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# A FLUORESCENT PROBE STUDY OF THE SOLUBILISATION OF CHOLESTEROL BY BILE SALT-PHOSPHOLIPID MICELLES.[1]

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SUMMARY. The fluorescent probe dansyl cadaverine has been shown to bind strongly to mixed bile salt-phospholipid micelles containing unsaturation in the fatty acyl chains. Incorporation of cholesterol into the mixed micelles reduces the number of molecules of bound dansyl cadaverine without altering the binding affinity. These results suggest a tighter packing of the hydrocarbon matrix of the micelles in the presence of cholesterol.

## INTRODUCTION.

The solubility of cholesterol in bile is critically dependant on the concentration of bile salt and phospholipid (1). Interest in this area stems from the fact that the precipitation of cholesterol from bile micelles results in the formation of gall stones. While phospholipid micelles and their interaction with cholesterol have been the subject of intense physicochemical investigation (2) relatively little is known about the structure of mixed bile salt-phospholipid micelles. Small has proposed

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[3] Abbreviations: EPL, soya lecithin; DPL, dipalmitoyl lecithin; EPL-BS, soya lecithin-deoxycholate micelles; DPL-BS, dipalmitoyl lecithin-deoxycholate micelles; Chl, cholesterol.

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disc shaped models of bile salt-phospholipid micelles with cholesterol imbedded in the hydrocarbon matrix (3). Recent ESR studies have suggested the presence of a different type of aggregate when large concentration of cholesterol was incorporated into the micelles (4). It has been demonstrated that the feeding of polyunsaturated fats to rats, alters the composition of the biliary phospholipids leading to greater unsaturation of the fatty acyl chains. This in turn leads to increased solubilisation and excretion of cholesterol through bile (5). In order to understand the molecular mechanisms of cholesterol solubilisation, we have attempted to study bile-salt-phospholipid-cholesterol micelles, using dansyl cadaverine as a fluorescent probe.

## MATERIALS AND METHODS.

Soyalecithin (EPL) containing about 70% linoleic acid was a gift from Nattermann and Co., Cologne, Germany and was purified by chromatography over alumina. Sodium deoxycholate and dipalmitoyl lecithin (DPL) were from Sigma Chemical Co., USA. Dansyl cadaverine was synthesised as described earlier (6). Bile from goats, sheep and chicken were collected by puncturing the gall bladder. Mixed micelles of bile salt-soya lecithin with (EPL-BS-Chl) and without cholesterol (EPL-BS) were prepared by a modification of the procedure of Saunders and Wells (7). Lecithin and cholesterol were dissolved in chloroform and the solvent was evaporated under vacuo. The last traces were removed by passing a stream of nitrogen. The residue was dissolved in 0.025M Tris-HCl buffer (pH 8.2) and sodium deoxycholate dissolved in the same buffer was added. The solution was mixed, cooled and sonicated for 45 sec using a Branson sonifier (20 Kcycles, 75W), and incubated for 2 hours at 37°C. The solution was then passed through a Millipore filter (0.45 μm). Mixed micelles without cholesterol were prepared in a similar fashion.

Fluorescence measurements were made using a Perkin-Elmer Model 203 spectrofluorimeter. All spectra are uncorrected. The excitation maximum for dansyl cadaverine was 340 nm.

## RESULTS AND DISCUSSION.

Dansyl cadaverine has recently been shown to be a useful

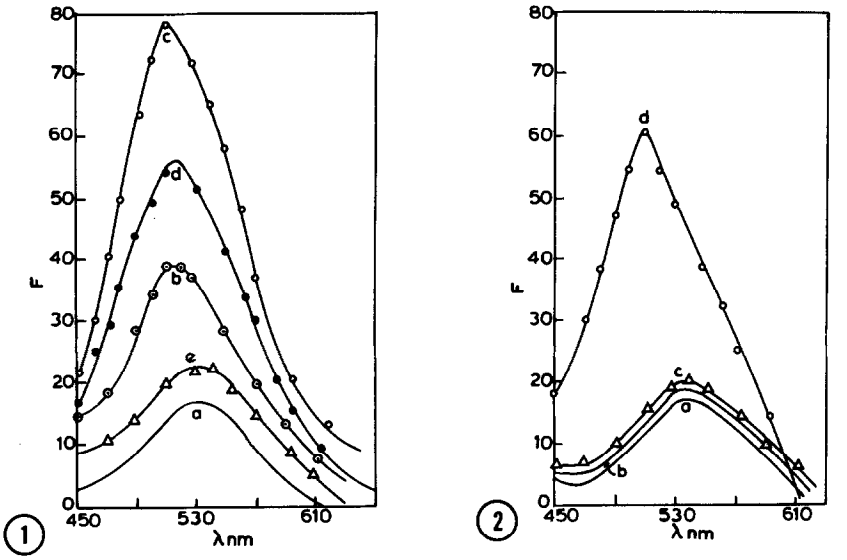


Fig. 1. Uncorrected fluorescence spectra of dansyl cadaverine in the presence of model micelles of deoxycholate, lecithin and cholesterol. All solutions were made in 0.025M Tris-HCl buffer pH 8.2, probe concentration was 4 μg/ml.

(a) Probe blank, (b) Probe + EPL-BS (100 μg/ml - 100 μg/ml). Probe was added externally after the micelles were prepared. (c) Probe + EPL-BS (100 μg/ml - 100 μg/ml). Micelles were prepared along with the probe. (d) Probe + EPL-BS-Chl (100 μg/ml - 100 μg/ml - 30 μg/ml). Micelles were prepared along with the probe. (e) Probe + DPL-BS (100 μg/ml - 100 μg/ml). Micelles were prepared along with the probe.

Fig. 2. Uncorrected fluorescence spectra of dansyl cadaverine in the presence of lecithin dispersions and deoxycholate micelles. All solutions were made in 0.025M Tris-HCl buffer pH 8.2. Probe concentration was 4 μg/ml and was added externally.

(a) Probe blank, (b) Probe + Deoxycholate (100 μg/ml), (c) Probe + DPL (100 μg/ml), (d) Probe + EPL (100 μg/ml).

probe for anionic sites on biological membranes (6). It has also been shown that dansyl cadaverine is displaced from erythrocyte membranes on addition of cations that compete for negatively charged binding sites (8). The probe may therefore be used to monitor structural perturbations in lipid micelles in low ionic strength buffers. The experiments described below were carried out in Tris-HCl buffer (0.025M, pH 8.2) as very little binding to

bile salt-phospholipid micelles could be detected in sodium phosphate buffer. Fig. 1 shows the fluorescence spectra of dansyl cadaverine in the presence of various lipid micelles. It is seen that incorporation of the probe into EPL-BS micelles is accompanied by a significant enhancement of emission intensity and a 20 nm blue shift of the fluorescence maximum. An interesting feature of Fig. 1 is that dansyl cadaverine shows almost no binding to DPL-BS micelles as evidenced by the lack of emission intensity enhancement. This suggests that probe molecules are more effectively incorporated into unsaturated phospholipid micelles, indicating that altered packing of the fatty acid chains facilitates penetration of the fluorescent probe. Two sets of experiments were carried out in which the probe molecules were incorporated internally by addition before sonication, and externally by addition after sonication. The degree of binding was substantially higher for internal incorporation suggesting that the mixed micelles are not freely permeable to the probe. Fig. 2 demonstrates that though the probe showed considerable binding to sonicated EPL dispersion, no binding was observed with similar DPL dispersion and with deoxycholate micelles.

Introduction of cholesterol into the EPL-BS micelles causes a dramatic reduction in the intensity of the probe fluorescence (Fig. 1d). This may arise due to a reduction in the number of probe molecules bound to the micelles or due to a fall in the emission quantum yield of the bound fluorophore or due to a combination of both factors. In order to assess the causes for the fluorescence decrease, titrations varying the lipid and probe concentrations were carried out. The quantum yield of dansyl cadaverine remained unaltered in the presence of cholesterol

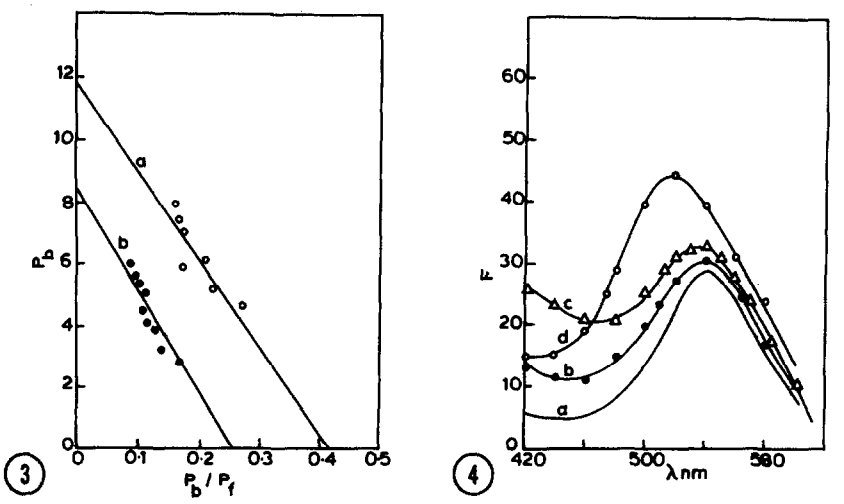


Fig. 3. Scatchard plots for the binding of the dansyl cadaverine to mixed micelles of lecithin, deoxycholate and cholesterol.

(a) EPL-BS, (b) EPL-BS-Chl. $P_b$ = Probe bound to micelles; $P_f$ = Probe free.

Fig. 4. Uncorrected fluorescence spectra of dansyl cadaverine in the presence of bile. All solutions were made in 0.025M Tris-HCl buffer pH 8.2. Probe concentration was 4 µg/ml.

(a) Probe blank, (b) Probe + Goat bile (60 µg/ml lipid conc.), (c) Probe + Sheep bile (60 µg/ml lipid conc.), (d) Probe + Chicken bile (60 µg/ml lipid conc.).

(Figure not shown). The binding data presented in the form of Scatchard plots (9) are shown in Fig. 3. It is seen that the addition of cholesterol to the EPL-BS micelles reduces the number of probe molecules bound without an appreciable change in binding affinities. Average dissociation constants ($K_D$) of 3.05 x $10^{-5}$M for probe binding were obtained. The extent of probe binding (n) varied from 8.9 µmoles $gm^{-1}$ in the presence of cholesterol to 11.9 µmoles $gm^{-1}$ in the absence of cholesterol. Similar studies were not carried out for DPL-BS micelles due to weak binding of the fluorescent probe.

Dansyl cadaverine was chosen initially with the aim of

probing the environment of anionic bile salt molecules. However, the small changes in fluorescence with bile salt micelles alone suggest a low affinity for the probe binding. Alternatively interactions between the charged carboxylate and amine functions in the micelles and probe respectively, may not result in large changes in emission, if the naphthalene ring remains largely exposed to solvent. This is conceivable in the case of the relatively small bile salt micelles. However in mixed phospholipid-bile salt micelles it is likely that the positively charged amine groups of the probe may bind to the carboxylate groups of deoxycholate while additional binding interactions of the hydrocarbon parts of the molecule are possible with the nonpolar regions of the mixed micelles. The relatively strong interaction with EPL-BS micelles on the other hand points to the possibility that dansyl cadaverine may serve mainly as a reporter of the unsaturated phospholipid environment. Weak interaction of the probe with DPL-BS micelles suggests that the fatty acid unsaturation of the phospholipid leads to a relatively loose packing of the hydrocarbon chain, which then allows facile penetration of the hydrophobic residues of the probe molecule.

The displacement of the probe on addition of cholesterol suggests that dansyl cadaverine and cholesterol pack in a similar region of the micelle. As a consequence presence of cholesterol reduces the accessibility of the probe to the micelles. High resolution $^{1}H$ NMR studies of the micelles of bile salt and phospholipid having different fatty acid composition suggest the possibility that the fatty acid chains in these micelles are in a relatively fluid state independent of the type of phospholipid (10,11). It is likely that solubilisation of cholesterol in this state reduces the fluidity of the hydrocarbon region. Preliminary

experiments using pyrene excimer fluorescence as a mobility monitor, confirms that cholesterol incorporation "freezes" the hydrocarbon matrix of the micelles. In this context it is interesting to note that in phospholipid vesicles, presence of cholesterol reduces the fluidity of the hydrocarbon matrix above the phase transition temperature, but enhances the fluidity below the phase transition (12).

The extrapolation of these studies to natural biliary micelles having different phospholipid composition yielded encouraging results. The fluorescence spectra of dansyl cadaverine in the presence of various bile samples show that, while goat and sheep bile produced small spectral changes, relatively large fluorescence changes are observed with chicken bile (Fig. 4). It is significant that bile lecithin of chicken is more unsaturated than that of goat and sheep bile (11). This suggests that dansyl cadaverine may prove a useful indicator of phospholipid unsaturation and that further investigations in progress using homologous probes may yield more detailed structural information on mixed bile salt-phospholipid micelles.

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REFERENCES.

1. Small, D.M. (1970) Adv. Int. Med. 16, 243-264.
2. Demel, R.A. and de Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109-132.
3. Small, D.M. (1971) in the "Bile acids - Chemistry, physiology and metabolism" (ed. Nair, P.P. and Kritchevsky, D.) 1, 249-356, Plenum Press, London and New York.
4. Stevens, R.D. (1977) J. Lip. Res. 18, 417-422.

5. Paul, R. and Ganguly, J. (1976) Chem. Phys. Lip. 17, 315-323.
6. Narayanan, R. and Balaram, P. (1976) Biochem. Biophys. Res. Commun. 70, 1122-1128.
7. Saunders, D.R. and Wells, M.A. (1969) Biochim. Biophys. Acta 176, 828-835.
8. Narayanan, R. and Balaram, P. (1977) Abstracts, National Symposium on Biological Membranes and Model Systems, Bangalore.
9. Azzi, A. (1974) Methods in Enzymol. 32B, 234-246.
10. Small, D.M., Penkett, S.A. and Chapman, D. (1969) Biochim. Biophys. Acta 176, 178-189.
11. Paul, R. (1977) Ph.D. Thesis submitted to Indian Institute of Science, Bangalore.
12. Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265-279.