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Structure of the liposome composed of lipid-heme and phospholipids

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The amphiphilic heme derivative, 5,10,15,20-tetra(α,α,α,α-*o*-(2′,2′-dimethyl-20′-(2″-trimethylammonioethyl)phosphonatoxyicosanamido)phenyl)phorphinato iron(II) (lipid-heme), formed a stable liposome (φ ≈ 400 Å) with phospholipids. Differential scanning calorimetry showed that incorporation of the lipid-heme in the liposome bilayer (lipid/lipid-heme > 25) causes no disordering of the bilayer structure. Ligation of a bulky ligand to the lipid-heme liposome indicated that the lipid-heme situates facing predominantly outwards in the liposome. The closed vesicle structure and the stability of the lipid-heme liposome were also confirmed by the encapsulating capability of the fluorescence compound.

Introduction

Metalloporphyrins play important roles in biological reaction systems such as chloroplast, cytochrome and hemoglobin. Models to mimic them have been studied by embedding metalloporphyrins in bilayer membranes [1–7]. For example, zinc- and magnesium-porphyrins were embedded in the bilayer of phospholipid membranes and their photoreactions were discussed in connection with the effects of the bilayer structure on the charge-transfer reactions [3–5]. An iron-porphyrin derivative was also incorporated into the bilayer of phospholipid liposome. It could bind molecular oxygen reversibly because the hydrophobic and non-polar environment around the iron-porphyrin protects its oxygen adduct from irreversible oxidation in aqueous media [6,7]. The position, distribution and orientation of metalloporphyrins in the bilayers were, however, ambiguous in the above-mentioned models. Relatively few studies have been devoted to the fine structure of the phospholipid bilayers involving metalloporphyrins.

Recently we have synthesized, by paying attention to the stereostructure and the hydrophilic–hydrophobic balance of the porphyrin, a novel and amphiphilic iron-porphyrin derivative having four alkanephosphocholine groups. 5,10,15,20-tetra(α,α,α,α-*o*-(2′,2′-dimethyl-20′-(2″-trimethylammonioethyl)phosphanatoxyicosanamido)phenyl)porphinatoiron(II) (abbreviated 'lipid-heme, Scheme I) [7]. The lipid-heme had high compatibility with the bilayer of phospholipid and formed a very stable liposome (abbreviated 'lipid-heme liposome'). The lipid-heme liposome could bind molecular oxygen reversibly under physiological conditions [8]. In this paper, the structure of the lipid-heme liposome has been studied by physicochemical methods and compared with that of the natural phospholipid liposome.

Experimental

Materials

5,10,15,20-Tetra(α,α,α,α-*o*-(2′,2′-dimethyl-20′-

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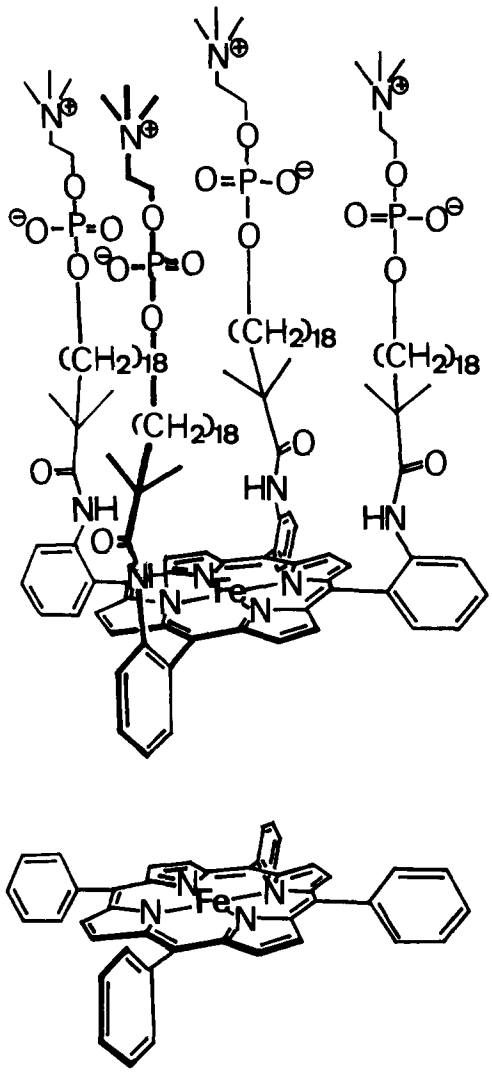


Scheme I. Above, lipid-heme; below, tpp-heme.

(2″-trimethylammonioethyl)phosphonatoxyicosanamido)phenyl)porphinatoiron(II) (lipid-heme) was prepared as in the literature [8,9]. 5,10,15,20-Tetra(α,α,α,α-phenyl)porphinatoiron(II) (tpp-heme, Scheme I) was also synthesized as in the literature [10]. Ligands, 1-laurylimidazole and 1-lauryl-2-methylimidazole, were synthesized as reported in Ref. 11. Egg yolk phosphatidylcholine (egg PC), L-α-1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), L-α-1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Sigma (special grade). Nitrosobenzene, as a bulky ligand to combine with the heme complex, and 5-carboxyfluorescein, as a fluorescent probe, were purchased from Aldrich and Eastman Kodak, respectively (special grade).

Preparation of lipid-heme liposome

The lipid-heme liposome was prepared as reported in Refs. 8 and 11. A thin film of the iron(III) derivative of lipid-heme (1 μmol) and phospholipid (50 μmol) with or without 1-laurylimidazole (3 μmol) or 1-lauryl-2-methylimidazole (20 μmol) was prepared on the glass wall of a large round flask. Oxygen-free phosphate buffer solution (pH 7, 20 ml) 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 lipid-heme liposome solution of iron(III) derivative thus prepared was reduced to the iron(II) derivative by addition of L-ascorbic acid (20-fold (mol) iron(III)). The red, transparent solution gave ultraviolet and visible absorption spectra with maxima at 426, 535 and 562 (shoulder) nm for the 1-laurylimidazole complex and 438, 535 (shoulder) and 562 nm for the 1-lauryl-2-methylimidazole complex, which could be assigned to the deoxy complexes (iron(II) state) of the lipid-heme [8,11].

Physicochemical measurements

Gel-permeation chromatography (GPC) was carried out with a Sepharose 4B column (Pharmacia Fine Chemical 2.2 cmϕ × 70 cm). The lipid-heme liposome was separated from the aqueous medium by ultracentrifugation (ultracentrifuge, Hitachi 65P-7). Transmission electron microscopy (transmission EM, Hitachi H-500) of the lipid-heme liposome was carried out by the negative stainning method using uranyl acetate (Tokyo-Kasei, special grade). Particle size distribution was measured with a modified dynamic light scattering method (Hiac/Royco, Nicomp Model 200). A thermogram to estimate the phase transition of the liposome was constructed using differential scanning calorimetry (DSC) (Seiko SSC-560). The phospholipid monolayer containing the lipid-heme was also evaluated by surface area–surface pressure curve measurement

(Shimadzu ST-1) [12,13]. The nitrosobenzene-binding reaction was measured using a stopped-flow spectrophotometer with a kinetic data processer (Unisoku SF-1000) [14,15]. Spontaneous leakage of the carboxyfluorescein encapsulated in the lipid-heme liposome was measured by monitoring the fluorescence spectrum of the carboxyfluorescein with fluorescence spectrophotometer (Hitachi HPF-4) [16].

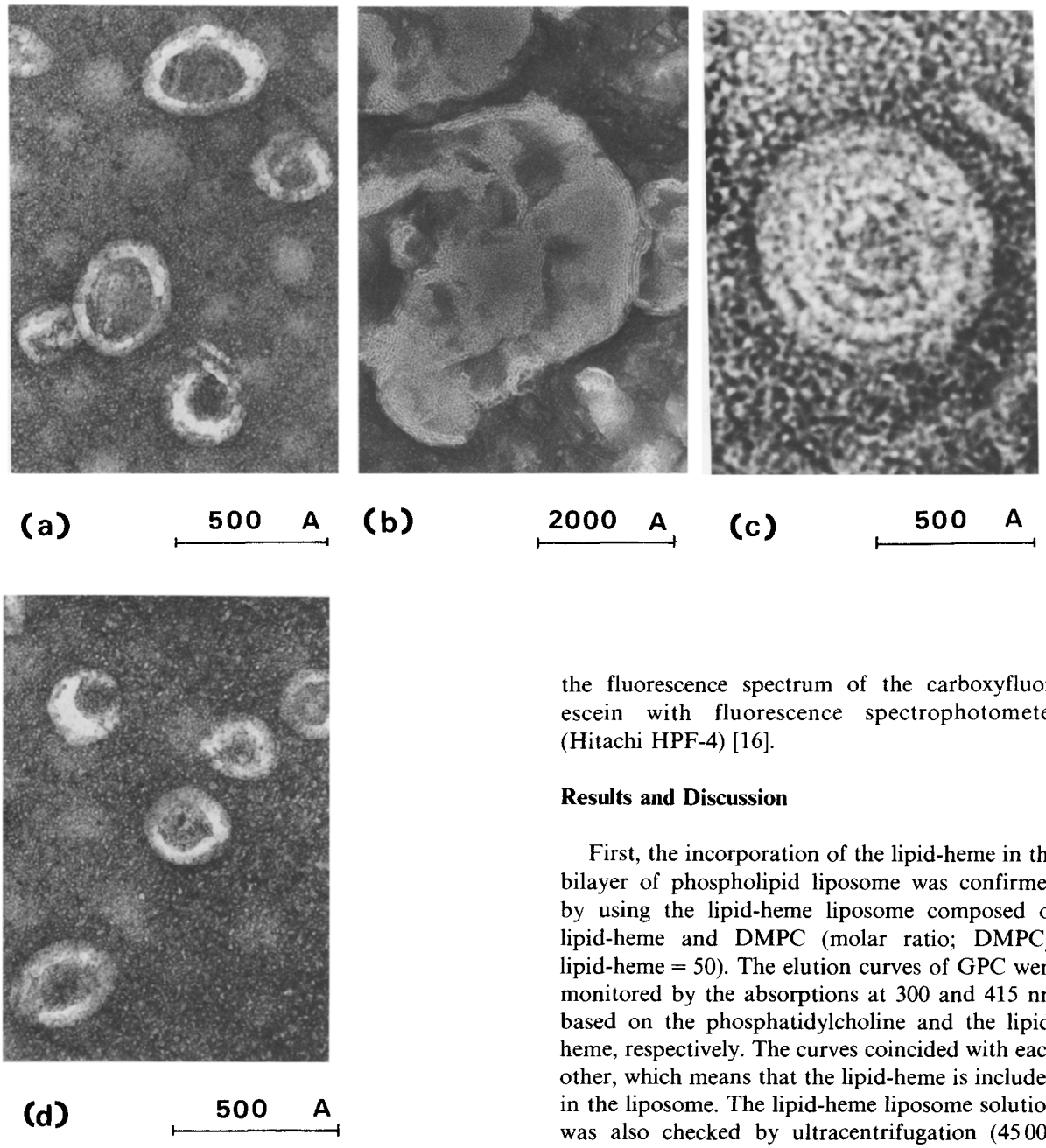


Fig. 1. Transmission electron micrographs of the lipid-heme liposome (a) and the tpp-heme liposome (b–d) stained by uranyl acetate. Molar ratios: (a) lipid/lipid-heme = 50; (b) lipid/tpp-heme = 50; (c) 100; (d) 200.

Results and Discussion

First, the incorporation of the lipid-heme in the bilayer of phospholipid liposome was confirmed by using the lipid-heme liposome composed of lipid-heme and DMPC (molar ratio; DMPC/lipid-heme = 50). The elution curves of GPC were monitored by the absorptions at 300 and 415 nm based on the phosphatidylcholine and the lipid-heme, respectively. The curves coincided with each other, which means that the lipid-heme is included in the liposome. The lipid-heme liposome solution was also checked by ultracentrifugation (45 000 rpm, 2 h, at 10°C). The solution remained transparent after ultracentrifugation and no precipitate containing the lipid-heme and/or the phospholipid developed, indicating that particles with a

diameter of more than 1000 Å did not exist. The average particle size of the lipid-heme liposome was also measured with a modified dynamic light scattering method; the most frequent diameter was determined to be 365 Å, with a sharp distribution pattern (±15 Å).

The structure of the lipid-heme liposomes with various compositions (molar ratio, lipid/lipid-heme = 50–200) was estimated by transmission electronmicroscopy. Every lipid-heme liposome looked like a small unilamellar vesicle or single-walled liposome (SUV) as shown in Fig. 1a. Lipid liposome forms SUV under this experimental condition. When the simple heme derivative, tpp-heme (Scheme I), was incorporated into the DMPC liposome (lipid/tpp-heme = 50), a plated and multi-lamellar liposome was observed (Fig. 1b). Decreasing the tpp-heme content, the structure of the tpp-heme liposome changed to a multi large lamellar vesicle or multi-walled liposome (MLV) for the molar ratio 100 and finally to SUV for the ratio 200 (Fig. 1c, d). The tpp-heme is not compatible with the phospholipid and causes structural distortion of the liposome. To avoid this destabilization, the liposome of tpp-heme and phospholipid tended to adopt the MLV or lamellar structure. On the other hand, the lipid-heme behaves like a phospholipid and gives only SUV.

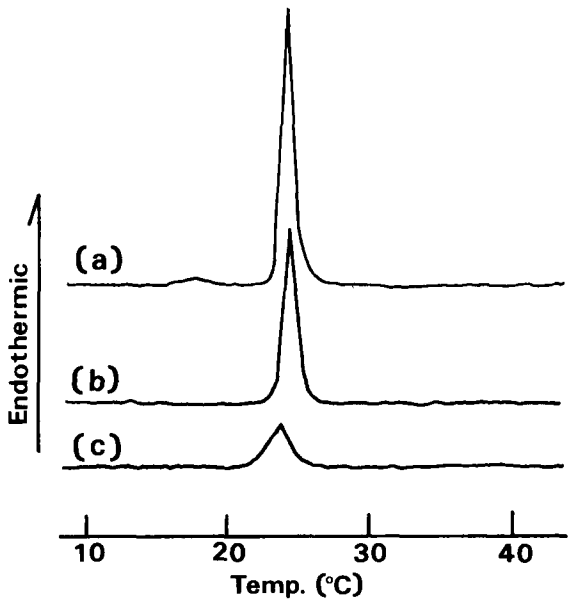


Fig. 2. Differential scanning calorimetric thermograms of the DMPC liposome (a), the lipid-heme/DMPC liposome (b) and the tpp-heme/DMPC liposome (c) at 1 Cdeg·min^{-1}.

The DSC thermogram (1 Cdeg·min^{-1}) of the lipid-heme liposome was taken to estimate the phase transition of the lipid-heme liposome (MLVs were prepared by incubating the corresponding SUVs and their thermograms were measured in order to enhance the phase-transition peaks) (Fig. 2). The DMPC liposome showed an endothermic peak at 24°C, which corresponded to the gel–liquid-crystal phase-transition temperature (T_c) of the liposome and agreed with that reported previously [11]. The peak for the lipid-heme/DMPC liposome was observed at the same temperature as for the DMPC liposome itself, 24°C. This suggests that compatibility of the lipid-heme with phospholipid is large enough to form a stable liposome. For the tpp-heme liposome, even with large lipid/tpp-heme ratio > 100, T_c and its phase transition endothermic peak were decreased and broadened, as shown in Fig. 2c. In contrast, the DSC thermogram of the lipid-heme liposome with a lipid/lipid-heme ratio of 50 showed a sharp endothermic peak and the T_c value corresponded to the lipid liposome itself (Fig. 2b). This suggests that the lipid-heme has a good compatibility with phospholipid and is packed well with phospholipid in the bilayer of liposome.

Table I shows T_c and enthalpy change, ΔH, for the gel–liquid-crystal phase transition. ΔH was scarcely influenced by the incorporation of the lipid-heme into the liposome. Upon increasing the degree of incorporation of lipid-heme, T_c remained constant ΔH decreased. By changing the structure of the lipid-heme complex (fourth (oxidized state), fifth (deoxy state), and sixth (carbon monoxide adduct) coordinated structure), the phase-transition profile was not affected. The phospholipid species also did not influence the phase-transition behavior of the lipid-heme liposome.

Fig. 3 shows the relationship of the lipid-heme content to T_c and ΔH. The T_c of the lipid-heme liposome of DMPC and DPPC were not influenced by the lipid-heme content. On the other hand, ΔH decreased with the content, demonstrating a break-point. When a small amount of the lipid-heme is incorporated in the liposome before the

TABLE I

T_c AND ΔH FOR THE PHASE TRANSITION OF THE LIPID-HEME LIPOSOME

Component of liposome (molar ratio)		T_c (°C)	ΔH (kcal/mol)
DMPC		23.7	6.47
DMPC/lipid-heme	(50)	23.8	5.06
	(20)	24.1	3.71
	(10)	24.1	2.93
	(5)	24.4	1.01
DMPC/lipid-heme/	(50 : 1 : 3)		
	(oxidized) [a]	23.7	2.87
1-laurylimidazole	(deoxy) [a]	23.7	2.67
	(CO adduct) [a]	23.8	3.60
tpp-heme/DMPC	(100)	22.0	2.39
	(50)	–	–

[a] Structure of the lipid-heme complex.

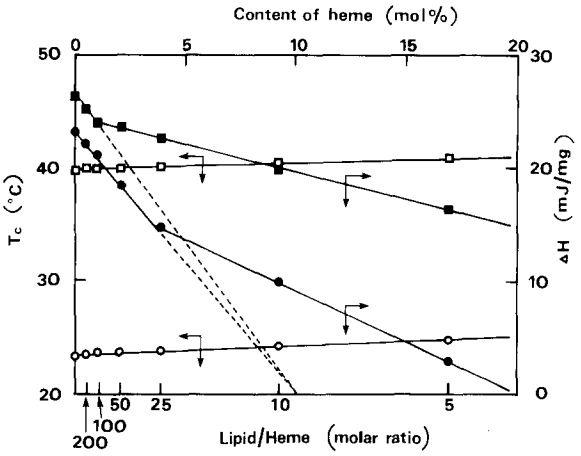


Fig. 3. Relationship of the gel-liquid phase transition parameters (T_c, ΔH) of the lipid-heme liposome to the content of the lipid-heme. Lipid-heme/DMPC liposome, ○; T_c, ●; ΔH, lipid-heme/DPPC liposome, □; T_c, ■; ΔH.

break-point, the lipid-heme is dispersed molecularly in the bilayer of DMPC- or DPPC-liposome. But where the lipid-heme content in the bilayer is above the concentration of the break-point, the lipid-hemes associates with each other to form a cluster in the bilayer. This consideration is supported by the following result in Fig. 3. Papahadjopoulos et al. [17] reported for their proteolipidapoprotein/DPPC membrane that the decrease in ΔH with increasing incorporated protein content is attributed to boundary lipid (the lipid molecule surrounding the guest molecule in the bilayer). In Fig. 3, the linear relationship at the smaller heme content is extrapolated to $\Delta H = 0$, giving a critical heme content of about 10 mol%. This means that the phase transition disappears and all the lipid of the lipid-heme becomes a boundary lipid when the liposome is formed with lipid-heme/lipid (1/9). This heme content indicates that there are about nine boundary-lipid molecules per one lipid-heme molecule. The boundary lipids around the lipid-heme agreed with those calculated by assuming structurally (CPK model) a base area of the lipid-heme of about 400 Å^2 (20 × 20 Å). The linear relationship at the higher heme content was extrapolated to $\Delta H = 0$, giving a second critical heme content of about 20 mol% for the DMPC system and about 40 mol% for the DPPC system. These values suggest that four lipid-heme molecules for the DMPC system and 36 lipid-heme molecules for the DPPC system form a cluster with each other. Anyhow, at the small incorporated content of lipid-heme the lipid-heme was dispersed molecularly in the lipid bilayer of the liposome.

The miscibility and compatibility of the lipid-heme with phospholipid were also confirmed from surface area-surface pressure isotherms of the lipid monolayer on a water surface (Fig. 4). The curve for the tpp-heme/DMPC monolayer shifted to right of the curve for the DMPC monolayer itself, which reveals the absence of effective packing of the lipid molecules in the monolayer. Against this, the curve for the lipid-heme/DMPC monolayer coincided with that for the DMPC monolayer itself; the lipid-heme has high compatibility with phospholipid and gives also a stable monolyer.

There is a question as to whether the lipid-heme is outward- or inward-facing in the liposome. The binding reaction of a bulky ligand such as nitrosobenzene to the lipid-heme liposome was measured with a stopped-flow method. The deoxy lipid-heme complex binds nitrosobenzene reversibly under an oxygen-free atmosphere. The nitrosobenzene binding-time–reaction curve was that of a monophase system for the lipid-heme liposome of SUV with a particle size of 400 Å, while the curve for the single-walled large lipo-

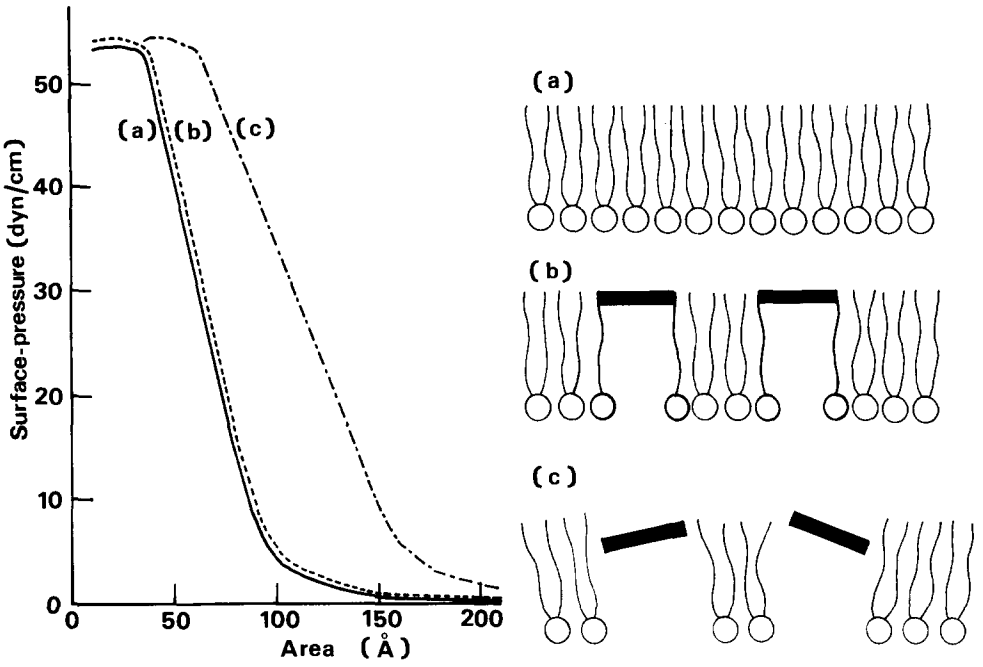


Fig. 4. Surface area-surface pressure curves of the DMPC- (a), the lipid-heme/DMPC- (b), and the tpp-heme/DMPC- (c) monolayers at 20°C. Molar ratios: (b) lipid/lipid-heme = 50; (c) lipid/tpp-heme = 200.

some (LUV) of about. 1000 Å was that of two-phase system (Fig. 5). The rapid reaction for the LUV liposome agreed with that for the SUV liposome, and the slower reaction corresponding to the ligand-binding where the bulky nitrosobenzene ligand at once crossed the bilayer and bound to the heme from the inner water phase (Fig. 5). It is considered that for SUV the lipid-heme exists preferentially facing outwards in the outer layer, because the SUV has a relatively high curvature and the lipid-heme has a somewhat cone-like structure (Scheme II). But for the LUV with a relatively small curvature, the lipid-heme is assumed to exist both in the inner and outer layers. By analyzing the two-phase reaction, the slower-reacting component, i.e., the lipid-heme fraction facing inwards, was estimated to be about 18 mol% for the LUV.

The closed vesicle structure and stability of the lipid-heme liposome was also studied by the following experiment (Fig. 6). A water-soluble fluorescent compound (carboxyfluorescein) was encapsulated into the inside water phase of the single-walled lipid-heme liposome. No fluorescence spectrum was observed because the encapsulated and concentrated fluorescence com-

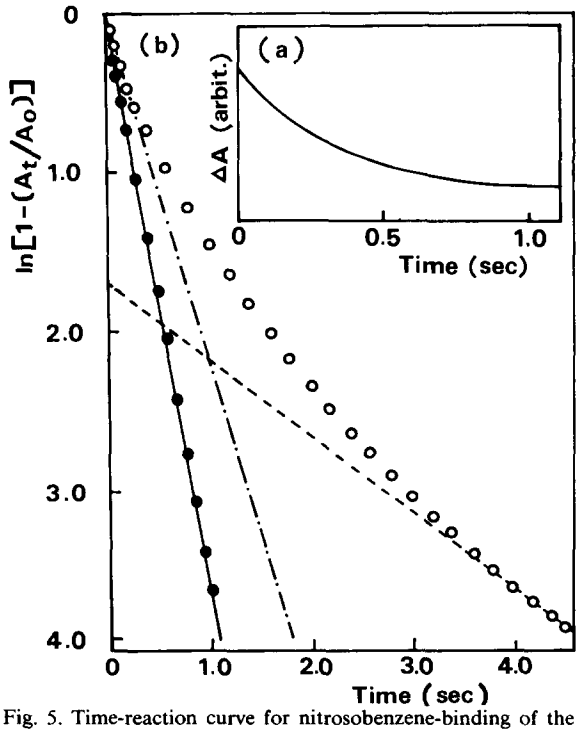
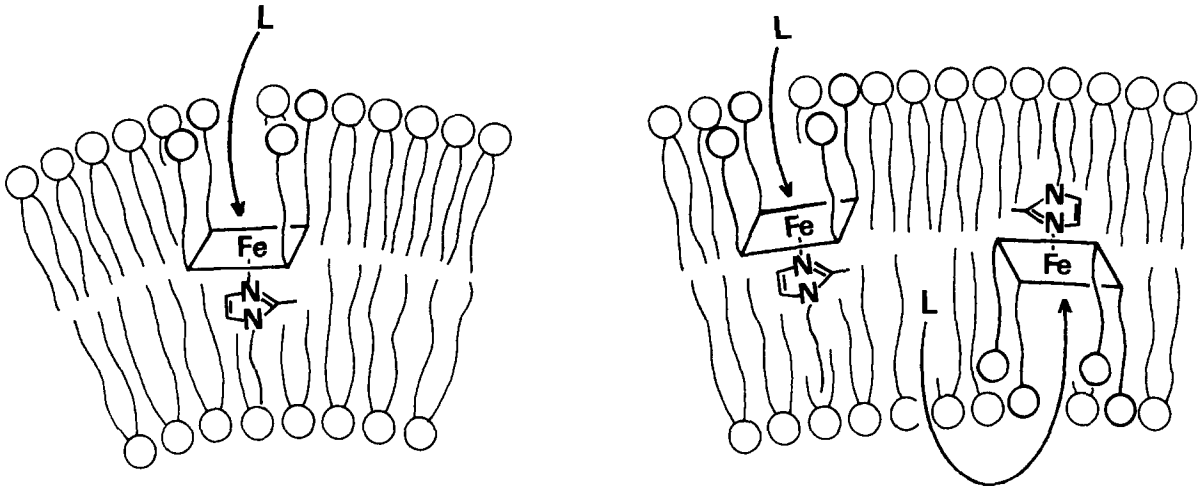


Fig. 5. Time-reaction curve for nitrosobenzene-binding of the lipid-heme liposome (a) and approximation to mono- and two-phase kinetics (b). ○; LUV, ●; SUV, lipid-heme concn. = $5.0 \cdot 10^{-5}$ M, molar ratio lipid/lipid-heme/1-lauryl-2-methylimidazole = 50/1/20, at 20°C. ΔA; differential absorbance, A_0 and A_t; differential absorbance at time 0 and t, respectively.



Scheme II. Left, SUV; right, LUV

pounds within the liposome quenched each other. This supports the closed vesicle structure of the lipid-heme liposome. The spontaneous leakage of the carboxyfluorescein across the bilayer occurred slowly. The rate of the leakage was much faster for the tpp-heme/egg PC liposome than for the lipid-heme/egg PC liposome. This means that the phospholipid forms a relatively stable liposome with lipid-heme. For the lipid-heme liposome with a lipid/lipid-heme molar ratio = 50 the leakage was suppressed to almost the rate of the egg PC liposome itself.

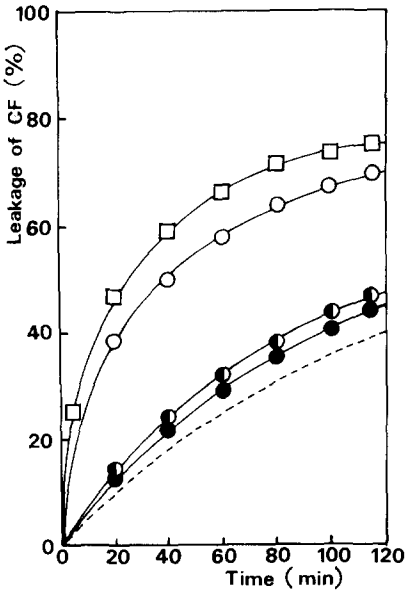


Fig. 6. Release of entrapped carboxyfluorescein from the lipid-heme liposomes. Control, egg-yolk phosphatidylcholine (egg PC). Lipid-heme/egg PC liposome, egg PC/lipid-heme = ○, 10; ◐, 20; ●, 50; tpp-heme/egg PC liposome, egg PC/tpp-heme = □, 50; –, control (egg PC liposome), at 50 °C.

It is concluded that the compatibility of the lipid-heme with phospholipid is large enough to form a stable liposome. The stereostructure and hydrophilic-hydrophobic balance of the lipid-heme are assumed to emphasize its compatibility with phospholipid.

The red and transparent solution of the deoxy lipid-heme liposome could bind molecular oxygen reversibly under physiological conditions (pH 7, 37 °C). The oxygen-binding of the lipid-heme liposome was very rapid (oxygen-binding rate, about 10^4 $M^{-1} \cdot s^{-1}$) and reversibly (oxy-deoxy cycle; more than 100-times). The oxygen-binding affinity was close to that of the red blood cell ($p_{1/2} = 25$ mmHg; red blood cell, $p_{1/2} = 27$ mmHg (37 °C)).

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