrophobic sites in the core are present in sodium cholate aggregates above the cmc region at 15 mM. Another critical point in the aggregation of cholic acid and sodium cholate occurs at 1 mM. Normal phase liquid chromatography determination of the cmc reversed micelles has also been performed, and the results obtained agree well with the spectroscopic studies.

Keywords: Cholic acid; Inverted aggregates; Normal aggregates; Transition concentrations

1. Introduction

Recent studies have explored a different class of amphiphilic compounds, namely the bile acids or their salts, as an alternative to the synthetic detergents or cyclodextrins to improve luminescence analysis [1,2].

The bile salts are biological compounds that are synthesized from cholesterol in the liver; they are typically composed of a steroidal backbone with one or more α-oriented hydroxy groups conjugated to an anionic chain, or tail. The α-orientation of the hydroxy groups places them on the concave side of the steroid skeleton, with the methyl groups positioned on the opposite, convex side; the polar or charged group on the aliphatic tail will therefore interact with the hydroxy groups on the concave surface [3]. The resulting bile salt conformation provides a hydrophobic (methyl-containing) surface on one side and a hydrophilic (hydroxy-containing) surface on the other side. This structure is distinct from that of a conventional detergent monomer, which has a hydrophilic head group and a long hydrophobic tail.

The bile salts also exhibit unique behavior with respect to self-association and molecular solubilization [4–7]. In conventional detergents, solubilization sites include the micellar surface, the palisade layer at the interface between the hydrophobic tails and the hydrophilic head groups and

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the hydrophobic inner core [8]. Analogous binding sites are not present in the smaller, more rigid, bile salt micelles. Instead, solubilization of hydrophobic compounds is accomplished through favorable interactions with the hydrophobic surfaces of the bile salt micelles [9,10]. The resulting solubilization microenvironments in the bile salt micelles are often highly apolar [11].

Micellar bile salt solutions have been reported to exhibit significant polydispersity with respect to aggregate size and structure [12], implying that the average aggregation number increases with surfactant concentration [13]. Thus, cmc determination is not a simple task.

Bile salts in aqueous solutions have been the subject of much interest and controversy in recent years. Sodium cholate, a trihydroxy bile salt, has been extensively studied by using different experimental techniques. Among them, we can mention conductivity, density, diffusion, equilibrium ultracentrifugation, ESR, fluorescence, light scattering, microcalorimetry, NMR, osmometry, X-ray, partition method, potentiometry, refractive index, solubilization, surface and interfacial tension, spectra shift, ultrasonic absorption and viscosity [14]. The very existence of true cmc values for bile salts has been questioned [15–17] because of the absence of well-defined critical points in experimental curves and the presence of aggregation, even at very low concentrations. Those cmc (or ''quasi cmc'') values reported for sodium cholate in water at room temperature range from 0.003 [18] to 0.018 M [19,20].

Bile salts have been used in micellar liquid chromatography [21,22] and as ion pair reagents in liquid chromatography [23]. The bile compounds are new chiral surfactants, and they have extended the scope of separation [24–26]. The analytical applications of aqueous micelle solutions and those of non-aqueous reversed micelles have been reviewed [27,28].

Reversed micelles offer more possibilities than simple aqueous micelles in analytical chemistry. The cmc of the reversed micelles is much more highly dependent on solvent, water content and temperature than normal micelles [29]. Since reversed micelles nanometer-scale water droplets are stabilized by a monolayer of surfactants in a bulk organic solvent, hydrophilic reagents are solubilized in the water pool but in a bulk organic solvent. The interior core of the reversed micelle provides a unique and versatile reaction field. Depending upon its water content (which also dictates the size of the aggregate), the microscopic polarity, microviscosity and the activity of the reagents induced in the core can vary markedly [30]. Thus, solubilization of enzymes (without loss of activity), and chemiluminescent reagents (luminol) [31], co-solubilization of the hydrophobic compounds constrained in the core and subjected to energy transfer between them, and occurrence of drastic changes and reactivity of solubilized reagents, provide an exciting field for analytical applications in reversed micelles [32].

Other authors have studied the aggregation state of the cholic acid and other derivatives in organic solvent as chloroform and carbon tetrachloride using vapour pressure osmometry [33].

The behaviour of a solution from the derivatives of bile acids is thoroughly different in each solvent. The type of aggregation of cholic acid in chloroform and carbon tetrachloride is that of a monomer–dimer–trimer, that is to say, it presents a number of small aggregations. Equally, bile acids in aqueous solution also present a number of small aggregations of three or four monomers in comparison with the SDS in aqueous solution, which presents an aggregation number of 62 [34].

We report here studies of cmc behavior of cholic acid in tetrahydrofuran (reversed micelles) and sodium cholate in water (normal micelles) based on fluorimetric measurements of several different fluorescent probes. The probes used include 1-pyrene butyric acid, rhodamine B, sodium salt 1,5-diaminoanthraquinone-1,6-disulfonic acid, quinine hemisulfate, monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid and pyrene. Measurements of fluorescence spectra were used to study the microenvironmental surfactant concentration. Scattered light measurements provide evidence of monomer aggregates similar to cmc in the micelles. Here, we include a study of the cmc determination by normal-phase liquid chromatography using cholic acid in tetrahydrofuran as a mobile phase.

2. Experimental

The fluorescence probes, including 1-pyrene butyric acid, rhodamine B, quinine hemisulfate salt and pyrene, were obtained from Sigma Chemical Co. (St. Louis, MO), sodium salt 1,5 diaminoanthraquinone-2,6-disulfonic acid was obtained as reported earlier [35], and monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid was obtained from Fluka Chemie AG (Buchs, Switzerland). The plant growth regulators 1-naphthylacetic acid (1-NAA), 2-naphthylacetic acid (2-NAA), indol-3-butyric acid (IBA), indol-3-propionic acid (IPA) and indol-3-acetic acid (IAA), and the chemicals cholic acid and sodium cholate were purchased from Sigma Chemical Co. Tetrahydrofuran and ethanol were gradient-grade Lichrosolv from Merck (Darmstadt, Germany).

Stock solutions of 1-pyrene butyric acid (1×10^{-3} M), rhodamine B (1×10^{-4} M), sodium salt 1,5-diaminoanthraquinone-2,6-disulfonic acid (1×10^{-3} M), quinine hemisulfate (1×10^{-3} M), and pyrene (2×10^{-3} M) were prepared by dissolving the appropriate weight of crystalline solid in ethanol and monosodium salt 8-amino-2-naphthol-3,6-disulfonic acid (1×10^{-3} M) in water.

Stock solutions of 1-NAA (5.37×10^{-3} M), 2-NAA (5.37×10^{-3} M), IBA (4.92×10^{-3} M), IPA (5.26×10^{-3} M) and IAA (5.71×10^{-3} M) were prepared by dissolving in methanol and stored at 4°C. Working solutions were prepared by diluting the stock solutions with tetrahydrofuran.

The cholic acid stock solutions (0.1 M) were prepared fresh on the day of use by dissolving the solid in tetrahydrofuran. The sodium cholate stock solutions (0.1 M) were prepared fresh on the day of use by dissolution of the solid in water. Lower concentration of cholic acid and sodium cholate were obtained by dilution of the stock solution in tetrahydrofuran and water, respectively.

Solutions of the probes in sodium cholate or cholic acid were prepared by passing a gentle stream of nitrogen gas over the appropriate volume of probe stock solution to evaporate ethanol, followed by dissolving the solid in the appropriate surfactant solution in a volumetric flask. The solutions were subjected to vibrations for at least 1 h in order to maximize the dissolution of the probe. The stability of the solutions was checked by fluorescence measurements, and the solutions were found to be stable for at least 8 h.

Fluorescence measurements were made with a Perkin Elmer LS50 Spectrofluorometer (Beaconsfield, UK) equipped with a xenon discharge lamp and two monochromators. Fluorescence Data Manager (FLDM) Software and an RS232C interface sent information to an external computer. For graphical recording, an NEC Silentwriter2 S60P laser printer was connected to the spectrofluorometer.

Scattered light measurements were made with an Aminco SLM 48000 S spectrofluorimeter equipped with a 450-W xenon lamp source, a Hamamatsu R298 photomultiplier detector tube, and a Pockel cell electrooptic modulator. The photocathode emission was minimized by cooling with a Peltier-cooled PMT housing. An IBM AT microcomputer was used for on-line data acquisition and data processing. The scattered light measurements used "100 average" mode, in which each measurement value was the average of 100 samplings, carried out automatically by the instrument circuitry in approximately 25 s. The excitation monochromator was set at 300, 450 and 550 nm, and the slits were set at 8 nm, with an 8 nm bandpass entrance.

A Merck-Hitachi (Darmstadt, Germany) liquid chromatograph, consisting of an L-6200 pump, AS-4000 autosampler, L-4250 UV–visible detector and D-6000 interface, was used. Instrumental parameters were controlled by Hitachi-Merck HM software. The LS-50 spectrofluorometer was used as an detector on line with the UV–visible spectrophotometer.

In descriptions of measurement conditions, the peaks correspond to the following excitation and emission wavelengths (λ_{ex} and λ_{em}, respectively): quinine hemisulfate salt, 240 nm and 363 nm; sodium salt 1,5-diaminoanthraquinone-2,6-disulfonic acid, 460 nm and 530 and 560 nm; 1-pyrene butyric acid, 342 nm, and 376 and 396 nm (vibronic bands I and III); rhodamine B, 556 nm and 575 nm; and pyrene, 335 nm, and 372 and 393 nm (vibronic bands I and III).

2.1. LC operating conditions

The plant growth regulators were chromatographed using an analytical column Spherisorb S5 amino normal-phase (25 cm × 4.6 mm; 5 μm particle size) from Phase Separations (Deesire, UK), cholic acid in tetrahydrofuran as mobile phase at 1 ml min^{-1} and fluorimetric detection (λ_{ex} = 280 nm and λ_{em} = 340 nm).

3. Results and discussion

3.1. cmc determination of inverted micelles with cholic acid in tetrahydrofuran

1-Pyrene butyric acid, rhodamine B, sodium salt 1,5-diaminoanthraquinone-2,6-disulfonic acid and quinine hemisulfate were selected as fluorescent probes because of their hydrophilic character. The excitation and emission spectra were collected for each probe in a series of cholic acid solutions varying the concentration range between 0 and 15 mM. Plots of peak intensity against cholic acid concentration are shown in Fig. 1. The results indicated that probes A and B give a cmc of 1 mM cholic acid, probe D a cmc of 5 mM cholic acid and probe C two cmc of 1 and 5 mM.

The absence of a break at 1 mM cholic acid with the probe quinine hemisulfate [Fig. 1(d)] may have been due to the less hydrophilic nature of the probe.

The distinct solvent dependence of vibronic fine structure intensities in the pyrene monomer structure [36–38] has been widely used in fluorescence probe studies of micellar systems. Since maximal

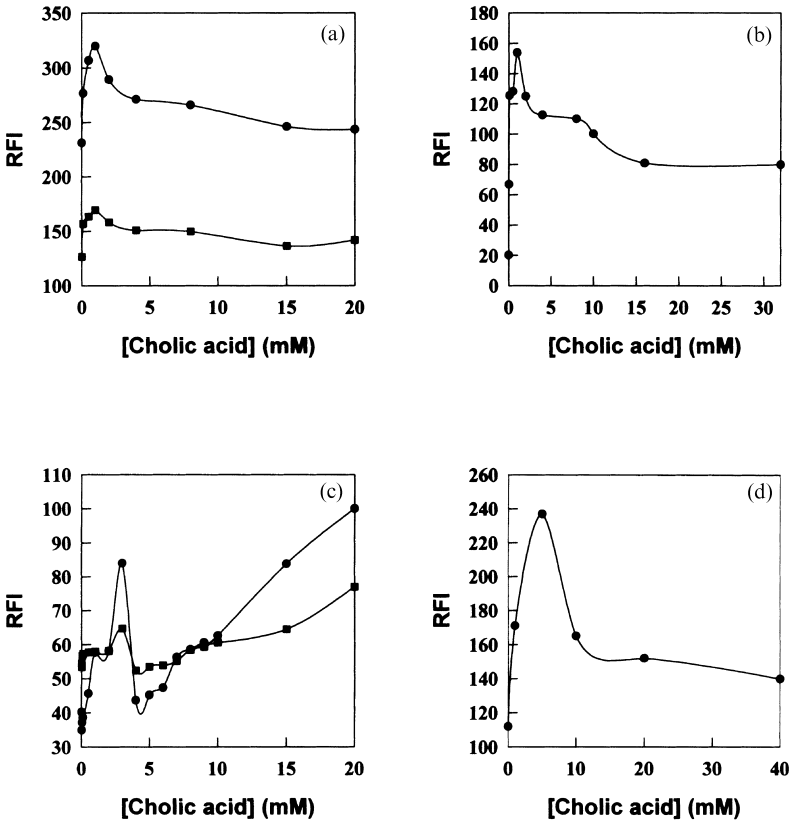

Fig. 1. Fluorescence intensity vs. cholic acid concentration. (a) 1-pyrene butyric acid (10^{-6} M), (b) 2×10^{-6} M), (c) sodium salt 1,5-diaminoantraquinone-2,6-disulfonic acid (10^{-5} M) and (d) quinine hemisulfate (10^{-5} M).

differences occur for the III/I band ratios when the probe moves from different microenvironments, this ratio was used to study both types of micelles, normal and reversed.

In reversed micelles with a polar core, we used 1-pyrene butyric acid because its hydrophilic character induces the solubilization in the core. Thus, the micellization process must be accompanied by significant changes in the III/I band ratios.

The excitation and emission spectra of the fluorescent probe 1-pyrene butyric acid were collected in a series of cholic acid solutions with concentrations ranging from 0 to 20 mM. The plot of the III/I vibronic band ratio gives an excellent indication of the microenvironment surrounding the probe. Fig. 2(a) shows variations of I/III band ratio against cholic acid concentrations. As can be seen, 1-pyrene butyric acid II/I vibronic band ratio (R) shows a sharp break at 1 mM cholic acid concentration. Although the variations in R values are small, the singular point seems to be the only one that can be marked.

However, R values of 0.53 are consistent with a polar microenvironment [35]. Below cmc (1 mM), R values are higher, corresponding to a non-polar microenvironment. Above the cmc, a lesser decrease of polarity up to 4–5 mM seems to indicate that a second cmc appears. From this point, the slope decreases and R changes little.

3.2. cmc determination of normal micelles (sodium cholate in water)

The III/I vibronic band ratio (R) of pyrene (hydrophobic fluorescent probe) gives a good confirmation of the 15 mM cmc values of normal micelles [Fig. 2(b)].

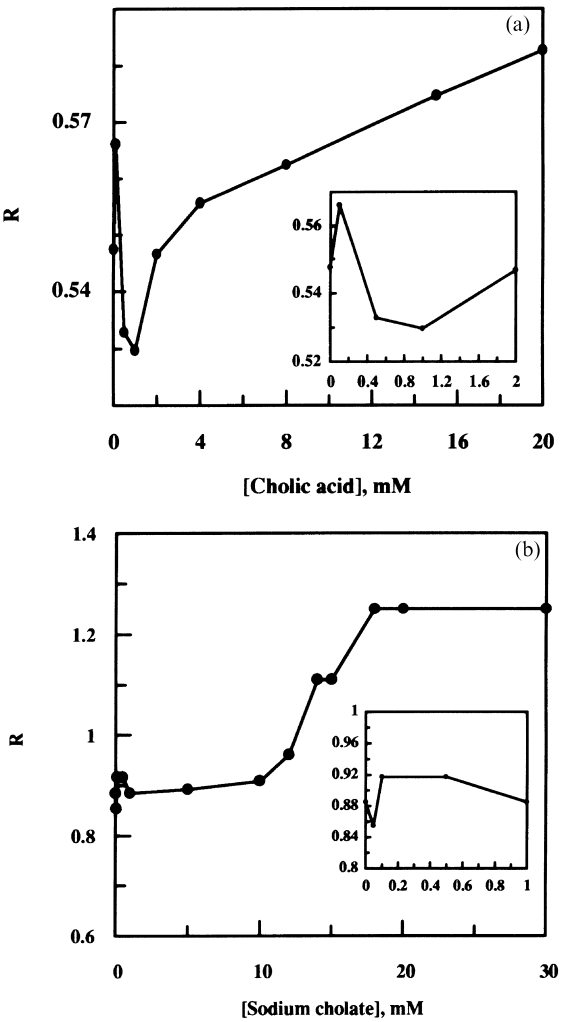

Fig. 2. III/I band ratio intensities (R) of the 1-pyrene butyric acid (10^{-6} M) (a) vs. cholic acid concentration and III/I band ratio intensities (R) of the pyrene (2×10^{-6} M) and (b) vs. sodium cholate concentration.

3.3. Effect of water content in reversed micelles

One of the most interesting aspects of reversed micelles is their ability to solubilize water in the interior of the micelle structure. The addition of water results in a rapid increase in the aggregation number and the size of the surfactant-entrapped water pool. The nature of the aqueous core of reversed micelles formed by cholic acid–tetrahydrofuran–water mixtures has been studied by monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid. The excitation and emission spectra of the probe at different percentages of water and 0.02 M cholic acid in tetrahydrofuran were collected.

We used a new fluorescent probe because it displays a strong affinity for the polar core of inverted cholic acid micelles. The fluorescence properties of monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid are extremely sensitive to the polarity of the microenvironment. Fig. 3 shows that the maximum emission wavelength

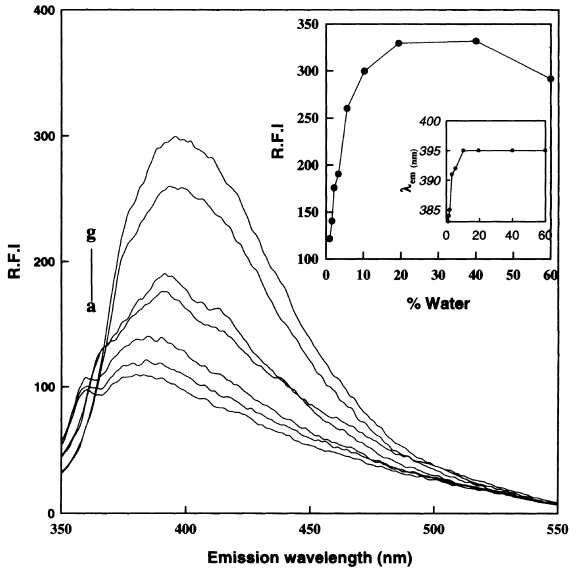

Fig. 3. Influence of water addition on the fluorescence and emission maximum wavelength of monosodium salt 8-amino-1-naphthol-3,6-disulfonic acid in cholic acid/tetrahydrofurn/water solutions. Water content: (a) 1, (b) 1.58, (c) 2.16, (d) 3.30, (e) 5.60, (f) 10.22 and (g) 19.44%.

ranges from 380 nm in tetrahydrofuran to 400 nm in water, and the fluorescence intensity of 10^{-6} M monosodium salt 8-amino-1-naphthol-3, 6-disulfonic acid ranges from 20 in tetrahydrofuran to 300 in water. A more polar environment leads to higher quantum yields and red shift of the emission.

Practically no fluorescence was obtained with 10^{-6} M monosodium salt 8-amino-1-naphthol-3, 6-disulfonic acid in pure tetrahydrofuran, indicating that the presence of inverted micelles is required to produce intense emission. This behaviour is different to other ionic dyes incorporated into the core of inverted micelles (e.g. 8-anilino-1-naphthalenesulfonic acid). The pronounced changes in fluorescence intensity and the red shift at a very low water content are interpreted as arising from an increment of polarity as water is incorporated into the micelles, maintaining a constant 20–50% water content.

3.4. Scattered light

The relative scattered light intensity vs. cholic acid concentration is shown in the Fig. 4(a) in the absence of a probe and measured at three different wavelengths of 300, 450 and 550 nm. From Fig. 4(a), it can be seen that the scatter curves have a maximum that is clearly differentiated to a cholic acid concentration of 7 mM and another maximum less intensified to a cholic acid concentration of 1 mM. These two break points in the aggregation of the cholic acid in tetrahydrofuran clearly indicate the existence of two cmc or a critical region with similar properties of two true cmc.

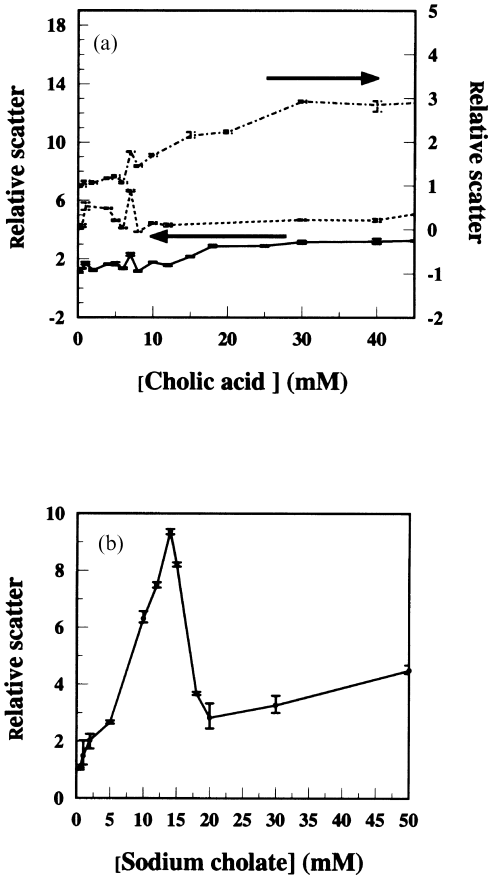

Fig. 4. (a) Relative scattered light intensity vs. cholic acid concentration measured at 300 nm (· · ·), 450 nm (———) and 550 nm (– · –). (b) Relative scatter light intensity vs. sodium cholate concentration measured at 550 nm.

Fig. 4(a) shows the relative scattered light intensity vs. sodium cholate concentration in the absence of a probe measured at a wavelength of 550 nm. From this, we can deduce that a maximum

at about 15 mM and another less pronounced maximum in a concentration range of 1–2 mM exist. These two critical points represent the cmc of sodium cholate in water.

3.5. Normal phase liquid chromatography determination of the cmc reversed micelles

Fig. 5 shows the capacity factor (log K) of the solutes IAA, IPA, IBA, 1-NAA and 2-NAA vs. cholic acid concentration in the tetrahydrofuran mobile phase, when an amino-bonded stationary phase was used. When the cholic acid concentration in the mobile phase passed through the cmc, we noted significant changes in retention times of the test solutes. The plots showed that the slope of the curve decreased close to the cmc, and if the two linear components of the curve were extrapolated, they would intersect near the cmc of the system under investigation. The average value was 5 mM cholic acid concentration in tetrahydrofuran.

This method has been previously used to determine cmc values for normal micelles used in reversed chromatography [39] and reversed micelles in normal chromatography [40].

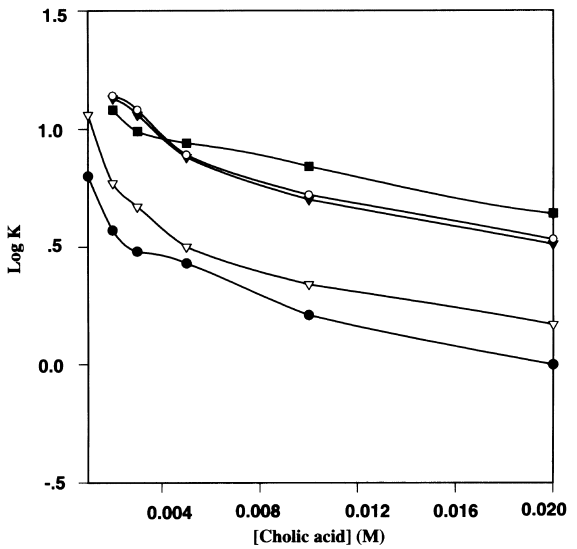

Fig. 5. Capacity factory of the fluorescent probes: 1, IBA (9.84×10^{-6} M) (●); 2, IPA (1.06×10^{-6} M) (▽); 3, IAA (1.04×10^{-5} M) (■); 4, 1-NAA (1.07×10^{-5} M) (▼); and 5, 2-NAA (1.07×10^{-5} M) (○) against cholic acid concentration.

The retention times of the plant hormones were 3.63 min for IAA ethyl ester, 6.43 min for 1-naphthalenacetamide, 10.1 min for IBA, 13.87 min for IPA, 19.8 min for 1-NAA, 22.6 min for 2-NAA and 27.23 min for IAA when cholic acid in tetrahydrofuran was used as the mobile phase and amino-bonded as the stationary phase [41]. However, the order of elution components was reversed when SDS in water was used as the mobile phase and alkyl nitrile-bonded as the stationary phase [42]. The reverse order of the retentions of the seven plant hormones obtained by the two chromatographic systems supports the hypothesis that reversed cholic acid micelles exist in the mobile phase.

From the results obtained, we can produce a bilayer tunnel-like spaces model [43] in which normal micelles (polar solvents) have the polar head (-OH) oriented to the outside of the internal channel and the tail part ($-CH_3$) directed to the inside of the internal channel. In inverted micelles, the apolar environment reoriented the tail to the external channel and the head to the interior channel. Transition points in both normal and inverted micelles can be associated with the minimum monomer elements that form a micelle with a sufficient stability to exist individually. A large chain, joined to monomers by hydrogen bonds, will have little stability. The existence of two transition points, 1 and 5 mM in inverted micelles, can be due to the association of several bidimensional layers forming a new aggregate with a larger number of monomers.

4. Conclusions

From steady-state fluorescence measurements, we have obtained valuable information about the cmc of normal and inverted micelles formed by cholic acid and its sodium salt in aqueous and non-polar media, respectively. Direct evidence of transition concentrations corresponding with aggregate formation was obtained by relative light scattering measurements. We also found that the liquid chromatographic determination of the cmc

values of inverted micelles accorded well with those obtained from fluorescence measurements. Finally, a new fluorescent probe for obtaining information within the polar core in inverted micelles has produced good results.

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