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Effect of hydrogen peroxide on the binding of Merocyanine 540 to human erythrocytes

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Abstract. The fluorescent dye Merocyanine 540 (MC540) is often used as a probe to monitor the molecular packing of phospholipids in the outer leaflet of biomembranes. In a previous study we showed that the increased staining of erythrocytes with a perturbed membrane structure was mainly due to an increase in the fluorescence yield of cell-bound MC540, rather than to an increase of the number of bound molecules. Erythrocytes and ghosts exposed to continuous fluxes of H_2O_2 exhibited pronounced lipid peroxidation. Further, red blood cells subjected to this form of oxidative stress also showed increased staining with MC540. It appeared that this was caused by a strong increase in binding of MC540, together with a slight red shift of the fluorescence emission maximum and a small increase in the fluorescence yield of bound MC540. The changed MC540 binding characteristics were not observed when lipid peroxidation was suppressed by the presence of the antioxidant BHT in the incubation medium. However, open ghosts exposed to H_2O_2 showed no increase of MC540 binding, excluding a direct involvement of lipid peroxidation. Measurement of fluorescence emission spectra and gel filtration studies showed that MC540 can bind to H_2O_2-exposed hemoglobin. Experiments with erythrocytes lysed in hypotonic medium after exposure to H_2O_2 revealed that peroxidation of lipids with H_2O_2 induced a non-specific permeabilization of the plasma membrane to MC540, thereby allowing MC540 to bind to the oxidatively denatured, more hydrophobic hemoglobin.
These results indicate that conclusions about packing of phospholipids in the outer leaflet of the membrane based on increased MC540-staining should be drawn with care.
Key words. Merocyanine 540; erythrocyte; oxidative stress; lipid peroxidation; hemoglobin.

Abbreviations. BSA = bovine serum albumin; BHT = butylated hydroxytoluene; MC540 = Merocyanine 540; PBS = phosphate-buffered saline; TBARS = thiobarbituric acid reactive species.

The fluorescent probe Merocyanine 540 (MC540) is a heterocyclic chromophore with a localized negative charge, that binds to the outer leaflet of the phospholipid bilayer of cellular membranes [1]. In model experiments with liposomes it was shown that MC540 binds preferentially to cholesterol-free phospholipid domains in membranes and to bilayers with relatively loose fatty acid packing [2–4]. Binding of MC540 to phospholipid bilayers can occur in at least two different ways depending of the packing of the phospholipids [5–7]. In closely packed lipids MC540 is mainly oriented parallel to the membrane and is largely in contact with the surrounding water phase. In this case the fluorescence emission maximum is at 572 nm. In more fluid lipid bilayers the dye molecule is predominantly in contact with the hydrocarbon matrix, with its long axis perpendicular to the membrane. In this configuration the fluorescence emission maximum is at 585 nm [5].
The phospholipids in the erythrocyte membrane are distributed asymmetrically, with most of the phosphatidylcholine and sphingomyelin in the outer leaflet and phosphatidylserine and -ethanolamine predominantly in the inner leaflet [8]. As the fatty acid chains of phosphatidylcholine and sphingomyelin are much more saturated than the acyl chains of the other phospholipids, the outer leaflet is much more tightly packed than the inner layer. Therefore, loss of the phospholipid asymmetry leads to a less tight packing of the outer leaflet and it could thus be expected that this would be accompanied by increased MC540 binding to the membrane. This appeared to be confirmed by experiments in which erythrocytes, exposed to the oxidative agent tetrathionate, lost their membrane phospholipid asymmetry and, concomitantly, exhibited increased MC540 staining [9]. Based on these observations MC540 was used in many subsequent studies as a probe to monitor the molecular packing of phospholipids in the outer leaflet of biomembranes [10–13]. In a previous study we showed that loss of phospholipid asymmetry, induced by tetrathionate in combination with ATP depletion, did not result in increased MC540 binding, but caused an increase in the relative fluorescence quantum yield of bound MC540, with a concomitant red shift of the fluorescence emission maximum [14]. These changes indicate penetration of the dye into more hydrophobic

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regions of the membrane [5] and can explain the increased cell-bound MC540 fluorescence as described under similar experimental conditions by Williamson et al. [9].

The same group also studied the binding of MC540 to erythrocytes that were subjected to oxidative stress by (bolus) addition of H_2O_2. The biological implication of this treatment was that these cells were more readily phagocytozed than untreated cells. The enhancement of uptake by macrophages was correlated with an increased staining of MC540 as measured by flow cytometry. Based upon this observation and on their previous results [9], it was concluded that peroxidation of membrane phospholipids resulted in a looser packing of the phospholipids in the exterior leaflet of the plasma membrane [12, 13].

The present study was initiated to characterize the fluorescence characteristics of MC540 bound to H_2O_2-treated erythrocytes in more detail. In order to mimic physiological conditions more closely, erythrocytes were exposed to a continuous flux, rather than a bolus addition, of H_2O_2. A completely different mechanism of staining from that of erythrocytes that had lost their phospholipid asymmetry, was found. It will be shown that peroxidation of lipids by H_2O_2 does not cause increased MC540 binding to the membrane, but leads to increased permeability of the membrane to MC540, thereby allowing MC540 to pass through the membrane and bind to the oxidatively denatured, more hydrophobic hemoglobin.

Materials and methods

MC540 was purchased from Eastman Kodak, Rochester, N.Y. Stock solutions (1 mg/ml) were prepared in 50% ethanol. Sephadex G25 was from Pharmacia, Uppsala, Sweden. [^{3}H(G)]-raffinose was obtained from DuPont NEN, Dordrecht, The Netherlands. All other chemicals were from Sigma, St. Louis, MO, USA or Baker, Deventer, The Netherlands.

Heparinized human blood was centrifuged shortly after collection. The erythrocytes were washed three times in PBS and resuspended in the same buffer. Open erythrocyte ghosts were prepared by hypotonic lysis as described by Girotti and Thomas [15].

Erythrocytes and ghosts were incubated for 5 min with 1.2 mM NaN_3 to inhibit endogenous catalase activity, prior to addition of hydrogen peroxide. Hydrogen peroxide was added as a continuous flux (30 $\mu l \cdot min^{-1}$; 1–20 mM, as measured from its optical density at 240 nm) by means of a perfusor pump, to 5 ml of a 4% hematocrit cell suspension. After 160 min of incubation at 37 °C with constant agitation, an aliquot was taken to determine the amount of lipid peroxidation and the remaining cells were washed once with PBS and resuspended to 2% hematocrit prior to further analyses. Erythrocyte ghosts were exposed to H_2O_2 in the presence of 25 μM hemoglobin [16]. H_2O_2 concentrations in the medium were assayed according to Pick and Keisari [17]. It appeared that under our experimental conditions the steady state H_2O_2 concentration always remained below the detection limit (1 μM).

Binding of MC540 to erythrocytes and ghosts was assayed as described by Allan et al. [18]. Briefly, a 1 ml erythrocyte suspension was incubated in the dark with 17.5 μM MC540 in the presence of 0.15% BSA for 5 min at 22 °C. After centrifugation in an Eppendorf centrifuge (14,000 r.p.m. for 5 min) the supernatant was removed. The pellet was resuspended in 1 ml PBS and a 100 μl sample of this suspension was added to 2 ml of n-butanol and vortexed for 10 sec. The amount of MC540 in the butanol layer was determined by measuring the fluorescence (excitation 540 nm, emission 580 nm) with a Perkin Elmer LS50B spectrofluorometer.

Relative fluorescence quantum yield and emission maximum of cell-bound MC540 were measured as described before [14]. Briefly, after incubation with MC540, cells and medium were separated by centrifugation. The cells were resuspended in fresh PBS (hematocrit 0.02%) and immediately assayed for fluorescence (excitation 540 nm). A second sample was treated identically but centrifuged immediately after resuspending. The fluorescence of the supernatant, representing MC540 released from the cells, was subtracted from the fluorescence of the cell suspension to obtain fluorescence data of cell-bound MC540. The relative fluorescence quantum yield is defined as the quantum yield of bound MC540 divided by the quantum yield of the dye at the same concentration in aqueous solution, both measured at the emission maximum [19].

Lipid peroxidation was assayed by measuring the generation of thiobarbituric acid reactive species (TBARS) as described by Miller et al. [20].

Uptake of raffinose was determined by incubating erythrocytes with 0.02 μCi ^{3}H-raffinose/ml at room temperature. After 10 min, cells were separated from the incubation medium by spinning them through a dibutylphthalate layer as described by DeBruijne et al. [21]. Radioactivity in the erythrocytes was measured in a Packard Tri-carb 4000 scintillation counter after bleaching hemoglobin with H_2O_2.

All experiments were carried out at least in triplicate, utilizing blood from different donors.

Results

Exposure of erythrocytes to various continuous fluxes of H_2O_2 for 160 min induced lipid peroxidation, as measured by the formation of TBARS (fig. 1). H_2O_2 exposure also affected the binding of MC540 to erythrocytes. Untreated cells bound 2.7×10^6 molecules MC540/cell, with a fluorescence emission maximum of

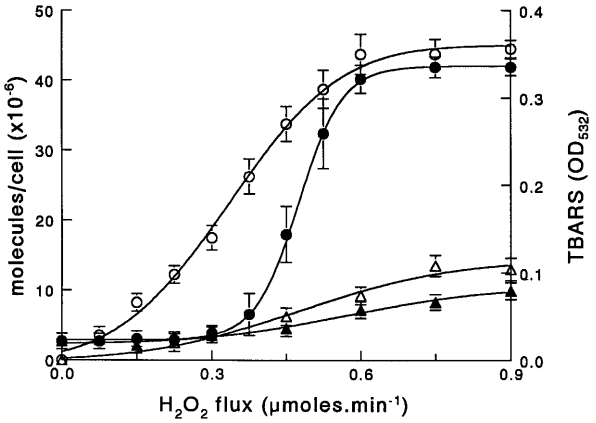

Figure 1. Binding of MC540 (closed symbols) and formation of thiobarbituric acid reactive species (OD_{532}, open symbols) after exposure of a 4% erythrocyte suspension to various continuous fluxes of H_2O_2 for 160 min at 37 °C. ● and ○, no further additions; ▲ and △, 0.1 mM BHT added. Values are expressed as mean ± S.E.M.

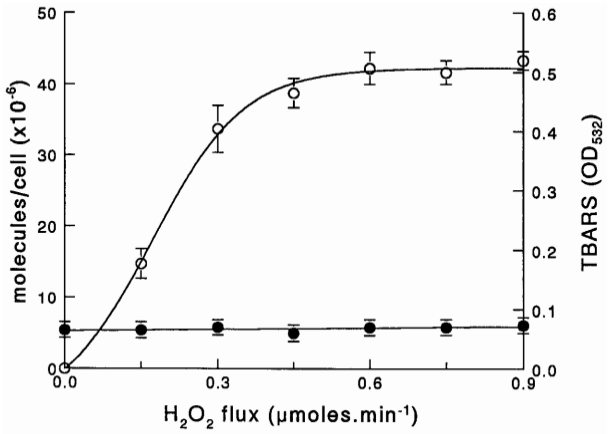

Figure 2. Binding of MC540 (●) and formation of thiobarbituric acid reactive species (OD_{532}, ○) after exposure of a 4% ghosts suspension to various continuous fluxes of H_2O_2 for 160 min at 37 °C. Values are expressed as mean ± S.E.M.

bound MC540 at 572 nm and a relative fluorescence quantum yield of 1. Treatment with fluxes of up to 0.15 μmoles $H_2O_2 \cdot min^{-1}$ had no effect on the binding characteristics of MC540. Higher fluxes produced cells which bound more MC540. Maximum binding (42×10^6 molecules/cell) was reached after exposure of the erythrocytes to 0.60 μmoles $H_2O_2 \cdot min^{-1}$ (fig. 1). This treatment also induced a shift in the emission maximum of bound MC540 to 578 nm and an increase in the relative fluorescence quantum yield to 2.6 (table 1). Addition of the chain-breaking antioxidant BHT to the incubation medium inhibited both the formation of TBARS and the increased binding of MC540 (fig. 1). The red shift in the emission maximum and the increased fluorescence yield of bound MC540 induced by H_2O_2 were also abolished by BHT (not shown).

To investigate whether oxidation of lipids was the main reason for the increased binding of MC540 to H_2O_2-treated red cells, ghosts were exposed to H_2O_2. This showed that the amount of H_2O_2-induced lipid peroxidation in ghost and intact erythrocytes is comparable (fig. 2). As shown before [14] open ghosts bind twice the amount of MC540 as compared to intact erythrocytes (5.4×10^6 molecules/cell). The emission maximum was shifted to 582 nm and the relative fluorescence quantum yield was increased to 4.7 (table 1). Treatment of ghosts with H_2O_2 fluxes of up to 0.90 μmoles $\cdot min^{-1}$ neither increased the binding (fig. 2) nor changed the fluorescence characteristics of MC540 (table 1). These results indicate that the increased binding and changed binding characteristics of MC540 to erythrocytes after H_2O_2 treatment cannot be explained by perturbation of the membrane structure alone.

Exposure of erythrocytes to H_2O_2 induces immediate oxidation of the heme iron, whereas prolonged incubation with H_2O_2 leads to denaturation of hemoglobin [22, 23]. To investigate whether there is an interaction between MC540 and oxidatively denatured hemoglobin, isolated hemoglobin was exposed to a continuous flux of H_2O_2. After the exposure to H_2O_2, MC540 was added and fluorescence emission spectra were recorded and compared with fluorescence spectra of MC540 in buffer and in the presence of untreated hemoglobin and methemoglobin. The results are depicted in figure 3. The emission maximum of MC540 in buffer was at 572 nm. Neither hemoglobin nor methemoglobin induced a change in the fluorescence spectrum of MC540. Oxidatively denatured hemoglobin, however, induced both a red shift in the emission maximum from 572 to 578 nm and an increase in the fluorescence yield of MC540. These results suggest that there is an interaction between oxidatively denatured hemoglobin and MC540. This interaction was confirmed by gelfiltration studies on Sephadex G25. MC540 was retained by Sephadex G25 (apparently by nonspecific adsorption) while hemoglobin, methemoglobin and oxidatively denatured hemoglobin were easily eluted from the column with PBS. When MC540 was applied on the Sephadex column together with either hemoglobin, methemoglobin or oxidatively denatured hemoglobin, neither normal hemoglobin nor methemoglobin had any effect

Table 1. Fluorescence characteristics of MC540 bound to erythrocytes, erythrocytes lysed after H_2O_2-treatment, and open ghosts; effect of exposure of the cells to a continuous flux of hydrogen peroxide (0.6 μmoles $\cdot min^{-1}$) for 160 min.

	H_2O_2	Emission maximum (nm)	Rel. fluorescence quantum yield
Erythrocytes	−	572	1
Erthrocytes	+	578	2.6
Lysed erythrocytes	−	582	4.6
Lysed erythrocytes	+	578	2.7
Ghosts	−	582	4.7
Ghosts	+	582	4.5

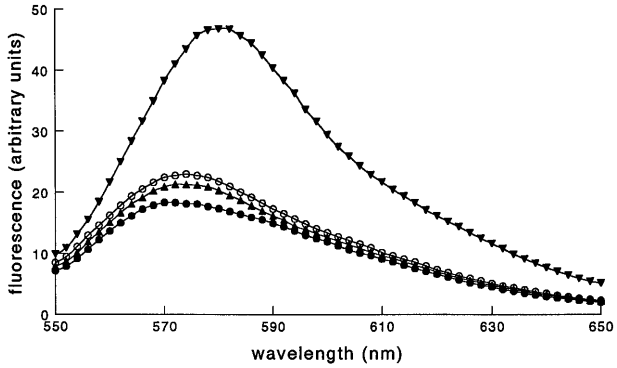

Figure 3. Fluorescence emission spectra (excitation 540 nm) of 1 μM MC540. ○, no further additions; ●, 10 μM hemoglobin added; ▲, 10 μM methemoglobin added; ▼, 10 μM oxidatively denatured hemoglobin added.

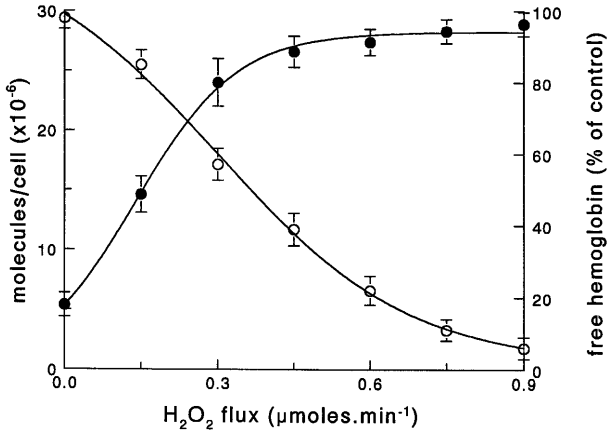

Figure 4. Binding of MC540 (●) and release of hemoglobin (○) after exposure of a 4% erythrocyte suspension to various continuous fluxes of H_2O_2 for 160 min at 37 °C followed by hypotonic lysis. Values are expressed as mean ± S.E.M.

on the retention of MC540. However, when MC540 was applied together with oxidatively denatured hemoglobin, MC540 eluted in the same fraction as the hemoglobin. These results clearly demonstrate that oxidatively denatured hemoglobin can bind MC540 while there is no interaction between MC540 and normal hemoglobin or methemoglobin.

Due to its localized negative charge MC540 is not able to cross the membrane of normal erythrocytes [1]. Therefore MC540 is not able to reach hemoglobin in intact erythrocytes. The increased MC540 binding is abolished by addition of BHT to the incubation medium (fig. 1). Since BHT inhibited lipid peroxidation (fig. 1) and has only a very small inhibitory effect on the oxidation of hemoglobin [22], it seems possible that oxidation of lipids causes a permeabilization of the membrane to MC540, thereby allowing MC540 to bind to denatured hemoglobin. To check this possibility erythrocytes exposed to H_2O_2 were lysed in hypotonic NaCl. Both release of hemoglobin from and binding of MC540 to the lysed erythrocytes were measured. H_2O_2 exposure decreased the release of hemoglobin after hypotonic lysis; untreated cells liberated almost all of their hemoglobin (98%) while after exposure to H_2O_2, 95% of the hemoglobin remained bound to the membrane (fig. 4). The increased binding of hemoglobin to the membrane was paralleled by an increased binding of MC540. BHT, present during exposure to H_2O_2, had no effect on either the decreased release of hemoglobin or the increased binding of MC540 to lysed cells (data not shown). The emission maximum of bound MC540 was blue shifted from 582 nm in the untreated lysed cells to 578 nm in cells treated with H_2O_2 while the relative fluorescence yield of bound MC540 decreased from 4.7 in untreated cells to 2.5 in H_2O_2-treated, lysed cells (table 1).

To clarify whether lipid peroxidation-induced permeabilization of the plasma membrane was specific for MC540, we studied the effect of H_2O_2 and BHT on the uptake of an impermeable sugar, ^{3}H-raffinose, by erythrocytes. As can be seen in figure 5, exposure of erythrocytes to H_2O_2 leads to uptake of raffinose. The uptake of the sugar was inhibited by addition of BHT to the incubation medium.

Discussion

In model experiments with liposomes it was shown that MC540 binds preferentially to bilayers with loose phospholipid packing [2–4]. Based on these observations MC540 is often used as a probe to monitor the molecular packing of phospholipids in the outer leaflet of biomembranes [9–13]. In a previous study we showed that the increased staining of erythrocytes with a perturbed membrane structure was due mainly to changed fluorescence properties of cell-bound MC540 (red shift of the emission maximum and increased fluorescence yield) and not to increased binding of MC540 [14].

As shown in the present studies, however, exposure of erythrocytes to hydrogen peroxide leads to a strong increase in binding of MC540 (fig. 1). Since the increased binding could be inhibited by addition of the chain-breaking antioxidant BHT to the incubation medium, it can be concluded that oxidation of lipids is involved in the increased binding of MC540 to H_2O_2-treated erythrocytes. However, open ghosts exposed to H_2O_2 showed no increase in MC540-binding, although a comparable amount of TBARS could be detected (fig. 2). This rules out a direct involvement of oxidation of lipids and suggests that hemoglobin is involved in the increased MC540 binding.

Measurement of fluorescence emission spectra of MC540 showed no influence of normal hemoglobin or methemoglobin on the fluorescence of MC540. However, oxidatively denatured hemoglobin red shifted the emission maximum (from 572 nm in the absence to 578 nm

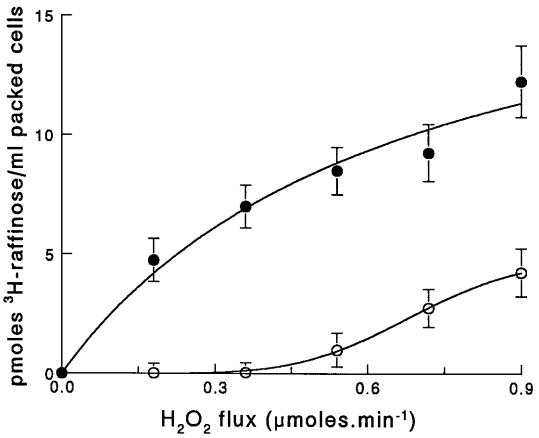

Figure 5. Uptake of ^{3}H-raffinose after exposure of a 4% erythrocyte suspension to various continuous fluxes of H_2O_2 for 160 min at 37 °C. ●, no further additions; ○, 0.1 mM BHT added. Values are expressed as mean ± S.E.M.

in the presence of H_2O_2-treated hemoglobin) and increased the fluorescence yield of MC540 (fig. 3). Both the emission maximum and the fluorescence yield are comparable with those found in H_2O_2-treated erythrocytes. Gelfiltration experiments confirmed the interaction between MC540 and oxidatively denatured hemoglobin. Neither normal hemoglobin nor methemoglobin had any effect on the retention of MC540, while in the presence of oxidatively denatured hemoglobin MC540 eluted in the same fraction as the hemoglobin.

The interaction between oxidatively denatured hemoglobin and MC540 was further confirmed by experiments with lysed erythrocytes. Erythrocytes lysed in hypotonic medium after exposure to H_2O_2 showed a correlation between binding of hemoglobin to the plasma membrane and MC540 binding. Both the emission maximum and the fluorescence yield of MC540 bound to H_2O_2-treated, lysed cells were comparable with those of MC540 in the presence of oxidatively denatured hemoglobin (table 1, fig. 3). In these experiments the presence of BHT during H_2O_2 exposure had no effect on either the changed MC540 binding characteristics or the decreased release of hemoglobin.

Exposure of oxyhemoglobin (Hb-$Fe^{II}O_2$) to H_2O_2 results not only in oxidation of the heme iron (to Fe^{III} and Fe^{IV}-OH) but also in protein damage [22, 23] This oxidatively damaged hemoglobin exhibits increased hydrophobicity, due to increased exposure of hydrophobic (Trp, Met) amino acid residues [24]. Since MC540 is a hydrophobic dye [1], this increased hydrophobicity of oxidatively denatured hemoglobin could be the basis for the interaction of MC540 and H_2O_2-treated hemoglobin. The emission maximum of the bound MC540 (578 nm) indicates that the hydrophobicity of the environment of the dye is comparable to that of ethanol [18].

Due to its negative charge MC540 is unable to penetrate intact cells [1] and thus unable to reach hemoglobin in erythrocytes. After exposure to H_2O_2 MC540 binds to oxidatively denatured hemoglobin, indicating that the membrane becomes permeable to MC540. Because BHT inhibited the increased binding of MC540 to H_2O_2-treated intact erythrocytes and had no effect on the binding to permeabilized erythrocytes, it can be concluded that lipid peroxidation permeabilizes the membrane to MC540. This lipid peroxidation-dependent permeabilization of the membrane is not specific to MC540, as it was also observed with raffinose (fig. 5). This H_2O_2-induced permeabilization of the membrane to MC540 and raffinose differs from the K^+-leakage induced by this agent. As shown previously, H_2O_2-induced K^+-leakage was not inhibited by BHT and appeared to be caused by oxidation of membrane protein SH-groups [16]. Similarly, Deziel and Girotti showed that photodynamically-induced release of glucose 6-phosphate from resealed erythrocyte ghosts was caused by lipid peroxidation, whereas the concomitant leakage of Na^+ was the result of protein damage [25]. To our knowledge this is the first time that it has been shown that, in addition to photoxidation-induced peroxidation, H_2O_2-induced, limited lipid peroxidation is also related to changes in the permeability of the membrane to larger molecules like raffinose and MC540.

It seems likely that other cell types may be similarly affected. For instance, Szabo Jr. et al. have shown that the plasma membrane of mouse spleen cells becomes permeable to MC540 upon oxidative stress induced by UV irradiation, thereby allowing the dye to bind to intracellular structures [26].

In previous studies it was concluded that the increased phagocytosis of H_2O_2-treated erythrocytes by macrophages was due to a looser packing of the phospholipids in the outer leaflet of the erythrocyte membrane. This conclusion was based on the observation that erythrocytes subjected to oxidative stress by treatment with H_2O_2 showed an increased staining with MC540 [12,13]. However, our present results show that the increased staining after treatment with H_2O_2 is due to binding of MC540 to oxidatively denatured, more hydrophobic hemoglobin, rather than to altered binding of the dye to the membrane. This indicates that changes in cell-bound MC540 fluorescence should be interpreted with the utmost care.

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