The Effects of Lipid Composition on the Rate and Extent of Heme Binding to Membranes*

W. Richard Light III‡ and John S. Olson§

From the Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251

The effects of membrane composition on heme binding to large unilamellar vesicles were examined using 30 separate phospholipid mixtures. Although there was some variation, most lecithins with \( T_m \) values \( \leq 20 ^\circ C \) showed overall equilibrium partition constants equal to \( \approx 5 \times 10^6 \) and association and dissociation partition rate constants equal to \( \approx 3 \times 10^5 \text{s}^{-1} \) and \( 7 \text{s}^{-1} \), respectively, for CO-heme binding at 30 °C. A sharp decrease in the association rate for CO-heme uptake was observed as the lipid vesicles changed from liquid-crystalline to the gel phase. The addition of dietyl phosphate or dimyristoylphosphatidylglycerol, which are negatively charged at neutral pH, decreased the affinity of the vesicles for CO-heme. The association rate and equilibrium partition constants for CO-heme uptake in unsaturated lecithins were unaffected by cholesterol content at levels up to 40% mol. The affinity of saturated dimyristoylphosphatidylcholine (DMPC) vesicles for CO-heme decreased with increasing cholesterol content at 30 °C. This effect appears to be related to the influence of cholesterol on the DMPC phase transition temperature \( (T_m) \) since at low temperatures \((\approx 20 ^\circ C)\) little CO-heme binds to vesicles composed of DMPC even in the absence of cholesterol.

Although several investigators have examined the effect of lipid composition on heme binding to membranes, the results have been conflicting (Tipping et al., 1979; Rose, 1982; Ginsberg and Demel, 1983; and Cannon et al., 1984). Tipping and co-workers (1979) used the spectral shift in the Soret region since at low temperatures \((\approx 20 ^\circ C)\) little CO-heme binds to vesicles composed of DMPC even in the absence of cholesterol.

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liposome composition on the rate and extent of heme binding was warranted. Unilamellar vesicles with 30 different compositions were prepared by the extrusion method, and the kinetics of CO-heme uptake and release by these membranes were determined by rapid-mixing, stopped-flow spectrophotometry. The temperature dependence of the rate of CO-heme binding was investigated using several saturated and unsaturated phosphatidylcholine vesicles to determine the effects of the gel to liquid crystalline phase transition on the rate parameters for heme binding (Table 1).

Although non-physiological, CO-heme was chosen because this complex remains monomeric at concentrations up to $1 \times 10^{-5}$ M and exhibits a well-defined absorption spectrum in water (Smith, 1959; Light, 1987). In aqueous solution, simple ferrous heme aggregates exhibiting broad, concentration-dependent absorption peaks, and under the same conditions, ferric heme (hemin) forms $\mu$-oxo oligomers which greatly complicate kinetic measurements (White, 1978). We have assumed that the rate constants for the uptake and release of the monomeric forms of these more physiological iron-porphyrins will show dependences on lipid concentration and temperature which are similar to those for CO-heme, even if the absolute values of the parameters differ.

**MATERIALS AND METHODS**

Liposomes were prepared and handled as described in Light and Olson (1990). Kinetic measurements were made with a Gibson-Durrum stopped-flow spectrophotometer that was interfaced to a microcomputer by OLIS, Inc. hardware and software. Measurements at 30 °C or higher in the stopped-flow device were often complicated by the formation of gas bubbles in the observation cuvette. The decrease in CO solubility with increasing temperature was particularly troublesome when both reactant syringes contained buffer equilibrated with 1 atmosphere of the gas at room temperature (~25 °C). When possible, evacuated buffer was used in one of the mixing syringes. This simple precaution eliminated the formation of gas bubbles. CO-heme uptake and release experiments were carried out as described by Light and Olson (1990). The gel filtration experiments were described in the Miniprint of their paper.

**RESULTS**

**Chromatographic Experiments**—Our preliminary observation that neither CO-heme or hemin bind to DMPC/Chol/DCP (5:4:1) vesicles was tested by examining heme binding to liposomes containing egg lecithin, DMPC alone, and mixtures of DMPC, cholesterol, and DCP using gel filtration chromatography. Fractions were collected from a Sephadex G-200 column at 4 °C and analyzed for lipid content by measuring light scattering at 280 nm. Hemin content was determined by measuring absorbance at 390 nm. Control experiments using egg lecithin liposomes without hemin and free hemin without lipids were also performed and showed that liposome vesicles eluted in the void volume of the column, whereas free hemin was greatly retarded by the Sephadex.

When small amounts of hemin were premixed with egg lecithin vesicles, all of the porphyrin eluted with the liposomes in agreement with previous work. In contrast, when a mixture of hemin and DMPC/Chol/DCP (50:40:10) liposomes was examined at 4 °C, all the heme eluted as free porphyrin, and none was found in the liposome fractions. Some hemin did comigrate with vesicles prepared from DMPC alone; however, the affinity was reduced with respect to egg lecithin liposomes since not all the hemin was bound. The extent of this binding was reduced further when DCP was added, and no hemin was bound to DMPC vesicles containing 40% cholesterol.

**Kinetics of CO-Heme Uptake by Liposomes**—Our initial gel filtration results suggested that the extent of heme binding to membranes depends markedly on the lipid composition, at least at 4 °C. To examine this effect quantitatively at more physiological temperatures, we carried out a series of stopped-flow rapid mixing experiments to measure the rates of CO-heme uptake and release by liposomes containing various purified lecithins, DCP, and cholesterol. Large unilamellar liposomes (100-nm diameter) were prepared by the extrusion method as described in the preceding paper (Light and Olson, 1990). In uptake experiments, CO-heme was mixed with liposomes, and the absorbance change accompanying the incorporation of aqueous heme into an apolar environment was followed. In release experiments, apomyoglobin was mixed with liposomes containing bound CO-heme, and the formation of CO-myoglobin was followed spectrophotometrically.

The uptake and release of CO-heme by membranes can be described by unimolecular partitioning phenomena:

$$
H^+_o \quad H^+_m \quad H^-_o \quad H^-_m
$$

where $H^+_o$ represents heme in the outer aqueous phase; $H^+_m$, heme in the outer lipid layer of the membrane; and $H^-_m$, heme in the inner layer. Complete derivations of the rates of uptake and release, the rates of transmembrane movement (i.e., $H^+_m \rightarrow H^-_m$), and the total extent of CO-heme binding are given in the Miniprint to Light and Olson (1990). In the present work, we have examined only the rates of partitioning into ($k_1$) and out of ($k_{-1}$) the external lipid layer of the membrane vesicles.

Under the conditions of our experiments, the observed rate of CO-heme uptake by the outer layer of liposomes is given by Light and Olson (1990, Miniprint)

$$
k_{\text{uptake}} = k_1 V_o C_f (R_o/(R_o^2 + R_i^2)) + k_{-1}
$$

where $R_o$ is the radius of the outer lipid layer; $R_i$, the radius of the inner layer; $V_o C_f (R_o + R_i)$ is the volume fraction of outer lipid layer; $V_o$ is the partial molar volume of the lipid; and $C_f$ is the concentration of lipid in mol/liter. For the vesicles used in our study, $R_o = 50$ nm, $R_i = 45.5$ nm, and $k_{\text{uptake}} = k_1 V_o C_f (0.55) / k_{-1}$. For mixtures, $V_o$ was computed from the partial specific volume, $V_p$, using the average molecular weight of the lipid mixture: $V_o = V MW_{mol}/1000$ ($V_p = 1.0$ ml/gm for all phospholipids). Small, 1986. $MW_{mol}$ was calculated from the mole fractions of the individual molecules, $f_i$, multiplied by their molecular weights: $MW_{mol} = f_1 MW_1 + f_2 MW_2 + \ldots + f_n MW_n$. The average molar concentration of lipid was computed from the phospholipid concentration, $C_f$: $C_f = C_{P}/f_P$, where $f_P$ is the mole fraction of phosphate-containing lipids. The final expression for the observed uptake rate is:

$$
k_{\text{uptake}} = k_1 V_o MW_{mol} C_{P} (0.55) / f_P + k_{-1}
$$

Thus, the rate of CO-heme binding should depend linearly on lipid phosphate concentration, and $k_1$ can be determined from the slope of $k_{\text{uptake}}$ versus $C_f$.

Time courses for CO-heme binding to liposomes were presented in the previous paper (Light and Olson, 1990), and sample plots of $k_{\text{uptake}}$ versus lipid phosphate concentrations are presented in Fig. 1. Unimolecular rate constants for heme movement from the aqueous to the membrane phase, $k_1$, were calculated using Equation 3 and are listed in Table II for a variety of lipid compositions at 30 °C.

**CO-Heme Dissociation and Electrostatic Factors**—In principle, it should be possible to determine the rate of heme dissociation from the $y$ axis intercept of linear plots of $k_{\text{uptake}}$ versus $C_f$ (Equation 3). However, Rose et al. (1985) have shown that significant deviations from linearity occur at low lipid to heme ratios due to electrostatic repulsion between the
propionate groups of neighboring porphyrins. They observed that the rate of heme uptake by small egg lecithin membranes became independent of lipid concentration at low phosphate levels. The limiting rate as \( C_p \) approached zero was 20–40 s\(^{-1}\) which was 5–10 times greater than the dissociation rate constant measured directly by mixing apohemoglobin with liposomes containing bound CO-heme. Similar curvature was observed for the rate of CO-heme uptake by large egg lecithin liposomes prepared by the extrusion method (Fig. 2). This curvature was more pronounced for the experiments with 3 \( \mu \)M CO-heme in the outer layer (see Light and Olson, 1990, Miniprint). Accurate accounting for interactions between bound heme molecules by attenuating the observed partition constant with an electrostatic factor that assumes uniform distribution of anionic charge along the surface of the membrane.

\[
K_p = K_0 \exp(-\Delta H_f^{\circ}/V_c C_v) \tag{4}
\]

where \( K_p \) is the ideal equilibrium constant at low levels of bound heme; \( H_f \) is the total solution concentration of bound heme in the outer layer (see Light and Olson, 1990, Miniprint); and \( A \) is a constant reflecting unfavorable electrostatic interactions which decrease the amount of heme that can partition into the bilayer. This was incorporated into the differential equation for heme binding by assuming that this unfavorable electrostatic potential increases the dissociation rate constant (Rose et al., 1985).

\[
\frac{dH}{dt} - h C_v C_v (0.55) (H - H_0)
- \left[ k_0 \exp(\Delta H_f^{\circ}/V_c C_v (0.55))\right] H
\tag{5}
\]

This equation was numerically integrated to generate theoretical time courses which were then fitted to a single exponential expression. The resultant theoretical uptake rates were plotted versus phospholipid concentration and are represented as the solid curves in Fig. 2. \( k_1 \) was taken from the slope of the observed rate data at high \( C_v \) values, and \( k_2 \) from release experiments in the presence of excess apomoglobin (open diamond, Fig. 2; Table II). A value of 8 M\(^{-1}\) s\(^{-1}\) for the \( A \) constant gave the best fit to the data. Rose et al. (1985) obtained a value of 15 M\(^{-1}\) s\(^{-1}\) for \( A \) at 10 °C using 20-nm vesicles of egg lecithin. The value of \( k_2 \) determined from release experiments in the presence of apomoglobin is significantly lower than the apparent \( y \) axis intercept generated either by numerically integrating Equation 6 or fitting the observed data in Fig. 2 to a straight line. Even a straight line fit to the region of the theoretical curve that appears to be linear ([phosphate] > 50 \( \mu \)M and [heme] = 1 \( \mu \)M, Fig. 2B) results in a \( y \) intercept that is somewhat higher than the rate measured by the apomyoglobin uptake method. In contrast, the apparent \( k_1 \) value from a least squares fit to either all the data or the entire

**TABLE I**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical name</th>
<th>Chain 1</th>
<th>Chain 2</th>
<th>( T_m )°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>Dilauroyl PC</td>
<td>12:0</td>
<td>12:0</td>
<td>~0</td>
</tr>
<tr>
<td>DTPC</td>
<td>Ditridecanoyl PC</td>
<td>13:0</td>
<td>13:0</td>
<td>15</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoyl PC</td>
<td>14:0</td>
<td>14:0</td>
<td>23.8*</td>
</tr>
<tr>
<td>cis-( \Delta )DMPC</td>
<td>Dimyrystoleoyl PC</td>
<td>14:1</td>
<td>14:1</td>
<td>&lt;0</td>
</tr>
<tr>
<td>DPDP</td>
<td>Dipentadecanoyl PC</td>
<td>15:0</td>
<td>15:0</td>
<td>34</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl PC</td>
<td>16:0</td>
<td>16:0</td>
<td>42</td>
</tr>
<tr>
<td>cis-( \Delta )DPPC</td>
<td>Dipalmitoleoyl PC</td>
<td>16:1</td>
<td>16:1</td>
<td>-36</td>
</tr>
<tr>
<td>POPC</td>
<td>1-Palmitoyl-2-oleoyl PC</td>
<td>16:0</td>
<td>18:1</td>
<td>&lt;0</td>
</tr>
<tr>
<td>OSPC</td>
<td>1-Oleoyl-2-stearoyl PC</td>
<td>18:1</td>
<td>18:0</td>
<td>~11</td>
</tr>
<tr>
<td>EL</td>
<td>Egg lecithin</td>
<td>Variable</td>
<td>Variable</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

*Light and Olson (1990) (Miniprint).

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FIG. 1. Observed uptake rates for CO-heme binding to liposomes as a function of lipid phosphate concentration. The reaction of 1 \( \mu \)M CO-heme with liposomes of various compositions were followed at both 408 and 420 nm in 0.05 M NaCl, 0.05 M Tris, pH 8.0, at 30 °C. The individual time courses were fitted to single exponential expressions, and the resultant rates were averaged and plotted as a function of total lipid phosphate concentration to determine the association partition rate constant, \( k_o \), as described in the text. The lipid compositions were egg lecithin (EL)/Chol (6:4) (open diamonds), cis-\( \Delta \)DPPC (closed squares), cis-\( \Delta \)DPDC/DCP (8:1) (open circles), POPC/DMPG (85:15) (closed triangles), and DPPC/DCP (9:1) (closed circles). The lines are the least squares fit to the points at lipid phosphate concentrations above 25 \( \mu \)M.

FIG. 2. Observed association rates for CO-heme binding to egg lecithin liposomes at low lipid phosphate concentrations. The reaction of CO-heme with egg lecithin liposomes was followed at both 408 and 420 nm, in 0.05 M NaCl, 0.05 M Tris, pH 8.0, at 30 °C. The average of the fitted first order rate constants (s\(^{-1}\)) were plotted versus total lipid phosphate. A, the data points for reactions with 1 and 3 \( \mu \)M CO-heme (closed circles and open circles, respectively). The solid lines are the theoretical rates (s\(^{-1}\)) determined using Equation 5. The open diamond on the y axis is value for \( k_0 \), measured by mixing liposomes containing bound CO-heme with an excess of apomyoglobin. B, data for the reaction of 1 \( \mu \)M CO-heme with the egg lecithin vesicles (closed circles). The straight line represents a least squares fit to the linear portion of the theoretical curve at [phosphate] ≥ 50 \( \mu \)M. The curved line was generated using Equation 5 and the open diamond is again the "true" value of \( k_1 \).
Heme Binding to Liposomes

TABLE II

Partition constants for the association and dissociation of CO-heme with various phosphatidylcholine liposomes mixtures at 30 °C in 0.05 M NaCl, 0.05 M Tris, pH 8.0

The rate parameters were determined in rapid mixing experiments using 1–3 μM CO-heme as described in the text, and abbreviations are given in footnote 1.

<table>
<thead>
<tr>
<th>Liposome composition</th>
<th>$k_0 \times 10^4$ s⁻¹</th>
<th>$k_+ \times 10^3$ s⁻¹</th>
<th>$K_0 \times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>2.7</td>
<td>3.3</td>
<td>0.81</td>
</tr>
<tr>
<td>DTFC</td>
<td>3.1</td>
<td>5.0</td>
<td>0.02</td>
</tr>
<tr>
<td>DMPC</td>
<td>1.7</td>
<td>10⁶</td>
<td>0.17</td>
</tr>
<tr>
<td>DMPC/DCP (95:5)</td>
<td>1.8</td>
<td>12⁶</td>
<td>0.15</td>
</tr>
<tr>
<td>DMPC/DCP (95:5) at 30 °C</td>
<td>(≤0.06)</td>
<td>(≤15⁶)</td>
<td>(≤0.004⁶)</td>
</tr>
<tr>
<td>DMPC/DCP (90:10)</td>
<td>1.7</td>
<td>13⁶</td>
<td>0.13</td>
</tr>
<tr>
<td>DMPC/DCP (85:15)</td>
<td>1.1</td>
<td>16⁶</td>
<td>0.07⁶</td>
</tr>
<tr>
<td>DMPC/Chol/DCP (8:1:1)</td>
<td>0.77</td>
<td>15</td>
<td>0.050</td>
</tr>
<tr>
<td>DMPC/Chol/DCP (7:2:1)</td>
<td>0.60</td>
<td>25</td>
<td>0.024</td>
</tr>
<tr>
<td>DMPC/Chol/DCP (5:4:1)</td>
<td>No binding observed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The $k_+$ values were not measured directly but estimated from plots of $k_{obs}$ versus lipid concentration and taking into account electrostatic effects (Equation 5).

Theoretical curve in Fig. 2B is only 10% less than the correct, theoretical value. In practice, data at phosphate concentrations ≥25 μM were used in the determination of $k_0$ values since curvature was only readily apparent at lower lipid levels.

Accurate determinations of $K_0$ were best obtained from release experiments carried out at high lipid to heme ratio. When apomyoglobin is mixed with liposomes containing bound CO-heme, the observed rate of release from the outer lipid layer is given by (Rose et al., 1985; Light and Olson, 1990, Miniprint):

$$k_{value} = \frac{k_0 \exp(\Delta H/V_C C_l (0.55))}{[1 + k_+ V_C C_l (0.55)/k_0(ApoMb)]}$$  \hspace{1cm} (6)

where $k_0$ is the bimolecular rate constant for heme binding to apomyoglobin (ApoMb) which is equal to $\sim 1 \times 10^9$ M⁻¹ s⁻¹ at 30 °C (Light, 1987). At high protein (≥100 μM) and moderately high lipid concentrations (≥200 μM phosphate) the observed rate of CO-heme release is independent of reaction conditions and equal to $k_+$, since $H/C_l$ approaches 0 and $k_0(ApoMb) >> k_+ V_C C_l (0.55)$ (see Rose et al., 1985).

The values of $k_0$ listed in Table II were determined either in direct release experiments or in uptake experiments in which Equation 5 was used to correct for electrostatic repulsion. $K_0$ was calculated from $k_0/k_+$ and represents the ideal partition constant in the absence of interactions between bound heme molecules (i.e. at low total heme/lipid ratios).

Effects of Temperature on the Rate of CO-Heme Uptake—The most dramatic result in Table II is the 10–20-fold difference between the $k_0$ values for DPPC and cis-Δ⁵DPPC containing liposomes at 30 °C. These differences are most likely due to DPPC membranes being in the gel state at 30 °C and cis-Δ⁵DPPC membranes being liquid-crystalline at the same temperature (Table I). The importance of the lamellar phase state was tested directly by comparing the temperature dependences of the rate of CO-heme binding to DMPC/PC vesicles ($T_m = 26 °C$, Olson and Light, 1990) and to cis-Δ⁵DMPC/DCP membranes ($T_m = 0 °C$, Table I) in the range 10–35 °C. An Arrhenius plot of the rate of CO-heme binding to cis-Δ⁵DMPC/DCP vesicles was linear between 20 and 35 °C, and the data were fitted to a straight line to obtain an activation energy (i.e. $k_{value} = A \exp(-\Delta H/RT)$). DLPC, DTPC, POPC, POPC/Chol (6:2 and 6:4), EL, EL/Chol (6:4), and OSPC/DCP (9:1) liposomes all behaved similarly, presumably because these phosphatidylcholines are liquid-crystalline in the observation range (Table I). The calculated activation energies for these lipids were all about 7 kcal/mol (Table III).
Table III
Activation energies for the binding of CO-heme to various liposomes in the region 20-35 °C

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>E_k kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>7.8</td>
</tr>
<tr>
<td>DTPC</td>
<td>7.8</td>
</tr>
<tr>
<td>cis-Δ^2DMPC/DCP (9:1)</td>
<td>8.1</td>
</tr>
<tr>
<td>POPC</td>
<td>4.9</td>
</tr>
<tr>
<td>POPC/Chol (8:2)</td>
<td>5.6</td>
</tr>
<tr>
<td>POPC/Chol (6:4)</td>
<td>5.3</td>
</tr>
<tr>
<td>EL</td>
<td>7.5</td>
</tr>
<tr>
<td>EL/Chol (6:4)</td>
<td>8.0</td>
</tr>
<tr>
<td>OSPC/DCP (9:1)</td>
<td>7.2</td>
</tr>
</tbody>
</table>

FIG. 3. Temperature dependence of CO-heme binding to DMPC-containing liposomes 0.05 M NaCl, 0.05 M Tris at pH 8.0. The reactions were followed in the stopped-flow apparatus at both 408 and 420 nm and the logarithms of the observed uptake rate constants (s^-1) were plotted versus the reciprocal of the temperatures of the reaction mixture. The symbols represent the observed rate constants (s^-1) for the reaction of 1 μM CO-heme with 125 μM DMPC/DCP (95:5) (open squares) or 95 μM cis-Δ^2DMPC/DCP (9:1) (closed circles).

The temperature dependence of the rate of CO-heme binding to DMPC/DCP liposomes was non-Arrhenius, with a steep change centered about the T_m, at -26 °C (Fig. 3). The rate of CO-heme uptake decreased 20-fold from 30 to 20 °C. The sigmoidal temperature dependence was a general characteristic of all DMPC-containing vesicles. The same patterns were found for DMPC/DCP (9:1), DMPC/Chol/DCP (8:1:1), DMPC/Chol/DCP (7:2:1), DTPC, and DPDPC liposomes, and the inflection point varied directly with the T_m of the lipid system. At temperatures higher than the T_m, the slopes of the Arrhenius plots were comparable to those found with the unsaturated lecithin residues.

Effects of Cholesterol—Cholesterol had little or no effect on CO-heme binding to liposomes when the measurements were carried out at 30 °C with membranes having T_m values ±15 °C (Table II). When cholesterol was added to DMPC/DCP liposomes at 30 °C, there was a decrease in the rate and extent of CO-heme binding. This appears to be due to broadening of the phase transition so that even at 30 °C not all of the membrane is liquid-crystalline. Precise measurements of the width of the non-Arrhenius rate transition were complicated by the reduced extent of heme binding, but the rate of uptake decreased dramatically at temperatures below the T_m with DMPC/Chol/DCP (8:1:1) liposomes, and the change in uptake rate occurred over a wider temperature range than that shown for DMPC/DCP vesicles in Fig. 3. Similar effects of cholesterol on the phase transition of lecithins were observed by Mabrey and Sturtevant (1978) and Presti and Chen (1982).

Discussion
Heme Binding and the Physical State of the Membrane—The results in Fig. 3 and Table II indicate that the most important factor governing the rate and extent of CO-heme binding is the physical state of the membrane. At 30 °C the equilibrium association partition constant for CO-heme uptake by liquid-crystalline cis-Δ^2DPPC vesicles was 16-fold greater than that for DPPC liposomes which are in the gel state at this temperature (Table I). As shown in Fig. 3, there is an almost 20-fold decrease in k_1 when DMPC containing membranes are cooled through their phase transition. This dramatic effect of the gel to liquid-crystalline transition on the rate and extent of binding confirms that the heme group is interacting directly with the acyl-chains of the lecithin molecules. Lenitz et al. (1982) observed a similar sigmoidal dependence of the logarithm of microviscosity on temperature for small sonicated DMPC vesicles. The absolute value increased from about 1 to 40 cp in the range 30 to 20 °C. If the rate-limiting step for heme uptake is intercalation into the outer lipid layer, then an increase in the microviscosity of the hydrocarbon phase would be expected to decrease k_1.

Dependence on Lipid Composition—The partitioning parameters for CO-heme binding varied little with fatty acyl-chain length or saturation as long as the measurements were made at temperatures well above that for the liquid-crystalline to gel phase transition. At 30 °C, k_1, k_m, and K_p for most of the lipid mixtures were 3-5 X 10^6 s^-1, 3-10 s^-1, and 5-8 X 10^5, respectively, (Table II). The K_p value is quite large and, assuming hemin shows behavior similar to that of CO-heme, suggests that the level of free aqueous iron-porphyrin is quite small in the cytoplasm of most cells. However, heme transfer between the outer layers of separate phosphatidylycholine membranes can occur readily through the aqueous medium and is limited only by the rate of efflux from the bilayer, k_m (see Ross and others, 1985). Heme is also readily available for binding to apoproteins by the same mechanism.

Adding negative charges to liposomes caused decreases in the equilibrium partition constant for CO-heme binding (Table II). A 3-fold decrease in K_p was generally observed at a level of 10% DCP. Similar results were obtained when dimyristoyolphosphatidylglycerol (DMPG) was added to POPC vesicles. The major effect of negative charge was an increase in the dissociation constant, k_m; smaller decreases in the association constant were observed. This result supports the assumption made by Ross et al. (1985) that electrostatic repulsion limits heme uptake by increasing the dissociation rate (see Equation 5.)

The addition of cholesterol to DMPC liposomes decreased the affinity of these vesicles for heme in a concentration dependent manner at temperatures near or below the T_m (Table II). This accounts for the inability of the DMPC/Chol/DCP (5:4:1) liposomes to take up heme in our initial absorption and chromatography experiments. In contrast, the addition of cholesterol to cis-Δ^2DPPC, POPC, or egg lecithin vesicles caused little or no change in k_1, k_m, or K_p at 30 °C (Table II), and thus the presence or absence of this sterol in biological membranes is not likely to be an important determinant of heme uptake and release.

Comparison of CO-Heme Uptake and Release with Transmembrane Movement—In the preceding paper, we showed that the interaction of CO-heme with liposomes involves two
distinct kinetic processes: 1) heme uptake or release by the outer lipid layer which is fast \((t_0 \leq 0.1 \text{ s})\) and governed by \(k_1\) and \(k_{-1}\); and 2) transmembrane heme movement which is slow \((t_0 \geq 3 \text{ s})\) and governed by \(k_2\) and \(k_{-2}\) (Equation 1). The temperature dependences of these processes near the gel to liquid-crystalline phase transition are markedly different and worth noting. The rate of transmembrane movement of heme increased sharply near the \(T_m\) value as DMPC vesicles were cooled and then dropped dramatically at lower temperatures. The net result was a peak in the Arrhenius plot (Fig. 4 of Light and Olson, 1990). The 10-fold increase in \(k_2\) at the \(T_m\) value was attributed to rapid rates of heme flipping through “gaps” in the membrane at gel liquid-crystalline interfaces. In contrast, a 20-fold decrease in \(k_1\) was observed when the same DMPC vesicles were cooled from 30 to 20 °C (Fig. 3). In addition, the rate of transmembrane movement depended markedly on the acyl-chain length of the lecithin used to prepare the liposomes. The value of \(k_2\) decreased from \(-0.5 \text{ s}^{-1}\) for \(C_{12}\) lecithin (DLPC) to \(-0.001 \text{ s}^{-1}\) for phosphatidylcholines with carbon chains \(\geq C_{16}\) (i.e. OSPC or egg lecithin). In contrast, \(k_1\) and \(k_{-1}\) showed little or no dependence on acyl-chain length as long as the measurements were made at temperatures significantly above the \(T_m\) value of the lecithin (Table II).

All of these observations support the two-step model for heme uptake and release given in Equation 1 and first proposed by Cannon et al. (1984). The initial binding of heme depends primarily on the charge at the outer surface of the liposome when the membrane is in the liquid-crystalline state. Gelation inhibits binding by simple exclusion from the “solid” hydrocarbon interior. The speed of transmembrane movement depends on the distance that the charged propionates must travel through the apolar hydrocarbon phase and hence on the length of the acyl-chains and the thickness of the membrane.

The physiological relevance of our results for CO-heme depends on the state of aggregation of iron-porphyrin complexes in vivo. The concentration of free aqueous hemin (or heme) is likely to be very small for two reasons: 1) various apoproteins and ligandins have very high affinities for porphyrins, and 2) the equilibrium constant for partitioning into membranes is also very large (Table II). Thus, in vivo uptake and release is probably governed by reactions of heme monomers. The key question is whether or not hemin and CO-heme show similar kinetic behavior for uptake, release, and transmembrane movement. Cannon et al. (1984) reported \(k_{-1}\) values (\(-2 \text{ s}^{-1}\)) for hemin efflux from egg lecithin vesicles which are similar to those shown for CO-heme in Table II and rates of transmembrane movement of heme which were within an order of magnitude of those measured for CO-heme under roughly comparable conditions (Light and Olson (1990) preceding paper). Even with the observed differences, it is clear that the rate of heme diffusion in cells is limited by the speed and number of required transmembrane movements. Heme transfer between membranes and uptake by apoproteins is rapid and governed primarily by \(k_{-1}\) (2–10 \text{ s}^{-1}, Table II; see also Rose et al., 1985). Ligandins, albumins, and other apoproteins probably serve to solubilize heme in aqueous phases, to keep the iron-porphyrins monomeric, and to prevent indiscriminant movement of heme from one membrane system to another (Jakoby, 1979; Rose et al., 1985).

**REFERENCES**


The effects of lipid composition on the rate and extent of heme binding to membranes.
W R Light, 3rd and J S Olson

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