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INTERACTION OF DIFFERENT FORMS OF HAEMOGLOBIN WITH ARTIFICIAL LIPID MEMBRANES

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Summary

The action of different forms of haemoglobin (oxy-, carboxy- and methaemoglobin) and myoglobin on the leakage of Rb^+ out of liposomes has been investigated. The results presented will demonstrate that only methaemoglobin is particularly effective in interacting with phospholipid vesicles by changing their permeability and catalyzing a peroxidation of their unsaturated hydrocarbon chains.

Model systems consisting of phospholipid liquid crystals have provided considerable insight into the mechanism of lipid-protein associations in membranes [1,2].

It has been recently shown that beef haemoglobin is the most effective among many proteins, in inducing a marked increase in the leakage of Rb^+ and other solutes out of negatively charged liposomes [3]. Since haemoglobin exists in different forms (oxyhaemoglobin, carboxyhaemoglobin, methaemoglobin, etc.) each with different, well characterized conformational states and functional properties, a more detailed analysis of their interaction with lipid membranes seemed of particular interest.

Human haemoglobin was prepared from fresh blood by the ammonium sulphate procedure and stripped of organic phosphates by passage through a Sephadex-G25 fine column equilibrated with 5 mM Tris·Cl and 100 mM NaCl, pH 7.6. Methaemoglobin was prepared by addition of a 10% molar excess of potassium ferricyanide to a solution of oxyhaemoglobin, followed by Sephadex-G25 gel filtration. Oxyhaemoglobin was converted to carboxy-

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haemoglobin by flushing with CO and allowing the solution to stand overnight in an atmosphere of CO. Horse heart myoglobin (ferric form) was from Sigma. The concentration of the haemoglobin solutions was determined using the mill molar extinction coefficients given by Antonini and Brunori [4]. All haemoglobin concentrations are expressed on a molar basis (M_r = 64 500). The method of liposome preparation was described in detail elsewhere [5,6]. Leakage was calculated by adding the counts lost by liposomes during each incubation period and expressing this loss as a percentage of the counts present in the liposomes at the beginning of each period of incubation.

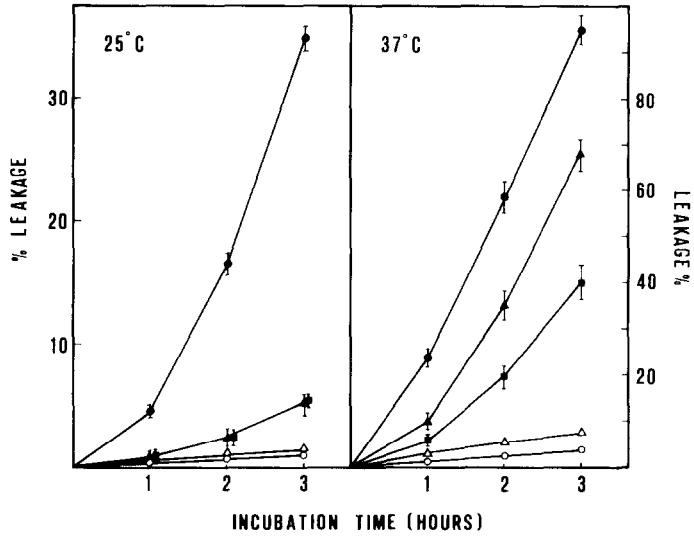

Fig.1. Effect of different forms of haemoglobin and myoglobin on Rb^+ leakage at 25° C and 37° C. Liposomes were prepared in 50 mM Tris—Cl, pH 7.6, containing 100 mM KCl and 1 mM EDTA and incubated in an air atmosphere at a final concentration of 3 μmol/ml. Proteins were $1.5 \cdot 10^{-6}$ M at 37° C and $2.3 \cdot 10^{-6}$ M at 25° C. ●—●, methaemoglobin; ▲—▲, oxyhaemoglobin; ■—■, carboxyhaemoglobin; △—△, myoglobin; ○—○, control.

The effect of different forms of haemoglobin on the diffusion of ^{86}Rb out of liposomes prepared with pure phosphatidylcholine, in physiological conditions of pH and ionic strength, is shown in Fig. 1. It can be seen that while at 25° C only the haemoglobin in its ferric state is particularly effective in inducing Rb^+ leakage, at 37° C the differential stimulation by the various forms is markedly reduced and tends to disappear with the time of incubation. Spectral measurements (Fig. 2) of oxyhaemoglobin-liposome mixtures performed in identical experimental conditions at 37° C, reveal that the protein undergoes a hypochromic shift of the Soret band from 414 nm to that typical of methaemoglobin i.e. 406 nm, while at 25° C no substantial shift is observed. The results shown in Fig. 1 can then be explained in part with the transformation at 37° C but not at 25° C of the ligand bound forms of haemoglobin to methaemoglobin, which behaves as the most effective form inducing ^{86}Rb diffusion. A tentative explanation for the autoxidation of

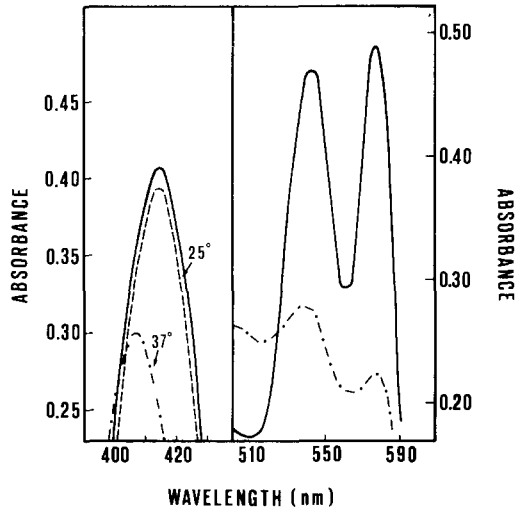

Fig. 2. Absorbance spectra of human oxyhaemoglobin after 90 min incubation in the presence of liposomes at two different temperatures (25° C and 37° C). Concentration of protein $1.0 \cdot 10^{-5}$ M (diluted 10-fold prior to recording the spectrum in the Soret region). Phospholipid concentration was $1.0 \cdot 10^{-3}$ M. Same buffer as in Fig. 1. Solid line refers to the spectra recorded at zero time. Controls without liposomes remained unchanged.

oxyhaemoglobin (Fig. 2) is that its interaction with liposomes induces a somewhat subtle alteration of the electrostatic equilibrium which normally hinders the dissociation of the superoxiferrihaem complex in the oxygenated state [7].

Interaction of haemoglobin with sonicated liposomes is followed by a marked increase in turbidity observed at 280 nm (Fig. 3b), which, in analogy with the leakage experiment, (Fig. 1) is most evident in the presence of methaemoglobin. It is noteworthy that a decrease in absorbance at the Soret peak parallels the turbidity increase described above (Fig. 3a). As sonicated liposomes are incapable of osmotic swelling or shrinking [1]; the change in light scattering observed must be ascribed to the formation of a protein-liposome aggregate. The decrease in absorbance in the Soret peak might arise, at least for methaemoglobin, from a progressive degradation of the haem group of the protein during stimulation of lipid peroxidation (see Fig. 4) [8, 11].

Several lines of evidence have been produced in past years involving haemoproteins as effective catalysts for peroxidase activity, especially when in their high spin state [9,11].

When the peroxidase activity of different haemoglobin derivatives was tested on liposomes prepared with egg lecithin (Fig. 4), it was found that only methaemoglobin had the property to catalyze the cleavage of fatty acid hydroperoxides while no effect was observed with dipalmitoylphosphatidylcholine liposomes both at 25° C and 42° C. Such a chemical action exibited by ferric haemoglobin on the liposome structure, however, is not the major

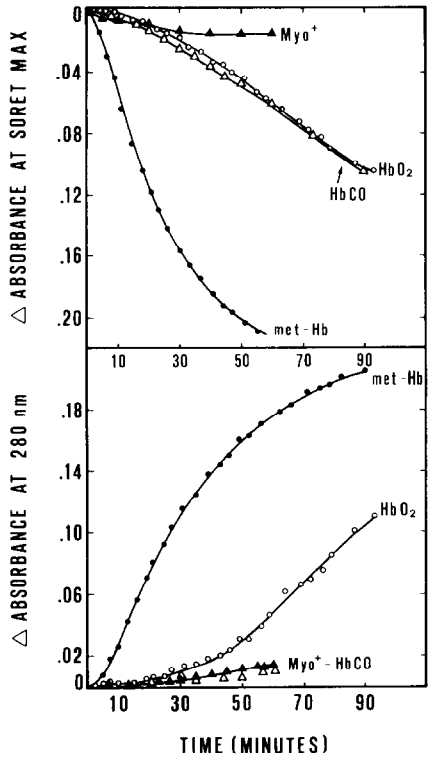

Fig. 3. Absorbance changes of haemoglobin derivative-liposome mixtures as a function of time. Proteins and phospholipid concentrations were $8.3 \cdot 10^{-7}$ M and $5 \cdot 10^{-4}$ M, respectively. Measurements were done in a Zeiss PMQ II spectrophotometer at 23°C. The Soret wavelengths for methaemoglobin, oxyhaemoglobin, carboxyhaemoglobin and myoglobin were 406, 415, 418 and 408 nm, respectively.

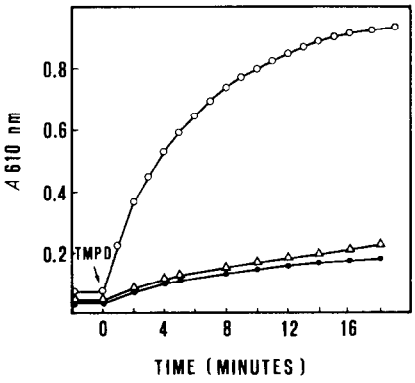

Fig. 4. Peroxidase activity of methaemoglobin on liposomes prepared with egg lecithin (Grade I Lipid Products, England). The assay was carried out at 23°C with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as hydrogen donor, using a system containing 50 mM Tris·Cl buffer, pH 7.5, 1 mM EDTA, 0.2 mM TMPD, 0.5 mM phosphatidylcholine and 2.3 μM protein as a catalyst. The rate of TMPD oxidation was followed spectrophotometrically at 610 nm, measuring the formation of Wurster's blue free radical [9]. ○—○, methaemoglobin, liposomes and TMPD; △—△, liposomes and TMPD; ●—●, methaemoglobin and TMPD.

cause for its greater effect on the liposome permeability. In fact, diffusion experiments performed at 37° C with liposomes prepared with dipalmitoyllecithin at 42° C, which contains only long chain saturated fatty acids, showed that the effect elicited by the different forms was the same as that induced on phosphatidylcholine liposomes at 37° C with methaemoglobin being the most powerful. The demonstration of peroxidase activity by methaemoglobin on membranes containing unsaturated fatty acid residues however, may represent a good model for the lipid peroxidation catalyzed by haemoproteins which, up to date, has been studied mainly with linoleic acid micelles [9,11].

On the basis of the experimental evidence presented here, no definitive explanation for the marked difference in leakage (5—10 fold) induced by ferric haemoglobin and ligand-bound haemoglobin can be unequivocally given. As the different behaviour of the two forms occurs mainly at 25° C where autoxidation is negligible (Fig. 2) and since methaemoglobin is equally effective on liposomes prepared with dipalmitoyllecithin where lipid peroxidation can be ruled out, the effectiveness of methaemoglobin must reside primarily on its conformation. We may assume that methaemoglobin has the most favourable conformation for an interaction with the lipid membrane, suitable to perturb its permeability to Rb^+. On the contrary, the conformational state of oxyhaemoglobin allows its binding but not penetration into the lipid bilayer. This latter event will occur only when it has been transformed, probably through some membrane induced autoxidation, into methaemoglobin. This hypothesis is substantiated by the time-lag observed for oxyhaemoglobin to produce leakage increase in light scattering (Figs 1 and 3). The failure of carboxyhaemoglobin to undergo the sequence of events mentioned above is probably due to its more stable conformation [4].

The negligible effect of myoglobin reported in these studies (Figs 1, 3), suggests that its difference in behaviour as compared to haemoglobin, besides the different primary structure, could be attributed to the lack of the quaternary structure typical of haemoglobin, which could provide hydrophobic interfaces between subunits. The high affinity of haemoglobin for lipid-water interfaces [10,12] suggests that its interaction with liposomes is largely nonpolar in nature. This conclusion is also supported by the high affinity exhibited for membranes made with phosphatidylcholine in the presence of an ionic strength (0.15M) which normally hinders Coulombic interactions. It is worth mention that most of the experiments described here, were performed with concentrations of haemoglobin in a range (0.1—0.2 mg/ml) in which dissociation into dimers is well known to occur [4].

Whatever is the final explanation for the unique effect exhibited by methaemoglobin, these experiments do suggest that the presence or an excess of this form of haemoglobin within the red blood cells (e.g. met haemoglobinemias) could markedly alter the physicochemical properties of their membrane.

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