The Effect of Albumin on Incorporation of Merocyanine 540 into Phospholipid Liposomes

L. ŠIKUROVÁ and R. FRANKOVÁ

Department of Biophysics, Comenius University, Mlynská dolina F1, 842 15 Bratislava, Slovakia

Abstract. Absorption and fluorescence emission spectroscopy was applied to study the changes in albumin modified incorporation of merocyanine 540 into liposomes composed of different lecithins (DMPC, DPPC, POPC, and egg PC). Our results confirmed high affinity of merocyanine molecules toward albumin and revealed that albumin competed with all phospholipids used for binding merocyanine 540 molecules. However, the extent of this competition was determined by the kind of phospholipid. Albumin competed very successfully with lecithins containing saturated fatty acid chains (DPPC, DMPC) and weakly with unsaturated lecithins (POPC, egg PC) for binding merocyanine 540 molecules.

Key words: Merocyanine 540 — Albumin — Phospholipid — Liposome

Introduction

Merocyanine 540 (MC 540) is a lipophilic fluorescent dye which has been used as a probe to investigate the structure and function of cellular membranes (Waggoner, 1976; Easton et al. 1978; Lelkes and Miller, 1980). This dye was evaluated for photodynamic purging of leukaemia cells from bone marrow explants and sterilization of blood products (Sieber et al. 1986; O'Brien et al. 1990). It has been reported that this dye binds avidly to and photosensitizes the inactivation of electrically excitable cells (Easton et al. 1978), certain classes of normal immature blood cells (Valinsky et al. 1978; Schlegel et al. 1980), leukaemia cells (Schlegel et al. 1980; Sieber et al. 1984), enveloped viruses (O'Brien et al. 1990; Dodd et al. 1991) and virally infected cells (Sieber et al. 1989; Smith et al. 1991). Cells that are highly susceptible to MC 540 sensitized photoinactivation tend to bind more dye than cells nearly resistant to MC 540 (Smith et al. 1991). The external membranes of normal intact cells, non-excitable cells and normal blood leucocytes bind the dye but this general low-affinity binding is abolished by the addition of serum (particularly its two components – albumin and lipoproteins) (Easton et al. 1978; Schlegel et al. 1980). It has been shown that the cells with low binding affinity to MC 540

do not fluoresce in the presence of serum. On the other hand, cells that bind MC 540 with high affinity may fluoresce even in the presence of serum (Easton et al. 1978; Valinsky et al. 1978). The reasons of different cells affinity for MC 540 are not clear.

Serum albumin shows to be one of the major modulators of MC 540 – mediated photoinactivation and provides a means to control the rate of reaction (Easton et al. 1978; Gaffney and Sieber 1988; O'Brien et al. 1990; Gaffney and Sieber 1992). Therefore most investigators perform the dye-sensitized photoinactivation of cells and viruses in the presence of serum or albumin (Sieber et al. 1986; Prodouz et al. 1991; Smith et al. 1991). In spite of this, it has not been identified which components of plasma membranes compete with albumin binding sites for MC 540 molecules. Since phospholipids are the main building components of membranes, it is important to compare dye binding between phospholipids and albumin. In this contribution the above mentioned competition was studied by absorption and fluorescence UV VIS spectroscopy using model membranes formed from various kinds of lecithins.

Materials and Methods

The fluorescent dye merocyanine 540 (MC 540) was obtained from Eastman Kodak Co. and was used without further purification. Two synthetic lecithins, dimyristoyl-L-α-phosphatidylcholine (DMPC, molecular weight 677.9) and dipalmitoyl-L-α-phosphatidylcholine (DPPC, molecular weight 734.1) were purchased from Fluka AG. The further phospholipid used, 1-palmitoyl-2-oleoyl-L-α-phosphatidylcholine (POPC, molecular weight 760.1) was prepared according to the method described by Herrmetter and Paltauf (1982). Natural egg lecithin (egg PC, molecular weight about 775) was prepared at the Kharkow State University, Ukraine. All lecithins were used in the preparation of "injection" liposomes according to the standard method (Vladimirov and Dobretsov 1980). The final lecithin concentration in the sample was 2 mg/ml. Bovine albumin (molecular weight about 69,000) obtained from Imuna, Slovakia, was added to liposome suspension. Bidistilled water, pH 6.2 ± 0.2, was used in all sample preparations. Fresh MC 540 solutions were prepared daily. The samples were equilibrated with the dye for 10 minutes at room temperature (24 ± 1 °C) and protected from light. Dye concentrations were in the range between 7×10^{-7} and 1×10^{-4} mol/l. The absorption spectra were recorded by a SPECORD M-40 spectrophotometer (Carl Zeiss, Germany). The fluorescence emission spectra were registered by a Perkin-Elmer MPF-4 (USA) spectrofluorimeter.

Results

Fig. 1 shows the visible absorption spectra of aqueous solutions of MC 540 in the absence and in the presence of albumin. Two absorption peaks at 500 nm (a dimer peak) and at 532 nm (a monomer peak) were recorded in the spectra of pure aqueous solution of MC 540. Titration of this solution by bovine albumin in

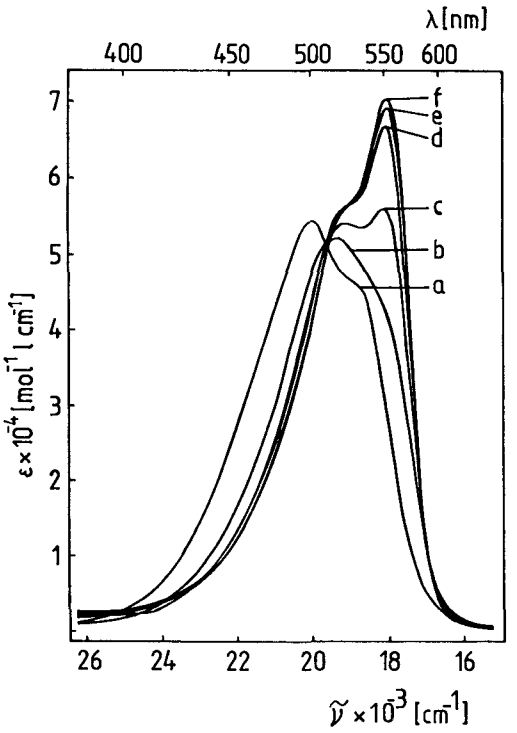

Figure 1. Visible absorption spectra of MC 540 (51 μmol/l) in aqueous solution of albumin. Concentrations of albumin (*a*) 0; (*b*) 12; (*c*) 29; (*d*) 73; (*e*) 116; (*f*) 145 μmol/l.

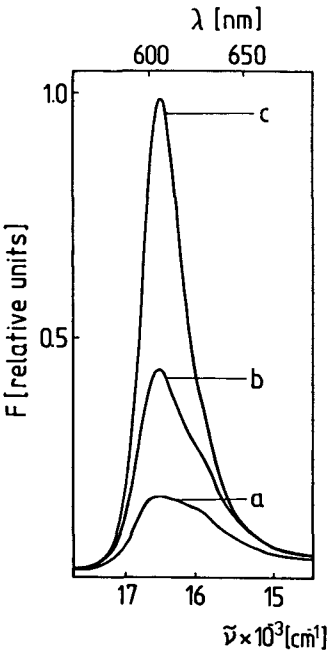

Figure 2. Fluorescence emission spectra of MC 540 (51 μmol/l) in aqueous solution of albumin (excitation at 550 nm). Concentrations of albumin (*a*) 0; (*b*) 15; (*c*) 43; (*d*) 87 μmol/l.

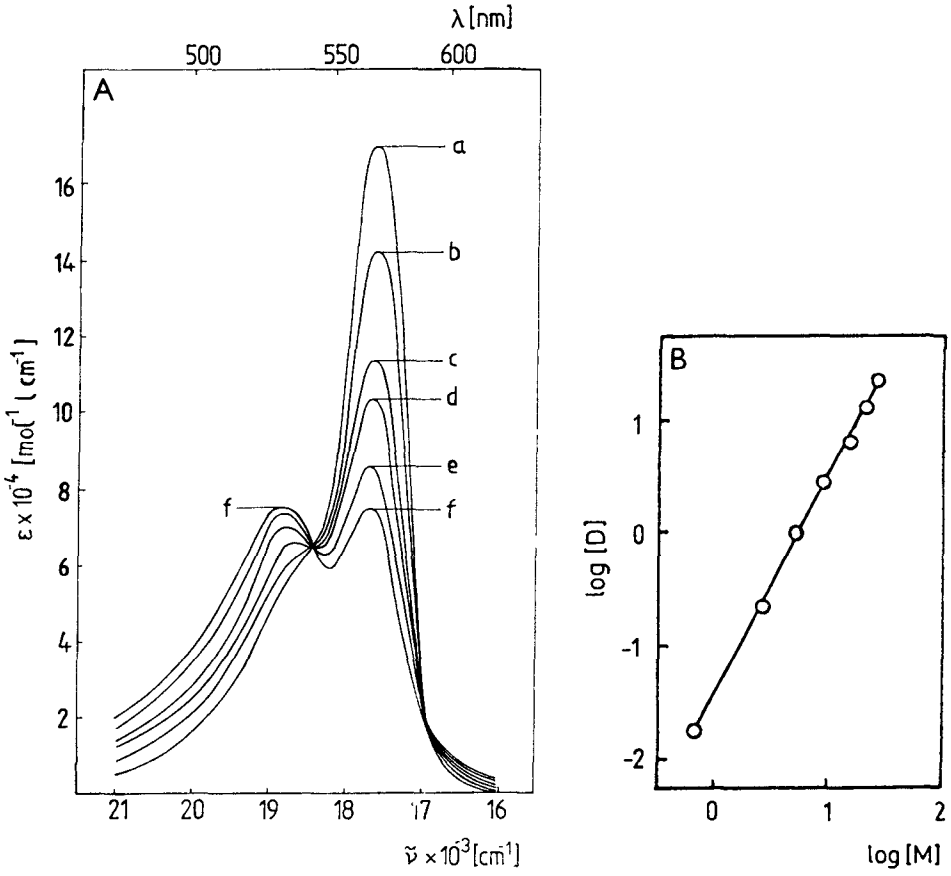

Figure 3. *A.* Visible absorption spectra of MC 540 stained POPC liposomes (total lipid concentration 2.63 mmol/l). MC 540 concentration (*a*) 0.70; (*b*) 2.81; (*c*) 7.02; (*d*) 14.00; (*e*) 28.10; (*f*) 49.10; (*g*) 70.20 μmol/l. *B.* Plot of log c_D vs. log c_M for MC 540 in POPC liposomes. The concentrations are given in μmol/l. The line is least square fitted with the correlation coefficient of 0.999 and its equation is $\log c_D = (1.945 \pm 0.022) \log c_M - (1.380 \pm 0.030)$.

the concentration range of 12–146 μmol/l caused several changes in the spectral appearance. Both original MC 540 absorption peaks (at 500 nm and at 532 nm) became less distinguishable unless they disappeared. A new band at 556 nm became resolvable with a vibrational shoulder at shorter wave-lengths. The intensity of this new band was systematically enhanced up to the saturation level (at albumin concentration of above 116 μmol/l) in which the molar absorption coefficient was found to be $\epsilon_A = (6.5 \pm 0.4) \times 10^4$ mol/l/cm. A non-sharp formation of one isosbestic

point can be seen in the family of the absorption spectra. Albumin did not show any absorption of visible light. The influence of albumin on the fluorescence spectra of water solutions of MC 540 is shown in Fig. 2. One fluorescence peak at 583 nm (a monomer peak) with a weak vibrational shoulder at longer wave-lengths was registered by excitation at 550 nm for pure aqueous solutions of MC 540. In the presence of albumin the new fluorescence peak at 598 nm appeared to retain a weak vibrational shoulder. The increase of albumin concentration was followed by the enhancement of fluorescence intensity at 598 nm.

An example of representative spectra of MC 540 in suspensions of lecithin liposomes is shown in Fig. 3*A*. MC 540 in POPC liposomes had a double-band absorption spectrum peaking at 568 nm (a monomer peak) and at 531 nm (a dimer peak). An increase of MC 540 concentration was manifested in an enhancement of the dimer peak absorbance as compared to a decrease of the monomer peak. Two isosbestic points were created at about 542 and 592 nm, respectively. In this family of spectra the monomer c_M and the dimer c_D concentrations were determined using the method of West and Pearce (1965). The values of c_M and c_D were plotted in logarithmic scales (Fig. 3*B*). This plot can be least square fitted with a straight line with a slope of two only for the MC 540 concentrations of up to 71 μmol/l.

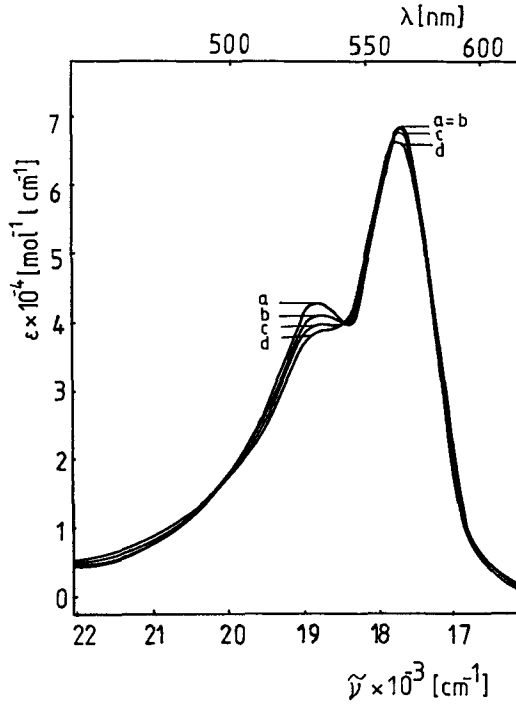

Figure 4. Visible absorption spectra of MC 540 stained POPC liposomes (total lipid concentration 2.63 mmol/l, total dye concentration 92 μmol/l) titrated by albumin at concentrations (*a*) 0; (*b*) 15; (*c*) 29; (*d*) 43 μmol/l.

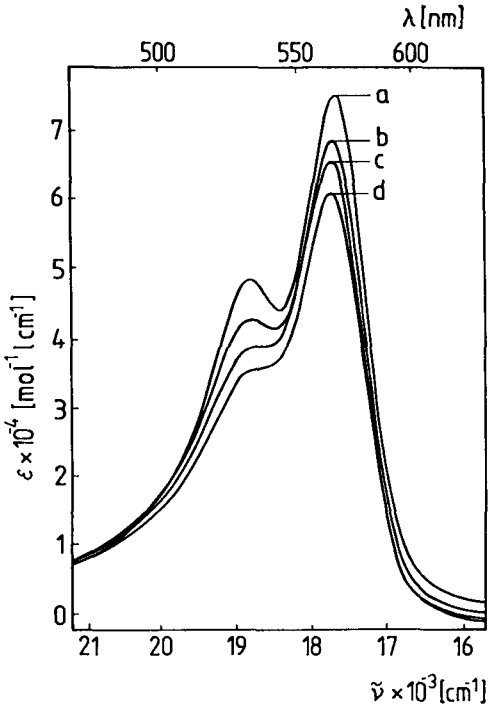

Figure 5. Visible absorption spectra of MC 540 (92 μmol/l) in suspension of POPC liposomes (2.63 mmol/l) incubated with albumin at concentrations (*a*) 0; (*b*) 29; (*c*) 87; (*d*) 116 μmol/l.

The effect of albumin on MC 540 incorporation into phospholipid liposomes was further investigated. Two different ways were carried out to prepare albumin-liposome suspensions: (1) Albumin was added to the liposomes that had previously been stained with MC 540, and (2) liposomes were incubated with albumin and then MC 540 was added.

Representative spectral changes for the sample prepared in the first way are shown in Fig. 4. Addition of albumin (up to 116 μmol/l) induced no significant spectral shifts; only the dimer absorbance decreased by about 9%. Alike, slight spectral changes were registered for other phospholipids used (egg PC, DMPC) for the samples prepared in the first way.

There is a different situation in the case of samples prepared in the second way. The absorption spectra of MC 540 in POPC incubated with albumin are shown in Fig. 5. The presence of albumin resulted in the changes of both shape and intensity of the spectra. The peak at shorter wave-lengths was red-shifted from 531 nm to 534 nm, whereas the peak at longer wave-lengths was blue-shifted from 568 nm to 562 nm. Albumin induced a stronger decrease of the peak absorbance at the shorter wave-lengths (by about 30%) than at the longer wave-lengths (by about 20%) (the data are valid for albumin concentration of 116 μmol/l). No absorption

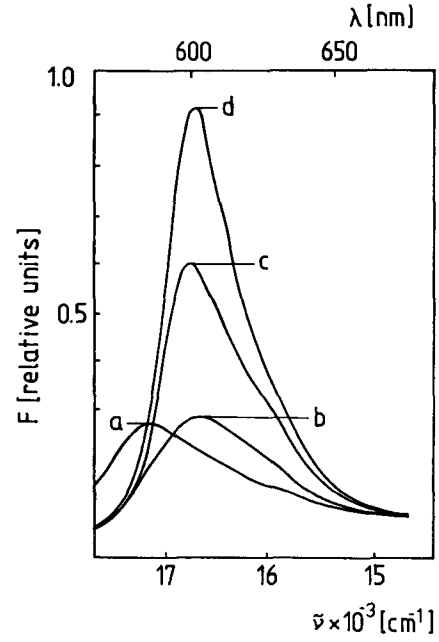

Figure 6. Fluorescence emission spectra of MC 540 (51 μmol/l) in suspension of POPC liposomes (2.63 mmol/l) incubated with albumin (excitation at 550 nm). Concentrations of albumin (*a*) 0; (*b*) 15; (*c*) 87 μmol/l.

band corresponding to MC 540 molecules in water nor higher aggregates were registered in the spectra for MC 540 concentrations of 0–80 μmol/l. Fluorescence spectrum of MC 540 in POPC liposomes (Fig. 6) shows one peak at 611 nm with a vibrational shoulder. The addition of albumin induced the fluorescence maximum shift to the shorter wave-lengths (by about 5 nm at albumin concentration of 116 μmol/l).

Quantitatively the same spectral behaviour was observed for MC 540 in egg PC and DMPC liposomes. The variety of the phospholipids was manifested only in quantitative differences.

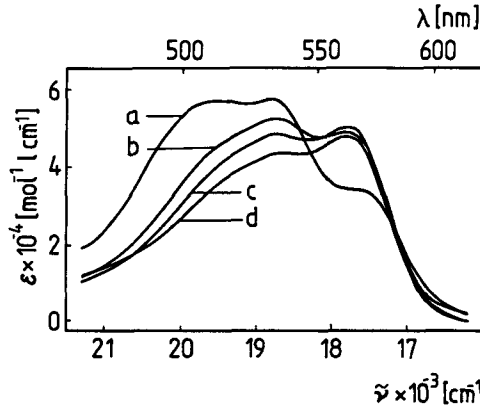

Figure 7. Visible absorption spectra of MC 540 (51 μmol/l) in suspension of DPPC liposomes (2.72 mmol/l) incubated with albumin at concentrations (*a*) 0; (*b*) 29; (*c*) 58; (*d*) 116 μmol/l.

Specific spectral behaviour was observed for MC 540 in suspensions of DPPC liposomes (Fig. 7) where three absorption bands were registered: at 510 nm (which pertains prevailingly to MC 540 dimers in water), at 531 nm (corresponding to MC 540 dimers bound to liposomes and MC 540 monomers in water), and at 568 nm (resulting from MC 540 monomers bound to DPPC liposomes). The presence of albumin in DPPC liposome suspensions changed markedly both the shape and the peak positions of spectra in the following way. The absorption bands of MC 540 in water became less distinguishable and the absorption band at the longest wave-lengths shifted from 568 nm to 561 nm and acquired a predominant position.

Discussion

The spectroscopic studies presented in this paper showed that spectral properties of MC 540 in water and liposome suspensions were strongly influenced by the presence of albumin. Titration of aqueous solutions of MC 540 by albumin caused the disappearance of both original "water" bands of MC 540 and the formation of a new absorption band. Since albumin does not show any absorption of visible light, the spectral changes observed can be attributed to the changes in MC 540 spectra. The presence of the isosbestic point and the tendency towards saturation with increasing albumin concentration (Fig. 1) suggest the appearance of a new absorbing species of MC 540 which probably is albumin-associated dye. The molar absorption coefficient of MC 540 associated to albumin has been estimated at $\epsilon_A = (6.5 \pm 0.4) \times 10^4$ mol/l/cm from the spectra of the saturated state. A new peak of MC 540 associated with albumin also appeared in the fluorescence spectra. In both absorption and fluorescence spectra of MC 540 in water solutions of albumin, the position of bands and spectrum resemblance to that observed for dye dissolved in some alcohols (Šikurová and Janíková 1987) indicate that MC 540 enters the interior of albumin which is less polar than that of water. This environment does not allow formation of dimers as it is possible in water, a highly polar solvent. Therefore the spectrum of MC 540 associated to albumin shows only the monomer band.

Since it has been postulated that MC 540 binds preferentially to disordered (rich in unsaturated lipids) bilayers (Williamson et al. 1981, 1983) we compared MC 540 incorporation into various phospholipids: DMPC and DPPC with saturated fatty acid chains, and POPC and egg PC with unsaturated chains. In the case of previously stained liposomes with MC 540, the addition of albumin induced only a small decrease in absorbance of the dimer band. These results indicate that MC 540 monomers incorporated into the hydrophobic interior of liposomes are protected from the contact with albumin by lipid matrix. MC 540 dimers localized near the membrane surface can be slightly influenced by the presence of albumin. There is a different situation for liposomes previously incubated with albumin and then

stained with MC 540. A pronounced decrease of absorbance and a shift to shorter wave-lengths were registered for both absorption bands (Fig. 5). The decrease of the dimer absorbance is stronger than that of monomers and therefore the monomer peak became predominant. Although these facts could indicate the decrease of relative contribution of MC 540 dimers versus monomers, the simple monomer-dimer equilibrium is not only valid as neither the presence of an isosbestic point nor the increase of the monomer peak were observed. The comparison of MC 540 absorption and fluorescence spectra in water, in water solutions of albumin, in POPC liposomes, and in the suspension of POPC liposomes in the presence of albumin indicates that the spectrum of MC 540 in POPC liposomes with albumin can be considered to be a superposition of the absorption bands of MC 540 bound to POPC liposomes and MC 540 associated with albumin. Thus we can suggest that albumin binding sites compete with phospholipid liposomes for binding MC 540 molecules. Although under our experimental conditions the "water" band of MC 540 in POPC liposome suspensions (with and without albumin) is not significant, it is possible that some of unassociated MC 540 molecules are present in water. As our aim was to estimate competition between albumin and lecithin for MC 540 binding, we evaluated only such experiments in which unassociated MC 540 molecules can be neglected. The results presented in Fig. 3*B* show that fully associated MC 540 molecules can be considered up to the concentrations of 71 μmol/l as for these concentrations the dependence of log c_D vs. log c_M is a straight line with a slope of two and therefore the mass low for the monomer-dimer equilibrium of fully associated dye molecules is fulfilled. For MC 540 concentrations of above 72 μmol/l the c_M and c_D values no longer confirm to the mass low and the dye molecules in water probably contribute to values of absorbance. However, for the concentrations of up to 71 μmol/l unassociated MC 540 molecules can be neglected and thus the total dye concentration c in albumin POPC suspensions should fulfil the following equation

$$c = c_L + c_A$$

where c_L and c_A are the concentrations of the dye bound to liposomes and albumin, respectively. Then the values of c_L and c_A can be calculated from the data of optical densities at the absorption maximum (Table 1). The same approach can be used for DMPC and egg PC liposomes. A different behaviour was observed for MC 540 in DPPC liposomes where the pronounced MC 540 "water" band is registered at the same concentrations used. This fact can result from a smaller dye binding ability to gell-phase vesicles than to fluid-phase ones (Williamson et al. 1981). These results indicate that albumin in DPPC liposome suspensions competes with DPPC for binding MC 540 molecules and also associates with MC 540 dissolved in water.

Our results reveal high affinity of MC 540 molecules towards albumin and consequently the ability of albumin to compete with all phospholipids used for

Table 1. Distribution of MC 540 between POPC and albumin in the suspension of POPC liposomes (2.63 mmol/l) and albumin (116 μmol/l). (c is the total dye concentration, c_L and c_A are the concentrations of MC 540 bound to liposomes and albumin, respectively).

c [μmol/l]	$\frac{c_L}{c} \times 100$ [%]	$\frac{c_A}{c} \times 100$ [%]
11	13	87
51	10	90
71	8	92

The results are expressed as the means of 5 independent experiments in which variability is less than 5%.

binding MC 540 molecules. However, the extent of this competition is determined by the kind of phospholipid. In the case of DMPC and DPPC liposomes stained with MC 540 (51 μmol/l) the presence of albumin at concentrations as low as 30 μmol/l induces association of at least 90% of MC 540 to albumin, whereas in POPC and egg PC liposomes the binding of more than 90% of the dye concentration to albumin is observed at albumin concentration as high as 110 μmol/l. These data show that albumin competes very successfully with lecithins containing saturated fatty acid chains (DPPC, DMPC) and competes weakly with unsaturated lecithins (egg PC and POPC) for binding dye molecules. It is therefore probable that the sensitivity of different cells towards MC 540 in the presence of albumin is related to various abundance of saturated and unsaturated fatty acid chains in the plasma membrane of different cells.

References

Dodd R. Y., Moroff G., Wagner S., Dabay M. H., Dorfman E., George G., Ribeiro P., Schumacker J., Benade L. E. (1991): Inactivation of viruses in platelet suspensions that retain their *in vitro* characteristics: comparison of psoralen-ultraviolet A and merocyanine 540-visible light methods. Transfusion **31,** 483—490

Easton T. G., Valinsky J. E., Reich E. (1978): Merocyanine 540 as a fluorescent probe of membranes: Staining of electrically excitable cells. Cell **13,** 478—486

Gaffney D., Sieber F. (1988): Antineoplastic effects of merocyanine 540: Role of serum and serum components. Proc. Amer. Assoc. Cancer Res. **29,** 497

Gaffney D. K., Sieber F. (1992): The role of serum and serum components in the merocyanine 540-sensitized photoinactivation of K 562 leukemia cells. Biochim. Biophys. Acta **1117,** 321—325

Herrmetter A., Paltauf F. (1982): Semisynthetic preparation of choline and ethanolamine plasmogens. Chem. Phys. Lipids. **30,** 47—53

Lelkes P. I., Miller I. R. (1980): Perturbations of membrane structure by optical probes: I. locations and structural sensitivity of merocyanine 540 bound to phospholipid membranes. J. Membrane Biol. **52,** 1—15

O'Brien J. M., Montgomery R. R., Burns W. H., Gaffney D. K., Sieber F. (1990): Evaluation of merocyanine 540-sensitized photoirradiation as a means to inactivate enveloped viruses in blood products. J. Lab. Clin. Med. **116,** 439—447

Prodouz K. N., Lytle C. D., Keville E. A., Budacy A. P., Vargo S., Fratantoni I. C. (1991): Inhibition by albumin of merocyanine 540-mediated photosensitization of platelets and viruses. Transfusion **31,** 415—422

Schlegel R. A., Phelps B. M., Waggoner A., Terada L., Williamson P. (1980): Binding of merocyanine 540 to normal and leukemic erythroid cells. Cell **20,** 321—330

Sieber F., Spivak J. L., Sutcliffe A. M. (1984): Selective killing of leukemic cells by merocyanine 540-mediated photosensitization. Proc. Nat. Acad. Sci. USA **81,** 7584—7587

Sieber F., Craig A., Krueger G. J., Smith R. E., Ash R. C. (1986): Autotransplantation of bone marrow after purging with merocyanine 540 and light. Blood **68,** 292a

Sieber F., Krueger R. J., O'Brien J. M., Schober S. L., Sensenbrenner L. L., Sharkis S. J. (1989): Inactivation of Friend erythroleukemia virus and Friend virus-infected cells by merocyanine 540-mediated photosensitization. Blood **73,** 345—350

Šikurová L., Janíková T. (1987): Effect of solvents on the absorption spectra of merocyanine 540. Stud. Biophys. **118,** 189—196

Smith O. M., Traul D. L., McOlash L., Sieber F. (1991): Evaluation of merocyanine 540 sensitized photoirradiation as a method for purging malarially infected red cells from blood. J. Infect Dis. **163,** 1312—1317

Valinsky J. E., Easton T. G., Reich E. (1978): Merocyanine 540 as a fluorescent probe of membranes: Selective staining of leukemic and immature hemopoietic cells. Cell **13,** 487—499

Vladimirov Yu. A., Dobretsov G. E. (1980): Fluorescent Probes in the Investigation of Biological Membranes. Nauka, Moscow (in Russian)

Waggoner A. S. (1976): Optical probe of membrane potential. J. Membrane Biol. **27,** 317—334

West W., Pearce S. (1965): The dimeric state of cyanine dyes. J. Phys. Chem. **69,** 1894—1903

Williamson P. L., Massey W. A., Phelps B. M., Schlegel R. A. (1981): Membrane phase state and the rearrangement of hemopoietic cell surface receptors. Mol. Cell. Biol. **1,** 128—135

Williamson P. L., Mattocks K., Schlegel R. A. (1983): Merocyanine 540, a fluorescent probe sensitive to lipid packing. Biochim. Biophys. Acta **732,** 387—394

Final version accepted October 28, 1994