A fluorescence study of the ternary system lecithin-sodium deoxycholate-water showing structural transformations

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A fluorescence method was applied to study structural transitions occurring in the ternary system lecithin-sodium deoxycholate-water. The method is based on binding of the hydrophilic probe rhodamine-B to the structures at molar ratios of lecithin and deoxycholate (L/D) ranging from 0.16 to 10.7. The relationship between the fluorescence intensity and L/D ratio was sharply altered at molar ratios of 0.9 and 4.5. Thus three L/D regions could be distinguished and inflection points were estimated for each one. It is suggested that the L/D values of the sharp transitions (0.9 and 4.5) and the inflection points (0.5, 1.5 and 5.5) indicate generation of new types of molecular arrangements in the ternary system.

Ternary system *Structural transformation* *Fluorescence method* *Rhodamine-B*

1. INTRODUCTION

Extensive physico-chemical studies have recently been carried out on the ternary systems lecithin-bile salt-water (LBW) (review [1–3]). The interest in these systems stems mainly from the fact that cholesterol is largely solubilised in bile due to its incorporation into mixed micelles [4] which are composed of 2 major constituents, i.e. lecithin and bile salts. The molecular arrangements and structural transformations occurring at variable molar ratios are essential prerequisites for the ability of these mixed micelles (MM) to solubilize cholesterol.

The available experimental data are still insufficient to give a reliable picture of the changes taking place in the molecular organisation of MM within a wider range of lecithin-bile salt molar ratios. The reasons for this on the one hand are the numerous factors governing transitions in the LBW systems and on the other the methods for their investigation allow only the formulation of molecular concepts based on circumstantial evidence. From a methodological point of view there are now 2 controversial lines of evidence. First, the molecular model of MM constructed by applying NMR spectroscopy ('Small' model) [1,5] is unlikely from the viewpoint of X-ray small-angle scattering analysis (XSAS) [6], quasi-elastic light scattering (QLS) [7], UV-absorption measurement and differential scanning colorimetry [8] which favour a 'mixed disk' model [7]. Second, according to QLS data MM of the mixed disk type can exist even at a lecithin-bile salt (L/B) molar ratio of less than 0.55, whereas from XSAS data it is claimed that such structures are likely to appear at values of L/B surpassing 0.55.

Taking these considerations into account we focused on the mutual structural transitions in the lecithin-sodium deoxycholate-water system (LDW) occurring in the range of physiological L/D values and higher, where liposomes can be generated. For this purpose, we resorted to the method of fluorescence analysis, employing the hydrophilic fluorescence probe rhodamine-B, not previously applied to this system, in order to compare the behaviour of MM and liposomes.

2. EXPERIMENTAL

2.1. *Preparation of mixed micelles and liposomes*

Freshly isolated egg yolk lecithin with a high degree of chromatographic purity was used. A

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value of 770 was accepted as an average molecular mass to calculate the *L/D* molar ratios. The MM were prepared from a stock solution of lecithin in chloroform-methanol which was then mixed with recrystallized sodium deoxycholate (Sigma) in methanol in defined v/v ratios. The organic solvents were evaporated and the residue was then suspended in distilled water to give a lipid concentration of 20 mM. The suspensions were subjected to ultrasonic vibration for 20 s. Single bilayer liposomes were prepared as described in [9]. All preparations were centrifuged for 60 min at 100000 × *g*. They were stored under nitrogen and were obtained at least 24 h before use.

2.2. *Fluorescence measurements*

Fluorescence intensity (*F*) was measured on an NF-44 Perkin Elmer fluorimeter. The samples contained 0.2 mg/ml lecithin and varying amounts of sodium deoxycholate. *F* of the rhodamine-B probe (Fluka) was read immediately after its addition to the MM or liposome samples. All measurements were performed at ambient temperature. The pH of the suspensions was approx. 7.0 and electrical conductivity varied from 5.4 to 6.1 $mS \cdot m^{-1}$.

3. RESULTS AND DISCUSSION

The study of the ternary system by fluorescence using the hydrophilic probe rhodamine-B showed an increase in *F* which was proportional to the decrease in the *L/D* ratio (fig.1). To compare the fluorescence data obtained at different *L/D* ratios, the maximal intensity of fluorescence (F_{max}) calculated in a reciprocal coordinate system was expressed as the normalized fluorescence (F_N). The relationship between F_N and the *L/D* molar ratio (fig.2) could be represented by 3 S-shaped curves falling within the molar limits 0.16–0.9, 0.9–4.5 and 4.5–10.7. Differentiation of these curves yielded inflection points at *L/D* 0.5, 1.5 and 5.5, respectively.

To check on the reliability of the method, single bilayer liposomes were used for comparison with samples from the *L/D* region where according to [5–7,10] bilayer micellar structures exist.

To achieve this, the second S-shaped curve was extrapolated to yield a limiting value of F_N when the concentration of sodium deoxycholate tended to zero. This value served to estimate the correction factors, thus allowing the recalculation of the intensities of fluorescence for any *L/D* molar ratio. By eliminating in this way the interference by the detergent, the fluorescence data for LDW and monolayer liposomes could be compared (fig.3). From the ratio of the ordinates it could be directly estimated that the F_{max} of LDW preparations was approximately twice that of the liposomes. This result might be solely explained by assuming that in the range of *L/D* molar ratios from 0.9 to 2.4 the LDW system really represented structures both surfaces of which are accessible for interaction with the hydrophilic probe rhodamine-B. This gives reason to assert that the approach followed in the analysis of the fluorescence data permits an accurate characterization of definite types of structures in LDW.

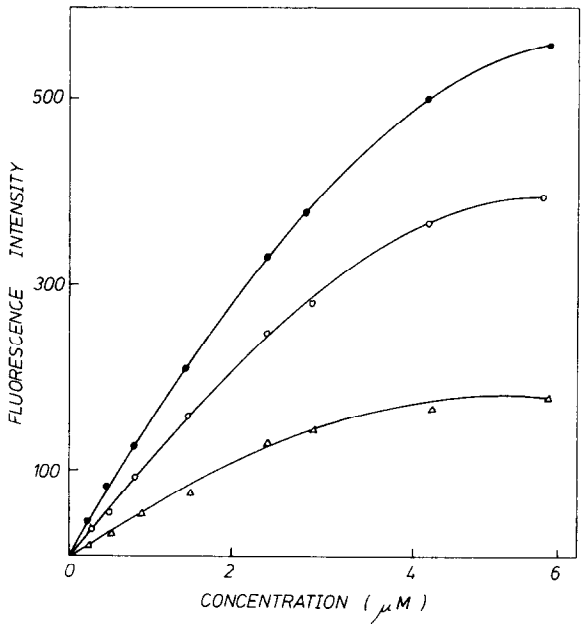

Fig.1. Fluorescence intensity of LBW preparation as a function of the rhodamine-B concentration by different molar ratios of *L/D*; 0.54 (●), 1.35 (○), 5.94 (△).

The comparison of the data from different authors for micellar systems is difficult because of the different experimental conditions (pH, ionic strength, bile salt type, concentration, mode of sample preparation, temperature, etc.). There still exist some controversies about the shape of the micelles, water molecule distribution, micelle-vesicle transitions, etc. due to contradictory ex-

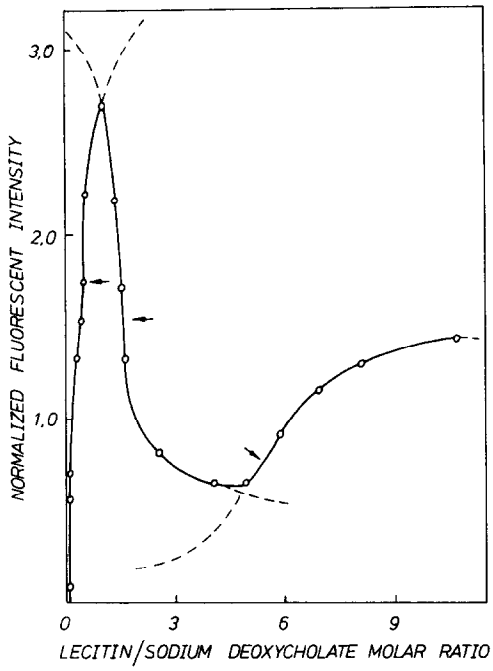

Fig.2. The normalized fluorescence (F_N) vs L/D molar ratio. The dashed lines suggest that the experimental curve could be represented as 3 S-shaped curves. The arrows indicate the inflection points.

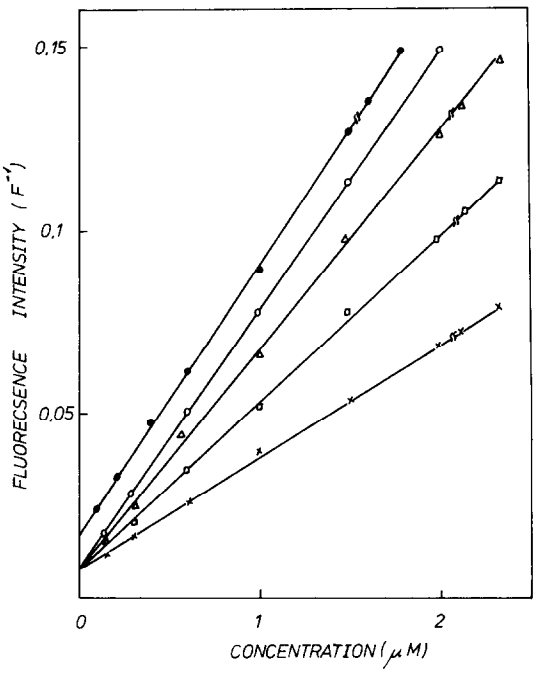

Fig.3. Two reciprocal coordination plots of fluorescence intensity as a function of rhodamine-B concentration by different molar ratios of L/D; 0.92 (○), 1.35 (△), 1.64 (□), 2.38 (×). The data were corrected as described in the text. Experimental points for single bilayer liposomes (●).

perimental evidence [11,12]. However, analysis of the graphic relationship for all the experimental points (fig.2) gives good grounds for some speculative conclusions: (i) The points from the first S-shaped curve (L/D from 0.16 to 0.9) fall in with the gamma boundary from the phase diagram for the taurocholate-lecithin model system (0.15 M NaCl, 20°C) [7]. The majority of the data indicate that in this region simple plus mixed micelles co-exist in varying proportions [7,13,14]; (ii) the literature data indicate that mixed disk type structures exist from L/D 0.5 to 1.5 [6,8]. These points showed full coincidence with the inflection points on the first and second S-shaped curves. On this basis we could propose that the inflection point at 5.5 also indicates the molar ratio at which definite structures in LDW are generated and/or cease to exist, for example, due to expansion of the bilayers and/or aggregation and fusion of the vesicles and also fluidization of the bilayers; (iii) the high L/D molar ratios ensure the formation of metastable liposomes [7,10]. Since the mechanism of vesicle formation is not known [16] an estimate of the L/D ratio at the micelle-vesicle transition is potentially important. Following the recent studies of Fromherz and Rüppel [10] it could be suggested that this point is somewhere in the second L/D region (0.9–4.5). The electron micrographs in this paper show discoid mixed micelles of egg lecithin-taurochenodeoxycholate at a molar ratio of 1.0. Other micrographs at L/D 3.1 reveal the primary formation of disk fragments by sonication and subsequent relaxation to closed shells. Analysis of the fluorescence data shows that in the region of the second S-shaped curve bilayers and metastable liposome co-exist. We suggest that the inflection point at L/D 1.5 may well indicate a critical L/D value for the formation of closed shells.

The sharp transition points of F_N at L/D 0.9 and 4.5 are of considerable interest. Most probably the first value indicates the L/D for complete solubilization in which all of the lipid is in mixed micelles. The second point shows another important value of L/D corresponding to the beginning

of the critical solubilization process. There the bilayers are saturated with detergent and onset of the lamellar to micelle transition occurs [2]. If these assumptions are valid it will be relatively easy to determine monomer concentration (see eqn 1 in [2]).

In the available papers considering LBW systems investigations were carried out at different ionic strengths, and mostly in unbuffered [5,10,19] or buffered suspensions at pH 7.0–8.0 [14,15,17]. Recently, it has been reported that at pH 7.0 the incomplete ionization of some bile salts exists [3] and the most probable model of the micellar aggregates there are helices ([18] and references therein). Our experiments at pH 9.0, 50 mM Tris-HCl lead to curves which show no essential difference as that presented in fig.2. The samples were also characterized by applying NMR-spectroscopic techniques and the results agree with the data of Lichtenberg and Zilberman [5] and Small et al. [19].

The results of the fluorescence investigations of the LDW system show that information concerning structural rearrangements within it can be derived from studies with a hydrophilic probe. With appropriate probes data for the hydrophobic core and fluidity of the structures could also be obtained. Further experiments could be of considerable importance for the better evaluation of the virtue of these methods.

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REFERENCES

[1] Small, D.M. (1971) in: The Bile Acids (Nair, P.P. and Kritchevsky, D. eds) vol.1, pp.249–356, Plenum, New York.
[2] Lichtenberg, D., Robson, R.J. and Dennis, E.A. (1983) Biochim. Biophys. Acta 737, 285–304.
[3] Carey, M.C. (1983) in: The Bile Acids in Gastroenterology (Barbara, L. et al. eds) pp.19–56, MTP Press, Lancaster, England.
[4] Carey, M.C. and Small, D.M. (1970) Am. J. Med. 49, 590–608.
[5] Lichtenberg, D. and Zilberman, Y. (1979) J. Magn. Reson. 34, 491–497.
[6] Müller, K. (1981) Biochemistry 20, 404–414.
[7] Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Biochemistry 19, 601–615.
[8] Claffey, W.J. and Holzbach, R.T. (1981) Biochemistry 20, 415–418.
[9] Gittis, A.G., Antonov, P.A., Kirazov, E.P. and Galabov, P.G. (1982) CR Acad. Bulg. Sci. 35, 343–346.
[10] Fromherz, P. and Rüppel, D. (1985) FEBS Lett. 179, 155–159.
[11] Wennerstrom, H. and Lindman, B. (1979) Phys. Rep. 52, 1–86.
[12] Menger, F.M. (1979) Acc. Chem. Res. 12, 111–117.
[13] Duane, W.C. (1977) Biochem. Biophys. Res. Commun. 74, 223–229.
[14] Higuchi, W.I., Su, C.C., Park, J.Y. and Gulari, E. (1981) J. Phys. Chem. 85, 127–129.
[15] Mazer, N.A., Schurtenberger, P., Carey, M.C., Preisig, R., Weigand, K. and Känzig, W. (1984) Biochemistry 23, 1994–2005.
[16] Bangham, A.D. (1982) in: Techniques in the Life Sciences, Lipid and Membrane Biochemistry B 420, pp.1–25, Elsevier, Shannon.
[17] Borgström, B. (1978) Lipids 13, 187–189.
[18] Campanelli, A.R., Candeloro De Sanctis, S., Giglio, E. and Petriconi, S. (1984) Acta Crystallogr. C40, 631–635.
[19] Small, D.M., Penkett, S.A. and Chapman, D. (1969) Biochim. Biophys. Acta 176, 178–189.