# Reverse Bohr Effect on the Oxygen-binding Affinity of Heme embedded in a Bilayer of Liposome as a Hemoglobin Model: pH-induced Oxygen Uptake and Evolution by Aqueous Synthetic Lipid-heme Solution †

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The oxygen-binding affinity ($p_{\frac{1}{2}}$; oxygen pressure at 50% binding for the heme) of a heme derivative embedded in a bilayer of natural phospholipid liposomes was influenced by the solution pH: the $p_{\frac{1}{2}}$ value of the liposome–lipid-heme increased with pH {Bohr coefficient, $r = d[\log(p_{\frac{1}{2}})]/d(\mathrm{pH}) = +0.09$ to $+0.32$}, which is the reverse of the Bohr effect for hemoglobin. This pH dependence was affected by the composition of the liposome–lipid-heme. Its mechanism is discussed in relation to the surrounding lipid bilayer structure and protonation equilibrium of the imidazole ligand. pH-induced oxygen-uptake and -evolution was observed, in a process which was sensitive to $\pm 1$ unit of pH change at pH 7.

The oxygen-binding affinity of hemoglobin (hb) decreases [the oxygen pressure at 50% binding ($p_{\frac{1}{2}}$) increases] with decrease in the pH of the medium. This phenomenon is called the Bohr effect of hb; hemoglobin releases oxygen more efficiently when the pH of the medium decreases in the presence of carbon dioxide.[1,2] This Bohr effect has been found to be triggered by a structural change accompanied by protonation of the globin chains of hb.[3–5] Few studies have been devoted to artificial gaseous molecule transporters or reservoirs which reversibly take up and release gaseous molecules, induced by pH changes, as does hb.

We previously found that hydrophobic and amphiphilic porphyrinatoiron derivatives incorporated into the bilayer of natural, synthetic, and polymerized phospholipid liposomes (abbreviated as 'liposome–heme') bind molecular oxygen reversibly under physiological conditions.[6–12] Recently we found and preliminarily reported[13] that the oxygen-binding affinity of 5,10,15,20-tetra{α-*o*-[2′,2′-dimethyl-20′-(2″-trimethylammonioethyl phosphonatoxy)eicosanamido]phenyl}porphyrinatoiron(II) (lipid-heme) embedded in polymerized phospholipid liposome was sensitively influenced by the solution pH. The relationship between the oxygen-binding affinity of the heme and the structure of the liposome was studied in order to elucidate the pH-dependent oxygen-binding affinity of the liposome–heme in comparison with that of hb.

## Experimental

*Materials.*—5,10,15,20-Tetra{α-*o*-[2′,2′-dimethyl-20′-(2″-trimethylammonioethyl phosphonatoxy)eicosanamido]-phenyl}porphyrinatoiron(II) (lipid-heme) and the ligands [1-laurylimidazole (lauryl = dodecyl) (li) and 1-lauryl-2-methylimidazole (lmi)] were prepared as reported.[6,9,14] Egg-yolk lecithin (eyl), L-α-dimyristoylphosphatidylcholine (myristoyl = tetradecyl) (dmpc), sphingomyelin (sm), and phosphatidylserine (ps) were purchased from Sigma (special grade). Lipid monomers, 2-*O*-octadecyl-*rac*-glycero-1-[9′-(*p*-vinylbenzoyl)-nonanoyl]-3-phosphocholine (lipid 1) and 1,2-bis(octadeca-2′,4′-dienoyl)-*sn*-glycero-3-phosphocholine (lipid 2) were synthesized as reported.[15–17]

*Preparation of Liposome–Lipid-heme.*—The phospholipid liposome–lipid-heme was prepared by modifying the normal liposome preparation method[18] and the previously reported method.[6,9,10] The porphyrinatoiron(III) bromide (lipid-hemin) (1 μmol) was reduced to iron(II) in the presence of li (3—6 μmol) or lmi (20—50 μmol) under hydrogen by mixing the methanol solution with Pd–C catalyst, and then carbon monoxide gas was bubbled through the mixture. The methanol solution was filtered and collected, dried with molecular sieves (4A), and added to the methanol solution of the natural or synthetic phospholipids (50—200 μmol) saturated with carbon monoxide. By evaporating the solvent under reduced pressure, thin films were prepared on the glass wall of the large round flask. This was dried *in vacuo* for *ca.* 1 h at 90 °C to remove carbon monoxide, giving the porphyrinatoiron(II) complex (lipid-heme). An oxygen-free aqueous solution [0.67 mol $dm^{-3}$ phosphate buffer solution (pH 5—9), 0.10 mol $dm^{-3}$ carbonate buffer solution (pH 9—10), physiological salt solution, dextran expander, or serum] (20 $cm^3$) was added, and the mixture was then shaken by a Vortex mixer. It was ultrasonicated and homogenized in an ice–water bath under nitrogen. The deoxy liposome–lipid-heme solution thus prepared was incubated at room temperature for 2 h. The red transparent solution showed a u.v.–visible absorption spectrum with maxima at 426 (absorption coefficient, ε $8.7 \times 10^5$), 535 ($8.8 \times 10^4$), and 562 nm ($2.7 \times 10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$) for the li complex, and 438 ($8.4 \times 10^5$), 535 ($4.5 \times 10^4$), and 562 nm ($7.9 \times 10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$) for the lmi complex, respectively.

Deoxy liposome–lipid-heme solutions were converted into their oxygen adduct solutions on exposure to oxygen [$\lambda_{max.}$ 422 (ε $= 5.6 \times 10^5$) and 545 ($6.0 \times 10^4$) for the li complex, and 422 ($5.4 \times 10^5$) and 546 nm ($5.9 \times 10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$) for the lmi complex, respectively]. The oxygen adducts were stable, and the adduct formation was rapid and reversible at 37 °C. The negative-stained liposome–heme looked like single-walled liposomes with an average diameter of *ca.* 400 Å according to transmission electron microscopy (Hitachi H-500). The average particle size of the liposome–heme was also measured as *ca.* 400 Å by dynamic light scattering (Hyac-Royco Nicomp model 200).

*Oxygen-binding and -dissociation Equilibrium Curve Measurements.*—Oxygen-binding and -dissociation equilibrium curves (o.e.c.s) and the oxygen-binding affinity ($p_{\frac{1}{2}}$) were determined by using a Shimadzu UV-3000 spectrophotometer and a Yellow Springs Institute YSI-5331 oxygen probe.[11,19] For reference, the o.e.c. of hb in a blood suspension in 0.67 mol $dm^{-3}$ phosphate buffer solution (pH 7.30—7.45) was measured with

† *Non-S.I. units employed:* 1 mmHg ≈ 133 Pa, cal = 4.184 J.

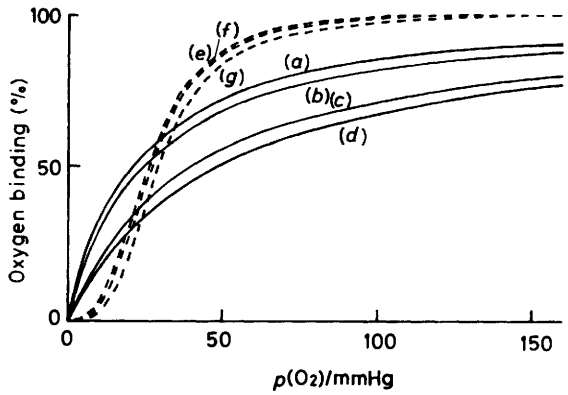



**Figure 1.** Oxygen-binding and -dissociation equilibrium curves of the eyl-liposome–lipid-heme (*a*)—(*d*) and hb in blood (*e*)—(*g*) at 37 °C. Lipid-heme:li:eyl = 1:3:50, [lipid-heme] = $5.0 \times 10^{-5}$ mol $dm^{-3}$; pH 6.0 (*a*), 7.0 (*b*), 8.0 (*c*), 9.0 (*d*), 7.45 (*e*), 7.40 (*f*), and 7.30 (*g*)

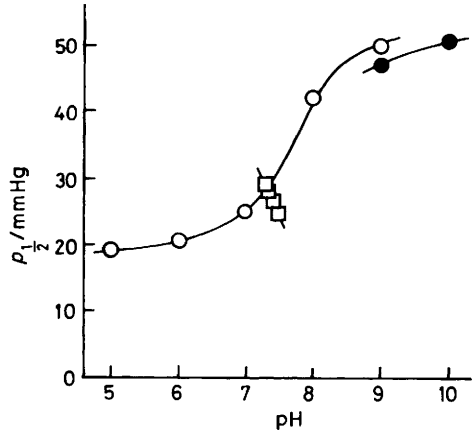



**Figure 2.** pH dependence of the oxygen-binding affinity ($p_{\frac{1}{2}}$) for the eyl-liposome–lipid-heme at 37 °C. Lipid-heme:li:eyl = 1:3:50, [lipid-heme] = $5.0 \times 10^{-5}$ mol $dm^{-3}$. (○), Liposome–lipid-heme in phosphate buffer solution; (●), in carbonate buffer solution; (□), hb in blood suspension

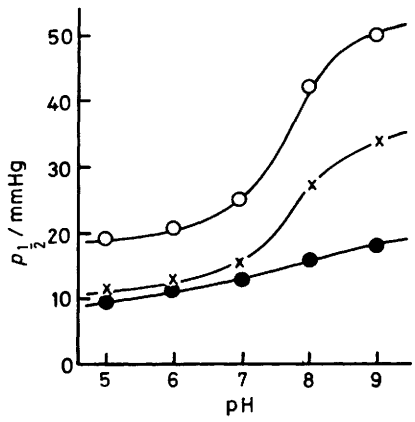



**Figure 3.** pH dependence of $p_{\frac{1}{2}}$ for the eyl-liposome–lipid-heme at various compositions at 37 °C. Lipid-heme:li = 1:3; [lipid-heme] = $5.0 \times 10^{-5}$ mol $dm^{-3}$. Lipid-heme:eyl = 1:50, (○); 1:100, (×); 1:200, (●); in phosphate buffer solution

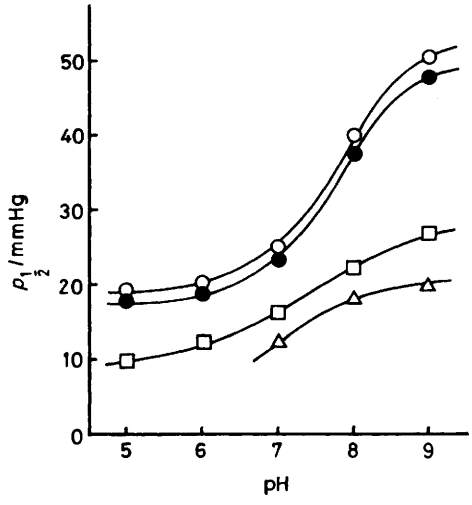



**Figure 4.** pH dependence of $p_{\frac{1}{2}}$ for the various lipid liposome–lipid-hemes at 37 °C: eyl, (○); dmpc, (●); sm, (□); ps (△)

the above-mentioned apparatus; it was consistent with the curve reported.[2,20,21]

*Oxygen-uptake and -evolution Volumes of the Liposome–Lipid-heme.*—The oxygen-uptake and -evolution volumes of the liposome–lipid-heme were measured by modified Warburg volummetry.[9] The physically dissolved oxygen amounts per 100 $cm^3$ of medium were 2.2 $cm^3$ for water and for the heme-free liposome solution, under an oxygen atmosphere at 37 °C.[9,22]

*Differential Scanning Calorimetry* (*d.s.c.*).—A liposome solution of phospholipid (2.5%) and concentrated liposome–lipid-heme solution were prepared in the same manner as above. The thermograms of these samples were measured with a differential scanning calorimeter (Seiko SSC-560C).[9]

## Results and Discussion

The oxygen-binding and -dissociation equilibrium curves (o.e.c.s) of the eyl-liposome–lipid-hemes were hyperbolic (Figure 1) like that of myoglobin (mb). The value of $p_{\frac{1}{2}}$ (oxygen pressure at 50% binding for the heme) for the eyl-liposome–lipid-heme, which has a molar ratio lipid-heme:li:eyl of 1:3:50, is *ca.* 25 mmHg in phosphate buffer solution (pH 7.0) at 37 °C. This value is close to that for hb in blood ($p_{\frac{1}{2}}$ = 27 mmHg under the same conditions). This oxygen-binding affinity of eyl-liposome–lipid-heme was independent of the medium, *e.g.* carbonate buffer solution, physiological salt solution, dextran expander, or human serum. It was however drastically changed by the pH in the range 5—9 (Figure 2). The $p_{\frac{1}{2}}$ value decreased with decreasing pH, especially in the range 6—8, *i.e.* eyl-liposome–lipid-heme binds molecular oxygen more strongly at lower pH.

The oxygen-binding affinity of hb decreases ($p_{\frac{1}{2}}$ increases) with decreasing pH, as shown in Figure 2. The pH dependence of $p_{\frac{1}{2}}$ for the eyl-liposome–lipid-heme is in contrast to the Bohr effect of hb {Bohr coefficient, $r = d[\log(p_{\frac{1}{2}})]/d(\mathrm{pH}) = +0.32$; $-0.60$ for hb in blood[2]}. In carbonate buffer solution, physiological salt solution, dextran expander, or human serum, the same pH dependence of $p_{\frac{1}{2}}$ was observed for the eyl-liposome–lipid-heme. The composition of the eyl-liposome–lipid-heme influenced this pH dependence (Figure 3). With increasing lipid content, the pH dependence was reduced (molar ratio lipid-heme:lipid = 1:50, $r = +0.32$; 1:100, $r = +0.20$; 1:200, $r = +0.04$). A similar pH dependence of $p_{\frac{1}{2}}$ was observed for liposome–lipid-hemes composed of various lipids such as eyl, dmpc, sm, and ps (Figure 4).

The pH dependence of $p_{\frac{1}{2}}$ for the li complex of the liposome–

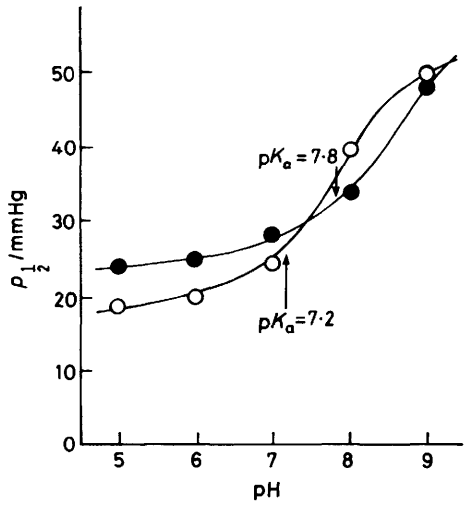



**Figure 5.** pH dependence of the oxygen-binding affinity for the eyl-liposome lipid-heme of the lipid-heme–li complex (○) and the lipid-heme lmi complex (●) at 37 °C

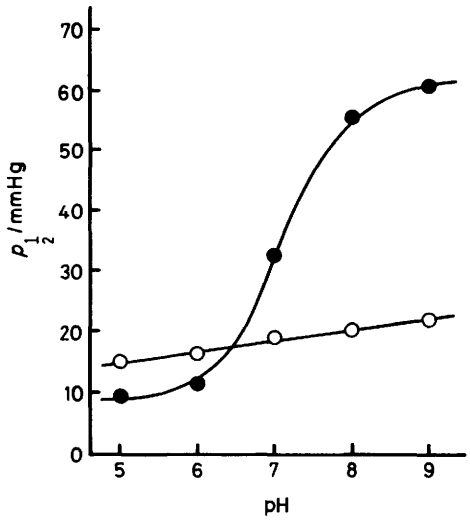
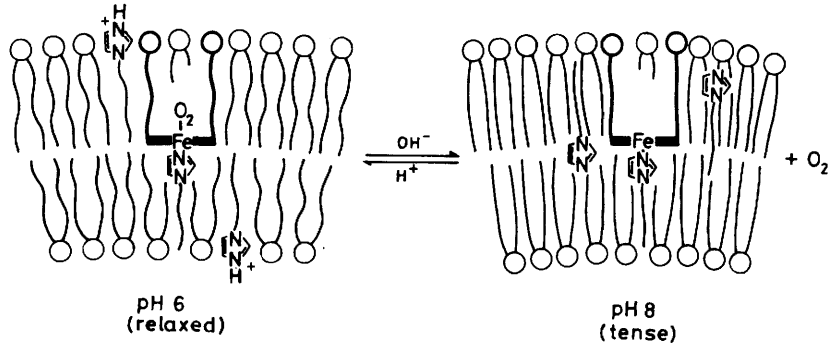



**Figure 7.** pH dependence of $p_{\frac{1}{2}}$ for the polylipid 2-(○) and polylipid 1-liposome–lipid-hemes (●) at 37 °C




**Scheme.**

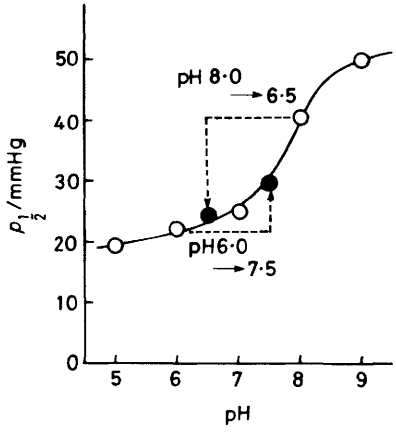



**Figure 6.** Response of the oxygen-binding affinity of the eyl-liposome lipid-heme to pH change at 37 °C. Responses were completed within 1 min

lipid-heme showed a midpoint (*i.e.* the largest slope in the curve) at pH 7.4. For the liposome–lipid-heme lmi complex, the curve shifted slightly to higher pH and the midpoint was at pH 7.9 (Figure 5). These midpoints are consistent with the $pK_a$ values of the imidazoles (li, $pK_a$ = 7.2; lmi, $pK_a$ = 7.8). This suggests that the pH dependence of $p_{\frac{1}{2}}$ is related to the protonation of the imidazole contained in the liposome–lipid-heme.

The pH dependence of $p_{\frac{1}{2}}$ for the liposome–lipid-heme could be explained as follows (Scheme). The liposome–lipid-heme contains a small excess of 1-laurylimidazole (li) or 1-lauryl-2-methylimidazole (lmi) as a ligand to complete the heme complex formation, and non-co-ordinated li or lmi is situated in the bilayer, directing the hydrophilic imidazole group outwards. This imidazole is protonated at lower pH ($pK_a$ of li, 7.2; lmi, 7.8), and is considered to increase the oxygen-binding affinity of the heme, for the following reasons. (*i*) Since oxygen binding involves some charge separation and the oxygen-binding affinity increases with increasing solvent polarity,[23,24] the oxygen bound to the liposome–lipid-heme is stabilized in the presence of the protonated imidazole. (*ii*) The protonated imidazole destroys the packing structure of the lipid bilayer or increases the mobility of the phospholipid in the bilayer. This change provides an environment around the heme similar to that provided by organic solvents such as benzene or toluene and allows the heme complex to adopt a relaxed structure in which it is stable, without a structural distortion caused by the surrounding lipid bilayer structure, and shows higher oxygen-binding affinity corresponding to that in organic solvents.

The lipid bilayer structure of the dmpc-liposome–lipid-heme was measured by differential scanning calorimetry (d.s.c.). While the gel–liquid crystal-phase transition temperature ($T_c$) of the dmpc-liposome–lipid-heme was consistent with that of the

**Table.** Phase-transition temperature and enthalpy change for the lipid bilayer of the dmpc-liposome–lipid-heme determined by differential scanning calorimetry (d.s.c.) at 1 °C $min^{-1}$

| System | d.s.c. $T_c$/°C | d.s.c. $\Delta H$/kcal $mol^{-1}$ |
|---|---|---|
| dmpc-liposome | 23.7 | 6.0 |
| dmpc-liposome–lipid-heme | | |
| pH 5.0 | 23.5 | 3.1 |
| pH 7.0 | 23.7 | 4.1 |
| pH 9.0 | 23.8 | 4.9 |

dmpc-liposome itself ($T_c$ = 24 °C) in the pH range 5—9, the endothermic peak of the phase transition was broadened with decrease in pH, *i.e.* the enthalpy change of the phase transition was decreased at pH 5 (Table). This result supports the effect of the surrounding lipid bilayer structure on the pH dependence of $p_{\frac{1}{2}}$ mentioned above.

The oxygen-binding affinity ($p_{\frac{1}{2}}$) of the eyl-liposome–lipid-heme responded very rapidly to the pH change (Figure 6). When the pH of the liposome–lipid-heme solution was changed from 6.0 to 7.5, $p_{\frac{1}{2}}$ changed correspondingly from 20 to 30 mmHg, which agreed with that determined from the pH *vs.* $p_{\frac{1}{2}}$ equilibrium curve. The reverse response was also observed ($p_{\frac{1}{2}}$ = 40 mmHg at pH 8.0 and $p_{\frac{1}{2}}$ = 23 mmHg at pH 6.5).

A concentrated solution of the liposome–lipid-heme was prepared with the lipid-heme complex of li embedded in the polymerized lipid 1 liposome (polylipid 1-liposome–lipid-heme).[10] To 100 $cm^3$ of this solution (pH 6), sodium hydroxide solution was added dropwise to increase the pH to 8. The colour of the solution changed simultaneously from dark red ($\lambda_{max.}$ 543 nm, 78% oxygen adduct) to brownish red ($\lambda_{max.}$ 539 nm, 43% oxygen adduct) and 3.6 $cm^3$ of oxygen were evolved {calc. 4.28 $cm^3$ per 100 $cm^3$ of solution at [heme] = 5 mmol $dm^{-3}$ and 25 °C}. In contrast, on decreasing the pH from 8 to 6, 3.6 $cm^3$ of oxygen per 100 $cm^3$ of the medium were taken up. This pH-induced oxygen-evolution and -uptake could be repeated several times at room temperature. On the other hand, the pH dependence of $p_{\frac{1}{2}}$ was reduced or cancelled for a 'rigid' liposome–lipid-heme. For example, $p_{\frac{1}{2}}$ for the polylipid 2-liposome–lipid-heme was not influenced by the solution pH ($r < +0.01$, Figure 7).

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