Construction and Function of Artificial Electron Channel in Bilayer Membrane

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Preface

The study presented in this thesis has been carried out under the direction of Professor Iwao Tabushi during April, 1983-March, 1987 and Professor Teruo Mastuura during April, 1987-March, 1988, at the Department of Synthetic Chemistry at Kyoto University.

In this thesis the author states the synthesis of artificial electron channel across the membrane and the sophisticated functionalization of artificial liposome systems. He is very happy that the present study made some contributions to a biomimetic chemistry including a membrane mimetic chemistry.

The author regrets that Professor Iwao Tabushi suddenly deceased on March 22, 1987. The author was always surprised by Tabushi's excellent insights into chemistry and prays for the repose of his soul. He wishes to express his supreme gratitude to the late Professor Iwao Tabushi for his kindest guidance, valuable suggestions and warm encouragement.

He also wishes to express his sincerest gratitude to Professor Teruo Mastuura for his leading and heartening advice, in succession to the direction of the late Professor Iwao Tabushi.

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The author also wishes to express much apprecation to Professor Thomas J. Meyer at the Department of Chemistry, University of North Carolina at Chapel Hill, for his giving the author an excellent opportunity to study with his group.

Finally, the author is grateful to his fiancee, Miss Yuumi Murakami for her typing this thesis and warm encouragment and his family for their constant financial support and deep affection.

March, 1988. Itaru Hamachi.

CONTENTS

General Introduction.

Biological Electron Transfer across a Biomembrane.

Electron transfer across biomembranes such as in respiration and photosynthesis, is one of the essential phenomena to keep the life of living organisms [1] . As the reaction field of electron transfer, biomembranes are composed of proteins, lipids and carbohydrates (glycolipids and glycoproteins). Based on many experimental data by X-ray diffraction, electronic micrography, Raman and IR spectroscopies, light scattering measurements and differential scanning calorimetry, it is now generally established that lipids in a biomembrane have a bilayer configuration and that its configuration is essential to maintain the membrane structure and its functions. The membrane-bound proteins are either located on the surface of the bilayer (extrinsic) or incorporated to various degrees into the lipid matrix (intrinsic) and both proteins play important roles in the electron transfer across the biomembrane.

In the biomembranes which tranport electrons, four major groups of components are responsible for the electron transport reactions [2] . These are (i) metallo-porphyrins which include chlorophylls and cytochromes, (ii) iron-sulfur clusters composed of none-heme iron and sulfur, (iii) quinones and (iv) flavins. In each chemical group, various degrees of chemical perturbations on the main structures exist and provide different

characters in the redox potentials, the mobility in membrane proteins and so on.

After the very short initial stage of the charge separation in the photosynthesis, the electron transfer system organized in the order of the redox potentials of its components is operated at the thylakoid membrane. This electron transfer system is similar, in principle, to that in the mitochondrial membrane of respiration. The respiration system uses a linear chain of redox components to transport electrons from reduced substrates (R-SH, H_2, NADH, etc.) to oxygen, while the photosynthetic reaction center provides both reducing and oxidizing potential arrays (electron and positive hole) for a series of redox components.

In the bioenergetics of oxidative- or photophosphorylation, the facilitated flow of electrons through the redox components across the biomembrane leads to the influx of protons into the cell interior from the external aqueous phase in the inner membrane of mitochondria or the cell membrane of bacteria or to the reversed efflux in the case of chloroplost. This electron flow produces a transmembrane potential. Together with the pH gradient (resulting from proton flux), the membrane potential produces the electrochemical proton gradient ($\triangle \mu H^+$) across the biomembrane. According to chemiosmotic theory by P. Mitchell [3], the electrochemical potential difference across the biomembrane thus produced can drive H^+ through the ATP-ase (proton-translocating ATP-ase) to synthesize ATP.

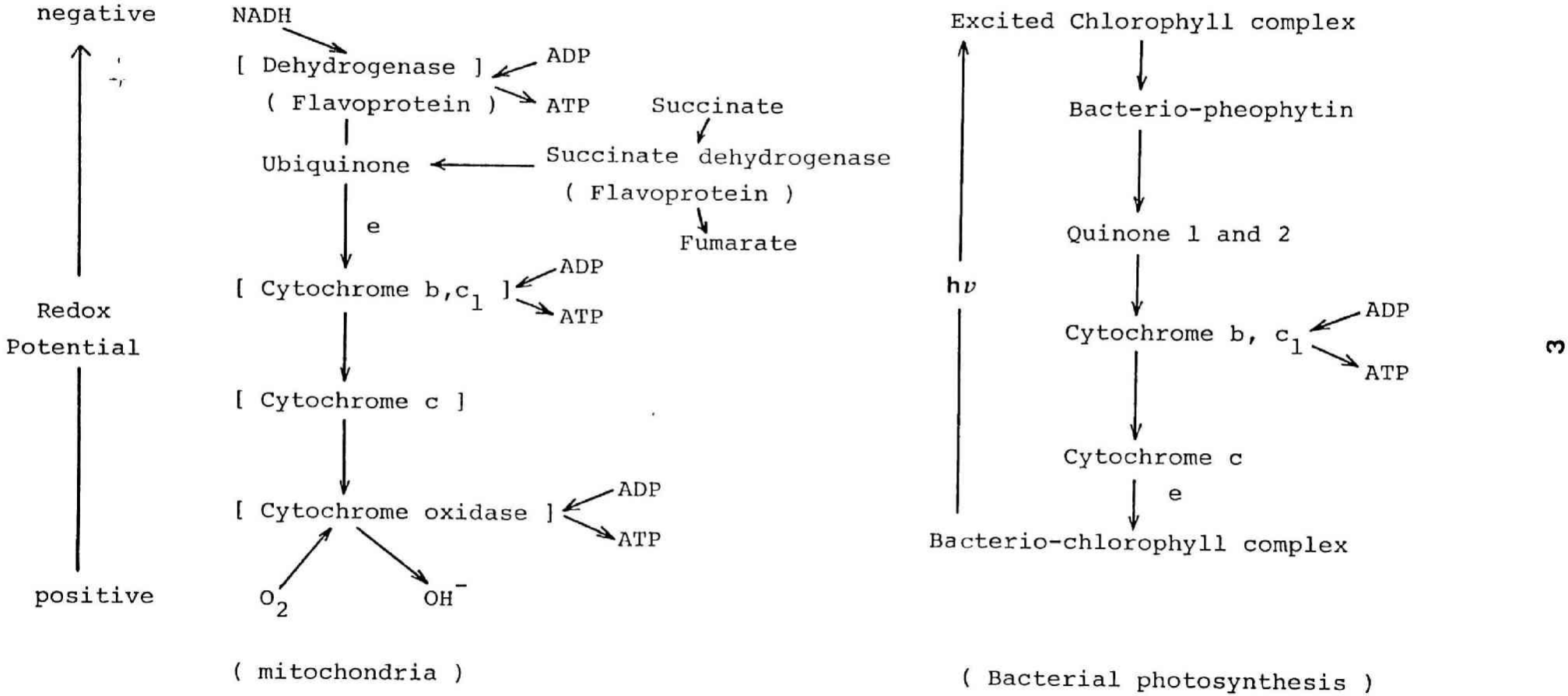


Fig. 1 Biological Electron Transfer System

Flavoprotein as Electron Transferase.

Flavoprotein is an important electron transducing enzyme in many metabolic systems, as amino acid oxidase, NADH dehydrogenase, NADPH cytochrome reductase and others where the flavin unit accepts electrons from various reducing substrates (NAD(P)H, aminoacid, R-SH etc.) and transfer them to the next acceptor, such as quinones, heme proteins and iron-sulfur clusters [4]. Here, the flavin unit can function not only as an efficient one and two electron transfer catalyst, but sometimes as a hydrogen transfer catalyst because half-reduced and fully reduced flavins have pKa's close to the physiological pH value [5].

As an another important electron-transfer flavoprotein, flavodoxin is known to be capable of replacing ferredoxin [6]. Flavodoxin which is known to transfer electrons in a one-electron step, utilizes its large negative redox potential (-400mV vs NHE) in the biological electron transfer systems [7].

Electron Transfer in Protein and Protein Complex.

Focused on the electron transfer through proteins and protein complexes, a complex between yeast cytochrome c peroxidase (ccp) and cytochrome c (cyt-c) was used by Hoffman and coworkers [8] to show a long-range electron transfer between heme centers at a fixed distance. In this complex, the spatial configuration of two proteins is well defined by X-ray

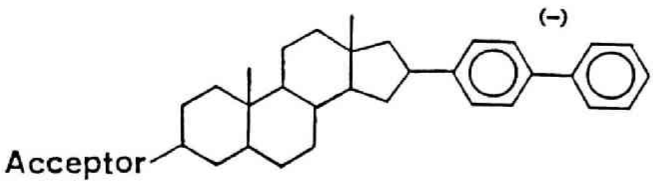
Table 1. Long-range electron transfer in proteins and proteins complexes.

Donor/Acceeptor	k (sec^{-1})	R (Å)
ccp (Fe^{2+}) /cyt-c (Fe^{3+})	0.3 ± 0.1	17
a_5 Ru/cyt-c (Fe^{3+})	30 ± 3	12
Zn-cyt-c/cyt-b_5 (Fe^{3+})	5 ± 1 × 10^5	8
Zn-Hb/cyt-b_5 (Fe^{3+})	8 ± 2 × 10^3	10
Zn-ccp/cyt-c (Fe^{3+})	138 ± 12	17
Zn / Hb (Fe^{3+})	100 ± 10	20
cyt-b_5 (Fe^{2+}) /cyt-c (Fe^{3+})	1.6 ± 0.7 × 10^3	8

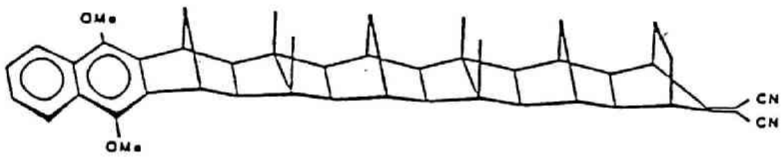
diffractions and the two heme plane are parallel and separated by 17～18Å (edge to edge distance). Gray and Isied modified cytochrome c and myoglobin (Mb) with ruthenium pentamine (a_5Ru) in order to investigate the long-range electron transfer through the protein interior [9]. The electron was found to be transferred over a long distance (12 Å) through the interior of cytochrome c from the ruthenated cyt-c (a_5Ru/cyt-c(Fe^{3+})) with a rate of 30 sec^{-1}. McLendon have also measured the rates of long-range electron transfer in zinc-hemoglobin and cyt-b_5 complex (Zn-Hb/cyt-b_5(Fe^{3+})) and Zinc-cytchrome c and cyt-b_5 complex (Zn-cyt-c/cyt-b_5(Fe^{3+})), using the excited triplet zinc-porphyrin having a strong reducing power [10] (See Table 1). Electron transfer processes across the separated donor and acceptor pair in protein matrices are going to be unveiled gradually.

Electron Transfer in the Model Systems.

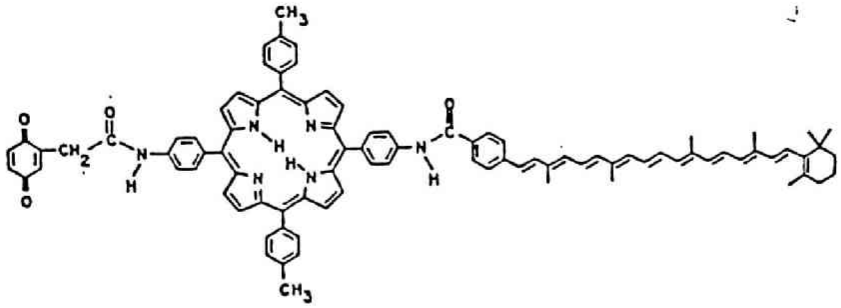
Among many synthetic approaches to long-range electron transfer, Closs and Miller prepared donor-acceptor molecules with a rigid steroid frame [11]. They observed the electron transfer from a biphenyl-radical anion to various organic acceptors separated by ～ 10 Å. This report is the first experimental demonstration of the inverted region of Marcus theory [12].



Paddon-Row and Hush, who made a series of donor-acceptor molecules separated by a rigid bicyclic bridge of a certain distance, showed an electron jumping from the donor, dimethoxynaphthalene, to the acceptor, dicyanoethylene over a distance of almost 15 Å [13].



The sophisticated donor-acceptor molecules which are composed of three components, carotene, porphyrin and quinone, were synthesized by Moore, Gust and coworkers [14]. The triad molecules by Moore et al. produced a photo-induced charge separation state with a long lifetime of microsecond order. Further interestingly, such a molecule was incorporated into a planer bilayer membrane and generated a transmembrane potential induced by light ($h\nu$).



Electron Transfer across a Bilayer Membrane.

In order to model a very fast electron transport across a biological membrane, a remarkable enhancement of the overall interphasical rate is desirable. This may be achieved by (i) an appropriate functionalization of the membrane and (ii) an increase of the surface area. In these viewpoints, lipid bilayer liposomes (or visicles) are satisfactory models of the biomembrane. Artificial liposomes prepared from a lipid show only very poor electron-transporting properties and appropriate modifications are necessary to facilitate the electron transport across the liposomal membrane. There seem conceivable two types of functionalizations : (a) carrier type and (b) channel type.

Hinkle modified a phospholipid liposome with an electron carrier such as benzoquinone and ferrocene to show the facilitated electron transport from the external ascorbate to the internal ferricyanide [15]. Tabushi used a series of alkylviologens, which are typical electron carriers across a membrane,and showed that the hydrophobicity (alkyl chain length)

strongly affects the electron transport rate across the liposomal bilayer [16]. Hauska investigated the electron transporting capacities of ubiquinones and plastoquinone which are naturally present in the electron transport chains of mitochondria and chloroplast, respectively [17]. Ubiquinones with a side chain of more than three isoprene units were far more efficient electron mediators than their analogues with a shorter-chain and such isoprenoid quinones were suggested to form separatly a quinone domains in the bilayer membrane.

In contrast with electron carriers, there are few successful examples on the construction of artificial electron channels. Tabushi measured a fast electron transport rate through a channel consisted of two cytochrome-c_3 molecules across a liposomal membrane [18]. Loach and coworkers prepared a membrane-bound polyimino-linked porphyrin and claimed the occurrence of an electron transport via a porphyrin channel [19]. Lehn synthesized a so-called molecular wire, caroviologen, bound in a liposome but no electron transport is reported so far [20]. In spite of the significance of electron channel in biological systems, detailed mechanisms seem still ambiguous due to limited informations on the localization of electron mediators, intermediater distances, the transmembrane diffusion rate of mediators and so on.

Abstract of Thesis.

In order to get a further molecular understanding of the transmembrane electron transport, the author prepared a new flavolipid which has a flavin unit as a redox center bound covalently to the vicinity of the polar head group of phospholipid (Chapter 1). The flavolipid was shown to be incorporated stably into a artificial liposome with a depth of 7 $\pm$ 1 Å. The flip-flop movement of the flavolipid seems suppressed to a similar order to the normal phospholipids. Detailed kinetics demonstrated that electrons were transferred via a transient channel consisted of two flavolipids (Chapter 2). Furthermore, the control of electron transport rate was demonstrated by the thermally induced phase transition of the dipalmitoylphosphatidylcholine liposome functionalized with the flavolipid (Chapter 3). In chapter 4, the author has shown that the present flavolipid accepted electrons from a hydrophilic NADH model, H_2, or dithiothreitol and transported them to Fe^{3+} across the bilayer membrane. Chapter 5 described that the present electron transport by the flavolipid was coupled to the proton transport to generate a pH gradient large enough to synthesize ATP by ATP-ase. New bis-flavosurfactants which were bound stably to a lipid membrane, were synthesized and gave a faster electron transport rate than the flavolipid half-channel did (Chapter 6). In addition, the oxidation of NADH by flavolipid was remarkably accelerated in ammonium (cationic)

bilayer, compared with that in phosphatidylcholine (zwitterionic) bilayer. It was demonstrated that the microenvironment in the vicinity of flavin determines the oxidation rate of NADH (Chapter 7).

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Chapter 1

Synthesis of Artificial Flavolipid and its Stable Incorporation into Artificial Liposome.

Abstract

An artificial flavolipid which has a flavin unit in the vicinity of the phosphatidylcholine moiety was newly synthesized as a model for membrane-bound flavoproteins and was proved to be incorporated stably into the bilayer membrane of the lecithin liposome by the gel permeation behavior and electronic spectra of the flavolipid in the liposome. The artificial liposome modified with this flavolipid exhibited stability similar to the egg lecithin liposome and a single-walled, bilayer liposome structure of ca. 360 Å in diameter based on dynamic light scattering and electron micrograph measurements. The present flavoliposome showed one of the most efficient electron transport rates (k_{obs} = 0.45 sec^{-1}) from the exterior dithionite to the interior ferricyanide across the bilayer membrane modified with the flavolipid.

Introduction

Membrane-bound flavoproteins are considered to play key roles in biological redox systems, such as respiratory chain of mitochondria [1], photosynthesis of thylakoid membrance [2], bioluminescence of luciferins [3] and many oxidation reduction reactions [4]. Especially important is the electron transport across the membrane aided by flavoproteins which catalyze the electron transfer from various electron donors (NADH, RSH, amino acid, etc.) to a flavin and further to the electron acceptor.

In order to understand the complicated and sophisticated function of the membrane-bound flavin in multienzyme systems, an appropriate simplified model system is necessary and important. The bilayer membranes of artificial liposomes are well known to provide an appropriate model of biomembranes. In this view point, a tightly bound flavin to a liposomal bilayer seems to be one of the best models for the membrane-bound flavoproteins.

In this chapter the author wishes to report the successful synthesis of the first member of the artificial flavolipid family, and its incorporation into the liposomal bilayer and electron transporting capacity.

Results and Discussion

Preparation and Structure of Flavolipid

As a reversible redox active site, we have chosen a flavin unit [5] which is incorporated into the bilayer membrane and facilitates the electron transfer across the membrane. The synthesis of the flavolipid (6) was outlined in Scheme 1. [6] [3-(10-Decyl-isoalloxazinyl)] acetic acid (4) was converted to its anhydride using ethyl chloroformate followed by

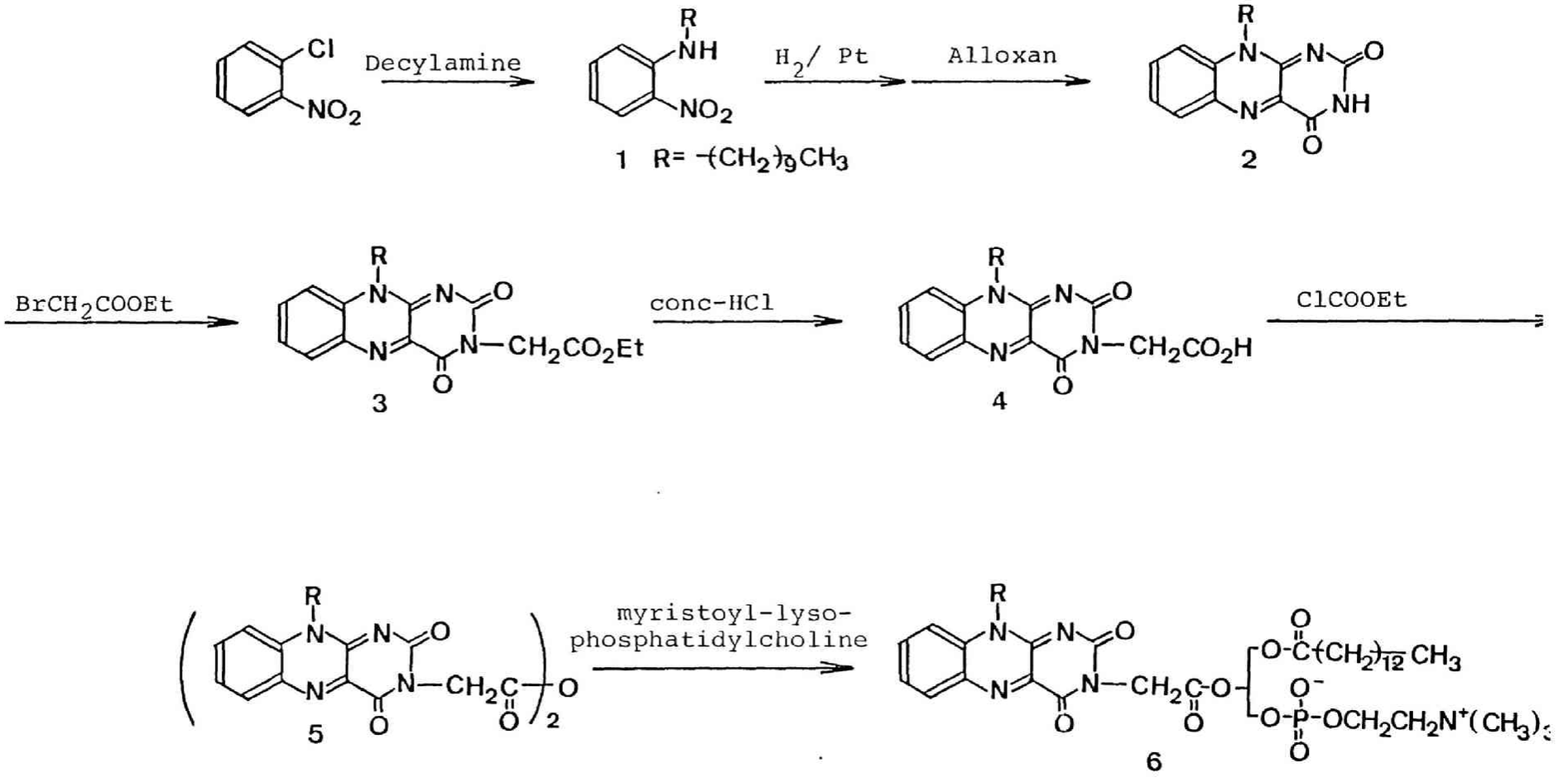
insert Scheme 1 here

esterification with myristoyl-L-α-lysophosphatidylcholine in the presence of 4-(N,N-dimethylamino)pyridine to produce 6. [7] The flavolipid (6) thus obtained has following characteristics:

(i) The flavolipid has a phosphatidylcholine moiety as a polar head group which should fit the membrane surface with minimal perturbation of the surface properties of the bilayer membrane of egg lecithin having the identical head group.

(ii) The flavin chromophore is bound covalently to the vicinity of the polar head group as a redox center in the membrane. The depth of the flavin unit from the interface was estimated as 7 ± 1 Å [8] judging from a CPK model, when extended into the membrane phase. This close distance from the interface is expected to function as an

Scheme 1


Cl
NO2
Decylamine
R
NH
NO2
1 R= -(CH2)9CH3
H2/ Pt
Alloxan
N
O
NH
2
BrCH2COOEt
N—CH2CO2Et
3
conc-HCl
N—CH2CO2H
4
ClCOOEt
5
myristoyl-lyso-
phosphatidylcholine
O-C-(CH2)12 CH3
O-P-OCH2CH2N+(CH3)3
6

efficient redox center to accept or release electrons from a reductant or to an oxidant, respectively, in the aqueous phase. (iii) The flavin part is attached additionally by a long alkylchain and the second acyl part of the lipid consists of a hydrophobic myristoyl residue. These two hydrophobic chains may serve to exhibit better affinity to the phospholipid bilayer and to stabilize the bilayer structure of the liposome.

insert Table I here

As shown in Table I, the electronic spectra of flavin chromphore are sensitive to the solvent used. Comparison of the electronic spectra of 10-pentyl-isoalloxazine in various solvents (H_2O, 0.25 M-Tris HCl, methanol,and chloroform) indicate that the absorption maxima of the flavolipid incorporated into the egg lecithin liposome appear at the almost identical positions to those in chloroform. In contrast, the absorptions appear at different positions in pure water or Tris buffer. Thus the flavin moiety should neither be located at bulk aqueous phase nor at the bilayer interface, but be incorporated into the membrane phase. The solvent shift of the fluorescence spectra of the flavin chromophore shows a trend similar to that of the electronic spectra.

Table I

Solvent Dependence of Electronic and Fluorescence Spectra of the Flavin Chromophore[a)]

Flavin	Medium	Electronic Spectrum (nm)	Fluorescence Spectrum[b)] (nm)
10-Pentyl-isoalloxazine	H_2O	347	433	512
	0.25M-Tris HCl[c)]	347	433	512
	MeOH	333	433	507
	$CHCl_3$	336	442, 415(s)[d)], 470(s)	498
1	liposome[c)]	336	444, 418(s), 472(s)	507

a) at 25°C, b) exitation = 340 nm, c) pH= 7.0, d) s means a shoulder

Structure and Stability of Artificial Liposomes Functionalized with Flavolipid.

After purification by centrifugation followed by gel filtration, artificial liposomes modified with flavolipid ($^{(o)}$ |fl-Lip| $^{(i)}$) [#] showed absorption maxima at 336 and 444 nm with shoulders at 418 and 472 nm. All of these absorptions are characteristic of the flavin chromophore. Artificial liposomes modified with the flavolipid incorporating ferricyanide in its interior aqueous phase showed absorptions at 304 and 425 nm, characteristic of ferricyanide, and 336 nm and 472 nm (shoulder), characteristic of the flavin chromophore, and diffraction at 215 nm, characteristic of the liposome. The characteristic absorption at 425 nm of ferricyanide was somewhat overshadowed by the tail from the absorption of the flavolipid at 444 nm. The quantitative determination of the concentration of both species was successfully achieved from the absorption at 425 nm with a help of the absorption at 472 nm which arises solely from the flavolipid.

insert Figure 1 here

[#] (o) : the outer aqueous phase

(i) : the inner aqueous phase

| | : membrane phase

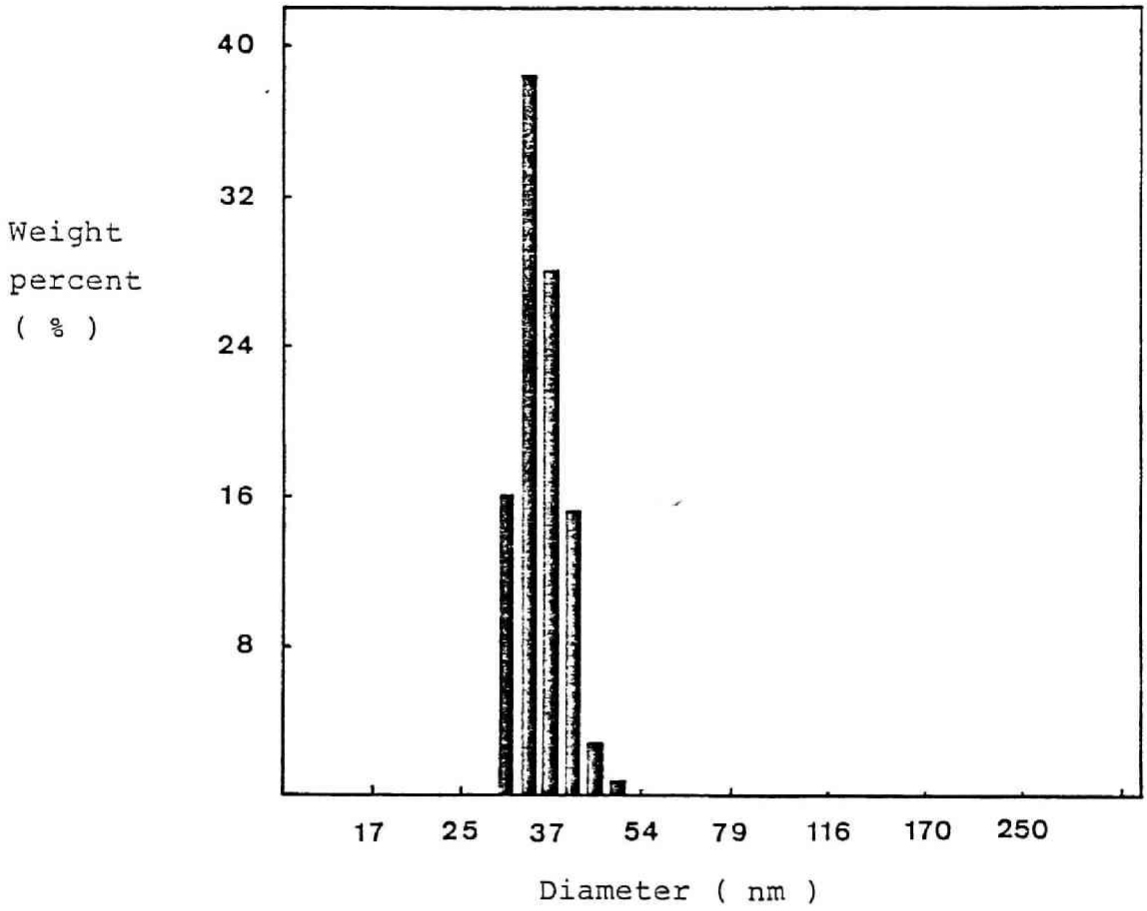


Fig. 1 Dynamic Light Scattering of the Artificial Liposome Modified with Flavolipid.
(3 mole % flavolipid, at pH 7.0, 25°C.)

Dynamic light sattering study of the purified liposomes ($^{(o)}$ |fl-Lip|$^{(i)}$, $^{(o)}$|fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$) indicated that the diameter of liposome particles was distributed from 300 Å to 500 Å with a mean value at ca 360 Å. The electron micrograph of the flavoliposome ($^{(o)}$ |fl-Lip| $^{(i)}$) demonstrated a closed, single compartment bimolecular liposomal structure with a diameter of ca 400 Å and a membrane thickness of ca 40 - 50 Å. Artificial liposomes modified with the flavolipid ($^{(o)}$ |fl-Lip | $^{(i)}$, $^{(o)}$ |fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$) showed a gel permeation behavior similar to the unmodified artificial liposomes ($^{(o)}$ |Lip| $^{(i)}$ $K_3Fe(CN)_6$), demonstrating that the flavolipid did not perturb significantly their structure and size. The artificial liposomes modified with the flavolipid by less than 5 mol % did not produce precipitates on standing 7 days after the preparation. Furthermore, the diameter distribution of the liposome modified with flavolipid showed no change for 7 days by a dynamic light scattering study. The flavolipid can be incorporated into the bilayer membrane in as large as 50 mol % quantity, being stable for at least 4 days. These results demonstrate that the present flavolipid shows a high affinity to the phospholipid.

Electron Transporting capacity of the Artificial Flavolipid

Electron transport rate from the exterior $Na_2S_2O_4$ to the interior $K_3Fe(CN)_6$ can be estimated by following the decrease of

Table Ⅱ. Reduction rate constant of Interior $K_3Fe(CN)_6$

Flavolipid (mM)	interior $K_3Fe(CN)_6$ (mM)	bulk aq $Na_2S_2O_4$ (mM)	k_{obs} (sec^{-1})
0.07	0.41	3.0	0.44 ± 0.02
0.07	0.41	7.2	0.45 ± 0.02
0.07	0.41	11.2	0.49 ± 0.02
0.07	0.41	13.4	0.52 ± 0.03

a) at 25 c, pH 7. b) Flavolipid/egg lecithin = 4/96

c) analytical concentration. interior concentration of $K_3Fe(CN)_6$ = 0.75 M.

the concentration of the interior $K_3Fe(CN)_6$ monitored by electronic absorption. The observed decrease of the ferricyanide concentration followed pseudo-first-order rate equation over the range of the dithionite concentration of 3.0 - 13.4 mM;

$$-d[Fe(CN)_6^{3-}] / dt = k_{obs} [Fe(CN)_6^{3-}]$$

The pseudo-first-order rate constants thus obtained were listed in Table Ⅱ. Interestingly, the observed first order rate constants were practically independent of the exterior dithionite concentration. The present electron transport rate constants are compared with those reported in the literatures (Table Ⅲ). The pseudo first order rates were listed at

insert Table Ⅲ here

different mediator concentrations. The direct comparison is difficult because the rates were measured at different concentrations. Although the concentration dependences are not exactly obtained in all cases, k_{obs} / [mediator] values were compared in order to obtain a rough measure. As shown in Table Ⅲ, the apparent electron transport rate for the flavolipid is much faster than those observed in any liposomes modified with ferrocene, [11] benzoquinone [11], and N,N'-di-n-butylviologen

Table III Electron Transport Rate across the Liposomal Membrane Modified with Various Electron Mediator

electron donor	electron acceptor	mediator[a]		k (sec^{-1})	k/[mediator] ($sec^{-1}\ M^{-1}$)	ref.
ascorbate	FeCl	chloroplast-pigment	[b]	0.007		9)
$Na_2S_2O_4$	$K_3Fe(CN)_6$	UQ_{10}	19.5	1.277	650×10^2	10)
		PQ	19.5	0.293	150×10^2	10)
		UQ_1	25	0.028	11×10^2	10)
ascorbate	$K_3Fe(CN)_6$	Ferrocence/Fccp.	20	0.013	6.5×10^2	11)
		benzoquinone	20	0.013	6.5×10^2	11)
$Na_2S_2O_4$	$K_3Fe(CN)_6$	C_4V^{2+}	4	0.0023	5.8×10^2	13)
		cyt-c	8.7	0.0075	8.6×10^2	13)
		cyt-c_3	10.8	0.230	210×10^2	13)
$Na_2S_2O_4$	$K_3Fe(CN)_6$	flavolipid	70	0.45	64×10^2	c)

a) abbreviations; UQ_{10}, ubiquinone with ten isoprene units, UQ_1, ubiquinone with one isoprene unit, PQ, platoquinone, FCCP, carbonylcyanide p-trifluoreomethoxyphenyl hydrazone, C_4V^{2+}, N,N'-di-n-butylviologen.

b) There is no description of the mediator concentration.

c) This work

(C_4V^{2+}),[12] which is the most efficient electron carrier of all alkyl viologens reported previously, or cytochrome-c which plays a key role in natural electron transfer systems.[13] The liposomes modified with ubiquinone-10, plastoquinone and cytochrome-c_3 [12] exhibited the fastest transmembrane electron transfer. All of the latter class of mediators are important oxido-reductive molecules in the respiratory chain of the mitochondria or the photosynthetic electron transfer of chloroplast. The present artificial flavolipid is almost equivalent to those natural systems as the fastest member of electron transporting media ers across the bilayer membrane. The comparison of electron transport rates by the change of reductants will be discussed in chapter 2.

Experimental Section

Instruments.

^{1}H-MNR spectra were obtained with a JEOL JNM PMX 60SI NMR spectrometer, a JEOL JNM FX90Q ET NMR spectrometer or a JEOL JNM GX400 spectrometer. Chemical shifts are given in δ values from Me_4Si. Mass spectra were obtained with a JEOL JMS-DX 300 mass spectrometer. IR spectra were recorded on a Hitachi model 260-50 spectrophotometer. Microanalyses were performed at Microanalytical Center of Kyoto University. Electronic absorption spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophotometer thermostated at 25.0 ± 0.1 ℃ with a circulation system, Type Handy Cooler TRL-108. Sonication was performed with an ultrasonic disruptor, Mode UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20III. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument. Dynamic light scattering studies were performed with a Otsuka Electronics DLS-700 spectrophotometer.

Materials.

Unless stated otherwise, all reagents and chemicals were obtained commercially and used without further purification. Egg yolk lecithin was carefully purified according to the literature method [14] and stored at -75 ℃ under Ar in the dark.

Dry dimethylformamide was obtained by stirring on BaO at room temperature overnight, followed by distillation under reduced pressure under Ar. Tetrahydrofuran was purified by refluxing first over Na for 12 h then on $LiAlH_4$ for 1 h, followed by distillation under N_2. Dry triethylamine was obtained by stirring over KOH overnight and refluxing with CaH_2 for 5 h, followed by distillation under N_2.

Preparation

(i) N-n-Decyl-2-nitroaniline (1).

In a 100 mL round-bottomed flask were placed 27.5 g (0.18 mol) of 1-chloro-2-nitrobenzene, 25 mL (0.13 mol) of n-decylamine and 15.2 g (0.19 mol) of anhydrous sodiun acetate and the mixture was stirred at 110 ℃ for 24 h. After cooling to room temperature, 40 mL of H_2O and 60 mL of diethylether were added to the orange-colored reaction mixture and ether layer was separated. The aqueous layer was extracted with two portions of 60 mL ether and the combined ether extracts were dried over anhydrous $MgSO_4$. Ether was distilled off and then excess 1-chloro-2-nitrobenzene was removed by distillation under reduced pressure (68 - 72 ℃/1 mmHg). Further distillation yielded 25.5 g (73 %) of N-n-decyl-2-nitro-aniline at 145 - 150 ℃ (1 mmHg). ^{1}H-NMR ($CDCl_3$), δ : 0.73 -1.05 (t-like, 3H, CH_3), 1.05 -1.90 (m,16H, $-(CH_2)_{-8}$), 3.27 (q-like, 2H, $-CH_2NH-$), 6.82 (dd, 1H, H_6, J_{65} = 8 Hz, J_{64} = 2 Hz), 6.58 (ddd, 1H, H_4, $J_{43} = J_{45}$ = 8 Hz,

J_{46} = 2 Hz), 7.41 (ddd, 1H, H_5, $J_{54} = J_{56}$ = 8 Hz, J_{53} = 2 Hz), 7.86 - 8.23 (broad-s, 1H, -NH), 8.13 (dd, 1H, H_3, J_{34} = 8 Hz, J_{35} = 2 Hz).

(ii) 10-n-Decyl-isoalloxazine (2) [6].

N-n-decyl-2-nitroaniline (13.9 g , 49.9 mmol) was dissolved in 80 mL of absolute ethanol and to this solution was added 0.15 g of platinum oxide. The catalytic reduction was conducted at room temperature at normal H_2 pressure in the dark with vigorous stirring for 10 h. When 3.6 L (0.15 mol) of hydrogen gas was absorbed at room temperature, 40 mL of 1N-HCl was added to the reaction mixture. Platinum powder was filtered off and the filtrate was placed in a 200 mL round-bottomed flask, to which was added 9.2 g (57.5 mmol) of alloxan monohydrate, and the mixture was refluxed in the dark for 15 min. After cooling to 0 ℃, brown precipitates formed were filtered and washed with 5 mL of cold ethanol to give 5.7 g of a yellow-green solid. The filtrate was concentrated to 30 mL to give 2.3 g of the yellow-green second crop. Total yeild of crude 10-n-decyl-isoalloxazine amounted to 49 %. The analyticlal sample was recrystallized from ethanol. ^{1}H-NMR (DMSO-d_6), δ : 0.73 - 1.03 (t-like, 3H, CH_3), 1.33 -1.96 (m, 16H, $-(CH_2)_{-8}$), 4.40 -4.76 (t-like, 2H, $-CH_2N$), 7.46 - 8.26 (m, 4H, isoalloxazine ring). IR(KBr) : ν_{NH} = 3580, 3540 cm $^{-1}$, ν_{CO} = 1720, 1670 cm $^{-1}$. mp. =245 - 247 ℃. Anal. Cald. for $C_{20}H_{26}N_4O_2$: C, 67.77; H,

7.39; H, 15.81; Found : C, 67.47; H, 7.21; N ,15.65. Mass: 354 (M+).

(iii) 3-Ethoxycarbonylmethyl-10-n-decyl-isoalloxazine (3)[15].

In a 100 mL round-bottomed flask were placed 5.0 g (14.1 mmol) of crude 10-n-decyl-isoalloxazine obtained by the procedure (ii), 5.9 g (42.7 mmol) of powdered anhydrous potassium carbonate and 60 mL of dry dimethylformamide. Ethyl bromoacetate (5.2 mL, 46.9 mmol) was added dropwise and the mixture was stirred at 40 ℃ for 6 h under Ar in the dark. Dimethylformamide and excess ethyl bromoacetate were distilled off under reduced pressure. To the residual brown solid were added 120 mL of dichloromethane and 100 mL of 1N HCl. The organic layer was separated, washed with two portions of 20 mL of H_2O and dried over anhydrous $MgSO_4$. Dichloromethane was evaporated to give 6.7 g of a brown crude product. Recrystallization twice from ethanol yielded 3.8 g (61 %) of pure yellow product. ^{1}H-NMR ($CDCl_3$), δ : 0.73 -1.03 (t-like, 3H, CH_3), 1.27 (t, 3H, $-OCH_2CH_3$, J = 7 Hz), 1.03 - 2.08 (m, 16H, $-(CH_2)_{-8}$), 4.24 (q, 2H, $-OCH_2CH_3$, J = 7 Hz), 4.50 - 4.90 (t-like, 2H, $-CH_2N$), 4.83 (s, 2H, $-N-CH_2COO$), 7.43 -8.42 (m, 4H, isoalloxazine ring). IR (KBr) : ν_{CO} = 1735, 1715,1660 cm^{-1}. mp. = 160 -163 ℃. Anal. Cale. for $C_{24}H_{32}N_4O_4$: C, 65.43; H, 7.32; N, 12.72; Found : C, 65.62; H,7.32; N, 12.52. Mass: 440 (M+).

(iv) 3-Carboxymethyl-10-n-decyl-isoalloxazine (4)[15].

3-Ethoxycarbonylmenthyl-10-n-decyl-isoalloxazine (3.15 g, 7.14 mmol) was dissolved in 12 mL of concentrated HCl and the solution was stirred at room temperature for 36 h in the dark. The solution was poured into 400 mL of cold water and yellow precipitates formed were collected by filtration, washed with 50 mL of H_2O three times and dried in vacuo [at room temperature, for 24 hours (0.1 mmHg) on P_2O_5] to yield 2.68 g (91 %) of the yellow carboxylic acid. Analytical sample was recrystallized from ethanol. ^{1}H-NMR (DMSO-d_6), δ : 0.71 - 1.01 (t-like, 3H, CH_3), 1.03 - 2.00 (m, 16H, $-(CH_2)-_8$), 4.38 -4.83 (t-like, 2H, $-CH_2N$), 4.58 (s, 2H, $-N-CH_2COO$), 7.58 -8.33 (m, 4H, isoalloxazine ring). IR (KBr) : ν_{OH} = 3450 cm^{-1}, ν_{CO} = 1750, 1655 cm^{-1}. mp. = 88 - 90 ℃. Anal. Cald. for $C_{22}H_{28}N_4O_4$: C, 64.06; H, 6.84; N, 13.58; Found: C, 64.10; H, 6.90; N, 13.50. Mass: 412 (M+).

(v) [3-(10-Decyl-isoalloxazinyl)] acetic anhydride (5)[7].

3-Carboxymethyl-10-n-decyl-isoalloxazine (0.54 g, 1.3 mmol) obtained above was dissolved in 8 mL of dry THF and into the solution was added 0.25 mL (1.5 mmol) of dry triethylamine. The resulting solution was cooled to -20℃ and a solution of 0.15 mL (1.6 mmol) of freshly distilled ethyl chloroformate dissolved in 5 mL of dry THF was added dropwise in a period of 15 min with external cooling. The mixture was further stirred

for 2 h, then warmed to room temperature and stirred for additional 1 h. The mixture was again cooled to ~-20℃ and 0.54 g (1.3 mmol) of 3-carboxymethyl-10-n-decyl-isoalloxazine in 8 mL of dry THF and 0.25 mL (1.5 mmol) of dry triethylamine in 3 mL of dry THF were added dropwise simultaneously. After the reaction mixture was stirred at room temperature overnight, the solvent was evaporated under reduced pressure. The crude product was dissolved in 150 mL of chloroform and the chloroform layer was washed with 50 mL of H_2O and dried over anhydrous $MgSO_4$. Removal of chloroform followed by drying [room temperature for 24 hours (0.1 mmHg)] afforded 0.96 g (92 %) of the yellow anhydride. The product was used for the next reaction without further purification. IR(KBr): ν_{CO}=1830, 1760, 1710, 1660 cm^{-1}.

(vi) 1-Myristoyl-2-[3-(10-decyl-isoalloxazinyl)acetoyl]-L-α-phosphatidylcholine (6)[7].

In a 30 mL round bottomed flask were placed 88 mg (0.19 mmol) of myristoyl-L-α-lysophosphatidylcholine and 308 mg (0.38 mmol) of the anhydride (5) dissolved in 10 mL of dry choloroform. To the mixture 50 mg (0.41 mmol) of 4-(dimethylamino)pyridine was added and the mixture was carefully degassed with Ar and stirred at 45℃ in the dark for 36 h. The solvent was evaporated under reduced pressure. The residual yellow solid thus obtained was dissolved in a 5 ml of 4:5:1 = $CHCl_3$: CH_3OH: H_2O (v/v) and the solution was passed through an AG-501-X8(D) column (1× 20 cm).

The lipid remained on the adsorbent was eluted with 100 mL of the eluent of the above composition. The fractions containing the product were then combined and the solvent was removed under reduced pressure. The crude lipid obtained as a yellow solid was applied further on a silica gel column (2.5 × 25 cm) and eluted successively with $CHCl_3$-CH_3OH mixture (gradient from 10:0 to 5:5). Fractions containing the main product (R = 0.30, 56:25:4 = $CHCl_3$:CH_3OH :H_2O(v/v)) were collected. Removal of the solvent under reduced pressure folllowed by drying in vacuo (0.1 mmHg) for 24 h at room temperature afforded 108 mg (68 %) of the yellow flavolipid. The flavolipid was further purified by reprecipitation from $CHCl_3$/hexane. ^{1}H-NMR ($CDCl_3$), δ : 0.87 - 0.89 (t-like, 6H, $CH_3 \times 2$), 1.23 - 1.86 (m, 38H, -(CH_2)-), 2.30 (t, 2H, -CH_2COO, J = 8 Hz), 3.39 (broad-s, 9H, -$N(CH_3)_3$), 3.84 - 5.28 (m, 13H, -$CH_2O \times 3$, -HC-O, -CH_2N and -N-CH_2-COO), 7.64 - 8.32 (m, 4H, isoalloxazine ring). IR(KBr) :ν_{CO} = 1730, 1705, 1660 cm^{-1}. Anal Cald. for $C_{44}H_{72}N_5O_{10}P \cdot 4H_2O$: C, 56.58; H, 8.63; N, 7.50, P, 3.32; Found : C, 56.99; H,8.49; N, 7.34, P, 3.24. FAB-Mass: 862((M+H)+). Electronic spectrum ($CHCl_3$) : 290, 336 and 444 nm's with a shoulder at 472 nm.

Prepatation of Artificial Single-Wall Bilayer Liposome.

Artificial egg lecithin liposomes functionalized with the falvolipid were prepared according to a slightly modified procedure of ours[12]. A solution of 75 - 80 mg of carefully

purified egg yolk lecithin and an appropriate amount of the flavolipid in 15 mL of $CHCl_3$ was gently evaporated under reduced pressure under Ar. The resulting thin film was dried in vacuo for 8 h in the dark at room temperature. Into the dried lipid film was added 5 mL of 5 mM aqueous Tris HCl (pH 7.0) solution and the lipid film was suspended in the solution. The suspended mixture chilled in an ice bath was sonicated in a box filled with Ar for 5 min. The sonication was repeated three times at intervals of 10 min. The resulting mixture was centrifuged (1.9×10^3 rpm) for 15 min at 4℃ and the yellow supernatant was applied on a Sepharose 4B column (1.0 cm × 40 cm) and eluted with 5 mM aqueous Tris HCl buffer (pH 7.0) at 4℃. Single-wall liposomes were obtained from eluents between 34 to 45 mL (± 2 mL). Artificial liposomes functionalized with the flavolipid in their membrane phase and containing $K_3Fe(CN)_6$ in their interior aqueous phase were prepared starting from the Tris HCl solution containing 0.75 M of $K_3Fe(CN)_6$.

Reduction of $K_3Fe(CN)_6$ in the Interior Aqueous Phase of the Functionalized liposome by $Na_2S_2O_4$.

Reduction of the inteior $K_3Fe(CN)_6$ by $Na_2S_2O_4$ was carried out by using the same method as described previously [12]. The reduction of the interior $K_3Fe(CN)_6$ was traced by the intensity change of the visible absorption at 425 nm. The absorption of flavolipid at 425 nm was ascertained not to interfare seriously

the above measurement based on the following facts: (i) The reduction rate of flavolipid was independently estimated by monitoring the intensity change of its absorpiton at 472 nm, characteristic of the flavin chromophore. Thus the net decrease of $K_3Fe(CN)_6$ was easily substracted from the overall change. (ii) The change in the $K_3Fe(CN)_6$ concentration was measured in the region where the concentrations of the oxidized and the reduced forms of flavolipid were practically constant in the steady state.

Dynamic Light Scattering.

Size distribution of the artificial liposome functionalized with the flavolipid was measured with an Otsuka Electronics DLS-700 spectrophotometer thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. The flavoliposome employed was freshly purified by gel-filtration using Sepharose-4B (Pharmacia). The single-wall liposome fractions were collected by monitoring electronic absorption spectra of every 1 mL fraction and diluted about 5 times with 5 mM-Tris・HCl.

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Chapter 2

Detailed Kinetics and Mechanism of Electron Transfer across a Bilayer Membrane Functionalized with an Artificial Flavolipid.

Abstract

Detailed kinetics of facilitated electron transport across a bilayer membrane modified with an artificial flavolipid from outside $Na_2S_2O_4$ to inside $K_3Fe(CN)_6$ was discussed. simple biphasic kinetics was observed in the reduction of the flavolipid present in the bilayer membrane. The faster process was ascribed to the reduction of the flavolipid in the outer half layer of the bilayer and the slower to the reduction of the flavolipid in the inner half layer of the bilayer. Saturation kinetics with respect to the concentration of $Na_2S_2O_4$ was also observed, which suggested that the rate determining step moved from the interphase influx ($Na_2S_2O_4 \rightarrow fl(out)$) to the fl(out)-fl(in) electron transfer step with increasing the concentration of $Na_2S_2O_4$. Furthermore, second order dependence with respect to the flavolipid concentration led us to propose a "half channel" mechanism, by which electrons were transported via a transient channel consisted of two flavolipid molecules.

Introduction.

Electron transports across the membrane play utmost important roles in the respiratory and photosynthetic systems in mitochondria and thylakoid membranes, respectively. This is a key step of bioenergetics for the energy storage processes utilizing light and O_2 as the ultimate energy sources. [1] Extensive investigations have been undertaken in these fields from various points of view. [2] The most difficult and interesting problems may be arising from the fact that the electron transports are often accomplished through multienzyme complexes which are tightly bound to the biological membrane. [3] [4] Dissociation of such a complex to single enzymes easily loses its original function. In spite of these difficulties, there have been reported recently several natural complex and artificial systems. [5] For example, the cytochrome c - cytochrome c peroxidase complex was isolated and demonstrated to transfer electron across as large as 20 Å distance with a significantly large rate.[6] Electron tunneling from one heme to another located at the protein active center is the suggested mechanism. There are also reported many artificial electron transfer systems which have attracted much interest in view of the Marcus theory. [7-10]

There are two possible mechanisms for the electron tranfer across the membrane: One is based on the electron carrier which

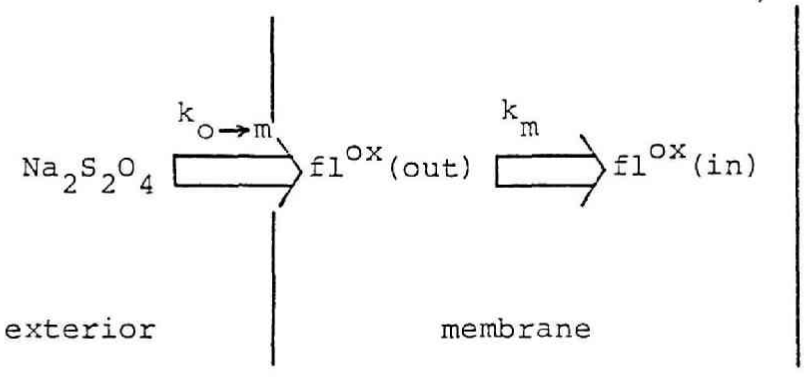
diffuses across the membrane, and investigated in detail for the case of alkylviologen, [11] benzoquinone, ferrocene and others. [12] The other is based on the electron channel through which electrons are transported via tunneling. The latter mechanism is interesting in view of its possible function in the biological membrane, where the electron mediator loses its mobility in a highly ordered matrix. Although there have been reported some indications of the system via the latter mechanism , no clear evidence has been reported.

Another interesting aspect of the electron transport across the membrane is visualized in the coupling of electron transport with proton or other material transport. [13] Examples could be referred to ATP synthesis, [14] vision systems or many others, [15] where the coupled proton transfer produces membrane potentials of H^+ for the driving force of ATP synthesis or the information transmission.

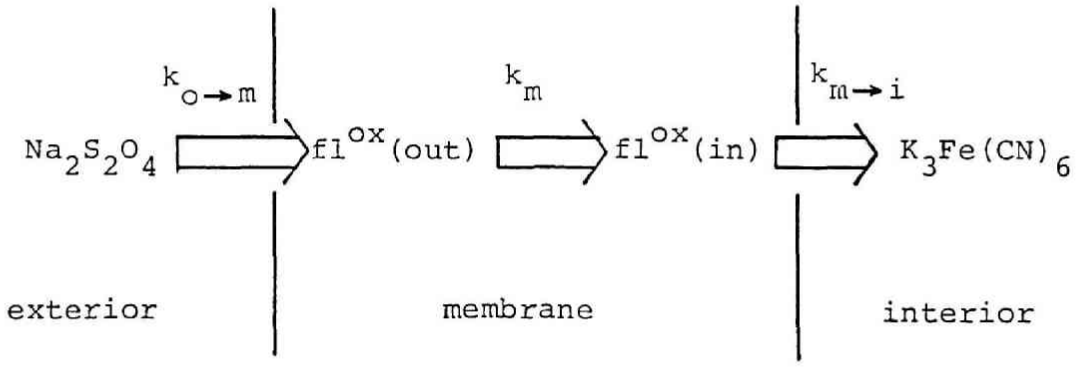
In continuation of the syudy described in chapter 1, we would like to report that the electron transfer across a bilayer membrane was successfully demonstrated to pass through the oxidation-reduction center of the flavolipid. This electron transport was shown by kinetic studies to be operated through a "half channel" mechanism, newly proposed.

Scheme 1.

(a) Reduction of Flavolipid in the Liposomal Membrane.



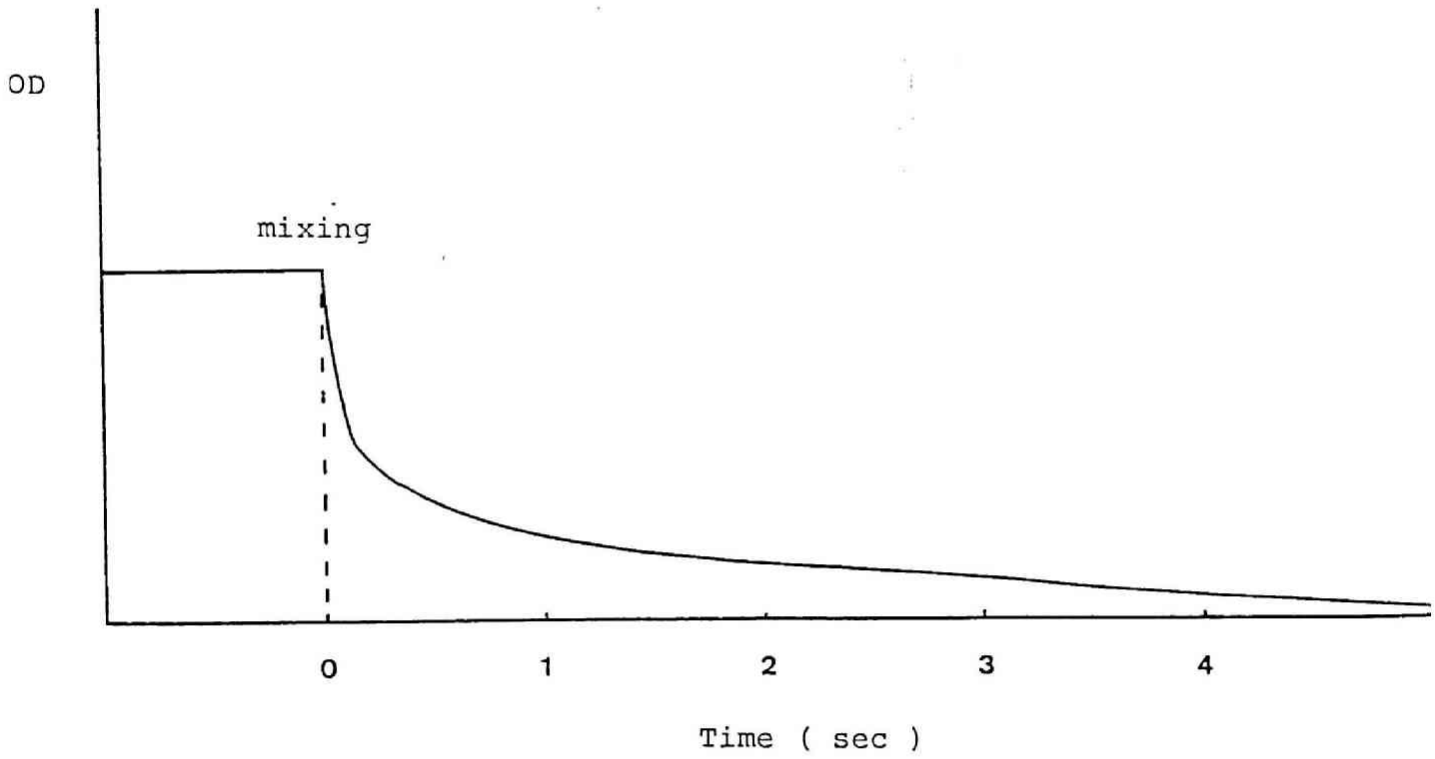
(b) Reduction of Interior $K_3Fe(CN)_6$ in Flavoliposome.



Results and Discussion.

Reduction of Flavolipid Incorporated into the Bilayer Membrane by Dithionite in the Exterior Aqueous Phase.

The solution of the liposome modified with the flavolipid($^{(o)}$ |fl-Lip| $^{(i)}$) was reduced with an external aqueous $Na_2S_2O_4$ solution. The decrease of the oxidized form of the flavolipid (fl^{ox}) in the bilayer membrane was monitored by the absorbance at 444 nm. As shown in Fig 1, a change of fl^{ox} concentration was observed to be clearly biphasic, where approximately 50 - 60 % of the total absorbance change of fl^{ox} decreased according to a faster process, followed by a slower one for the disappearance of the remaining 50 - 40 % of total fl^{ox}. The present method for the preparation of artificial liposomes modified with the flavolipid gives a distribution of the flavolipid both in outer and inner half layer of the membrane according to that of egg lecithin (about 6/4 = outer/inner). The flavin moiety locates at a fixed distance near the surface (7 Å inside the membrane from P atom of the phosphatidyl unit).[18] Therefore the redox sites should locate at innermembrane, one near to the exterior surface and the other to the interior. In such a situation, the topological difference of flavin moiety in the membrane may be the controlling factor in the reduction of flavolipid by external $Na_2S_2O_4$ although microenvironments and/or different binding sites in a liposomal membrane were suggested in the



g. 1. Reduction of Flavolipid in the Liposomal Membrane.
Change of absorption at 444 nm, characteristic of fl^{ox}.

interphasical reaction of several substrates in liposomes. [16] The above experiment shows that both of flavolipids in outer and inner half layer are completely reduced by external $Na_2S_2O_4$. The flavolipid located at the outer half layer of the membrane (fl^{OX}(out)) seems to be easily reduced. Therefore the faster process could be ascribed to the interphasical electron flux, $J_{o \to m}$ from exterior aqueous phase into the membrance phase (Scheme 1).

insert Scheme 1 and Table I here

On the other hand, the reduction of the flavolipid located at the inner half layer of the membrane (fl^{OX}(in)) follows a slower process of pseudo 1st order. All rate constants here obtained were given in Table I. Careful evaluation of the rate at different initial concentrations of $Na_2S_2O_4$ indicates that the rate of the faster process is 1/2 order with respect to $Na_2S_2O_4$. With decreasing the initial concentration of $Na_2S_2O_4$, the reduction of fl^{OX} was observed to be almost monophasic and 1st order with respect to fl^{OX}. This may be explained by the idea that the rate determining step is moved from the flavin→flavin electron transfer to the interphasical electron influx from external $Na_2S_2O_4$ to fl^{OX} (out) in membrane. In the biphasic region, the rate constants of the slower process were independent of the initial concentration of $Na_2S_2O_4$.

Table I

Biphasic Reduction Rate Constants of Flavolipid Incorporated into the Bilayer Membrane

temp (℃)	fl^{OX}	$Na_2S_2O_4$ (mM)	$k_{o\rightarrow m}(sec^{-1})$	$k_m (sec^{-1})$
25	0.037	1.0	6.2	0.63
5	0.037	1.0	2.9	0.56
5	0.019	0.052	0.34	a)
5	0.019	0.26	0.95	a)
5	0.019	0.52	1.48	0.13
5	0.019	1.0	1.85	0.14

a) monophasic reduction

$$-d[fl^{OX}] / dt = k_1 [fl^{OX}]$$

$$k_1 = k_{o\text{-}m} + k_m$$

$$k_{o\text{-}m} = k_1 [Na_2S_2O_4]^{1/2}$$

$$k_m = k_2 [fl^{red}(out)]$$

On the basis of the following reasons, this slow reduction is reasonably ascribed to a long distance electron transfer from fl^{red} (out) (the reduced form of the flavolipid at the outer half layer of the membrane) to fl^{OX} (in): (i) The flavolipid having a polar head group is a constituent of the bilayer membranne, and its estimated vertical diffusion i.e., flip-flop rate, $k = 10^{-5}$ sec^{-1} for the normal phospholipid is too slow to be accounted for the observed rate process. [17] (ii) Direct reduction of fl^{OX} (in) by the external $Na_2S_2O_4$ seems to be improbable because the distance between exterior interface and the nearest edge of fl^{OX} (in) is about 30 Å from CPK model. [18] (iii) Lateral diffusion of the flavolipid remains relatively free (10^{-8} - 10^{-9} cm^2 / sec) [19] and certainly accommodates the idea of fl^{red} (out) - fl^{OX} (in) electron transfer. (IV) The nearest edge-edge distance between fl^{red} (out) and fl^{OX} (in) is about 16 ± 1 Å from CPK model. The observed rate constants are compatible with those of

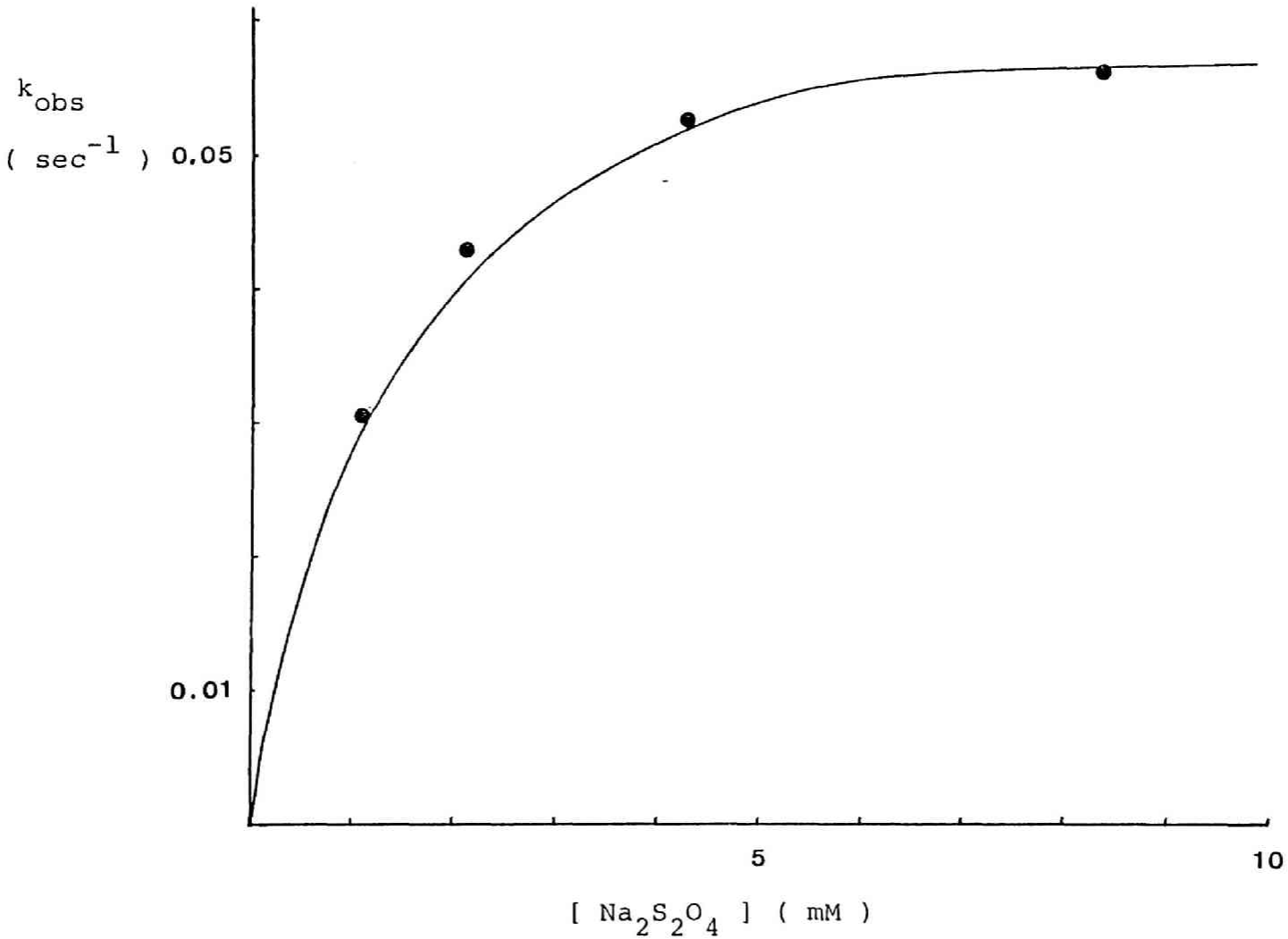


Fig. 2. $Na_2S_2O_4$ Dependence of Reduction Rate Constants of Interior $K_3Fe(CN)_6$

elctron transfer across a similar distance of the separation of porphyrin complexes such as a myoglobin modified with ruthenium, [5] a cyt c-cyt c peroxidase complex. [6]

Electron Transport across the Bilayer Membrane Modified with Flavolipid.

Artificial liposome modified with flavolipid containing ferricyanide in its interior aqueous phase ($^{(o)}$ |fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$) was treated with $Na_2S_2O_4$ added to the external aqueous phase. As described previously [20], a rapid reduction of fl^{OX} to fl^{red} after mixing of the liposome solution with an aqueous solution of $Na_2S_2O_4$ was observed to be followed by a slower pseudo first order decrease of ferricyanide. Under pseudo first order conditions, the rate dependence on the concentration of $Na_2S_2O_4$ was investigated. The pseudo first order rate constant was found to be of a 1/2 order with repsect to $Na_2S_2O_4$ in the range of 1.0 - 4.0 mM. Therefore, the total rate expression is given by eq (2):

$$-d[Fe(CN)_6^{3-}] / dt = k [Na_2S_2O_4]^{1/2} [K_3Fe(CN)_6] \qquad \text{eq. 2}$$

The half order dependence on $Na_2S_2O_4$ concentration is normally observed in homogeneous solution as well as in heterogeneous reductions (micelle or liposome) of a variety of substrates. [21] This is interpreted by a very rapid homolytic cleavage of $S_2O_4^{2-}$

Table Ⅱ

Reduction Rate Constnts of $K_3Fe(CN)_6$ in the Flavoliposome

concn			k_{obs}
flavolipid (mM)	$Na_2S_2O_4$ (mM)	$K_3Fe(CN)_6$ (mM)	(sec^{-1})
0	4.3	0.40	0.001
0.0015	4.3	0.40	0.0025
0.0068	4.3	0.40	0.025
0.011	1.1	0.40	0.031
0.011	2.1	0.40	0.043
0.011	4.3	0.40	0.053
0.011	8.4	0.40	0.056
0.014	4.3	0.40	0.083
0.070	3.0	0.41	0.44

a) All reactions were performed at 25℃, pH 7.0.

b) Analytical interior local concentration of $K_3Fe(CN)_6$ = 0.75 M.

to $2 \cdot SO_2^-$ followed by the electron transfer process from the latter. In the range of concentration higher than 4.0 mM of $Na_2S_2O_4$, a saturation kinetics with respect to $Na_2S_2O_4$ was observed (see Fig 2). The results are summarized in Table II and Fig. 2. The rapid reduction of fl^{OX} to fl^{red} above cited was clearly observable

insert Fig 2 and Table II here

only when the saturated range of $Na_2S_2O_4$ (more than 4 mM) was employed. The amount of fl^{red} corresponded to about 50 - 60 mol % of the total fl^{OX} initially employed. This value corresponded approximately to the flavolipid in the outer half layer of the membrane. At a lower concentration range of $Na_2S_2O_4$ (less than 4 mM), the initial rapid reduction of fl^{OX} to fl^{red} was not clearly discernible. The reduction of ferricyanide proceeded without the apparent fast reduction of fl^{OX}. Therefore, at the lower concentration range, the rate determining step of the reduction of the interior ferricyanide must be the interphase influx ($J_{o \rightarrow m}$). At the higher $Na_2S_2O_4$ concentration range, the interphase reduction becomes faster compared with the fl^{red} (out) $\rightarrow$ fl^{OX} (in) electron transfer, i.e. now the rate determining step. On keeping the higher concentration of $Na_2S_2O_4$ (4 mM) and flavolipid (70 μM), the rapid reduction of fl^{OX} to fl^{red} proceeded further to accumulate till 80 mol % of

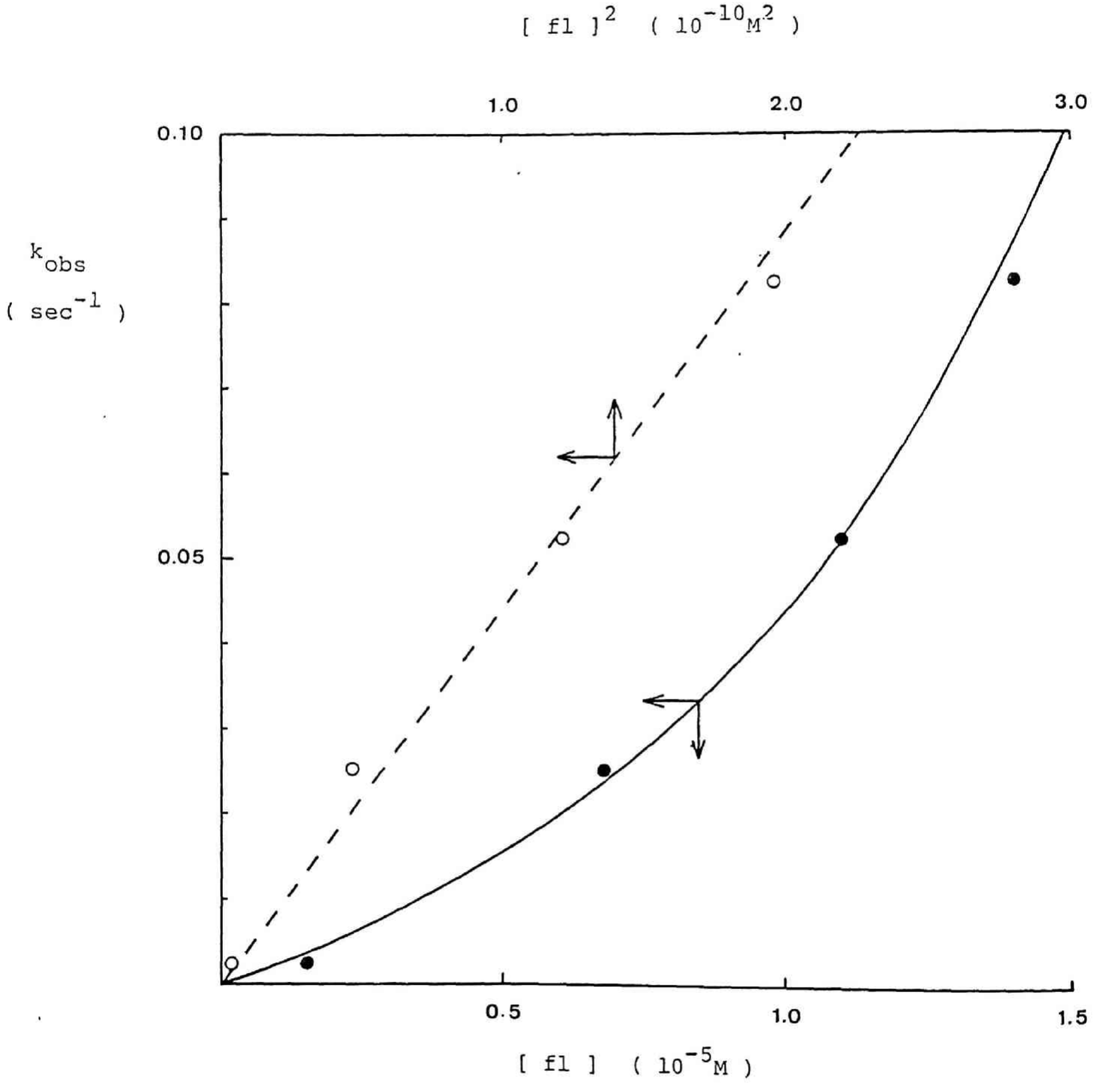


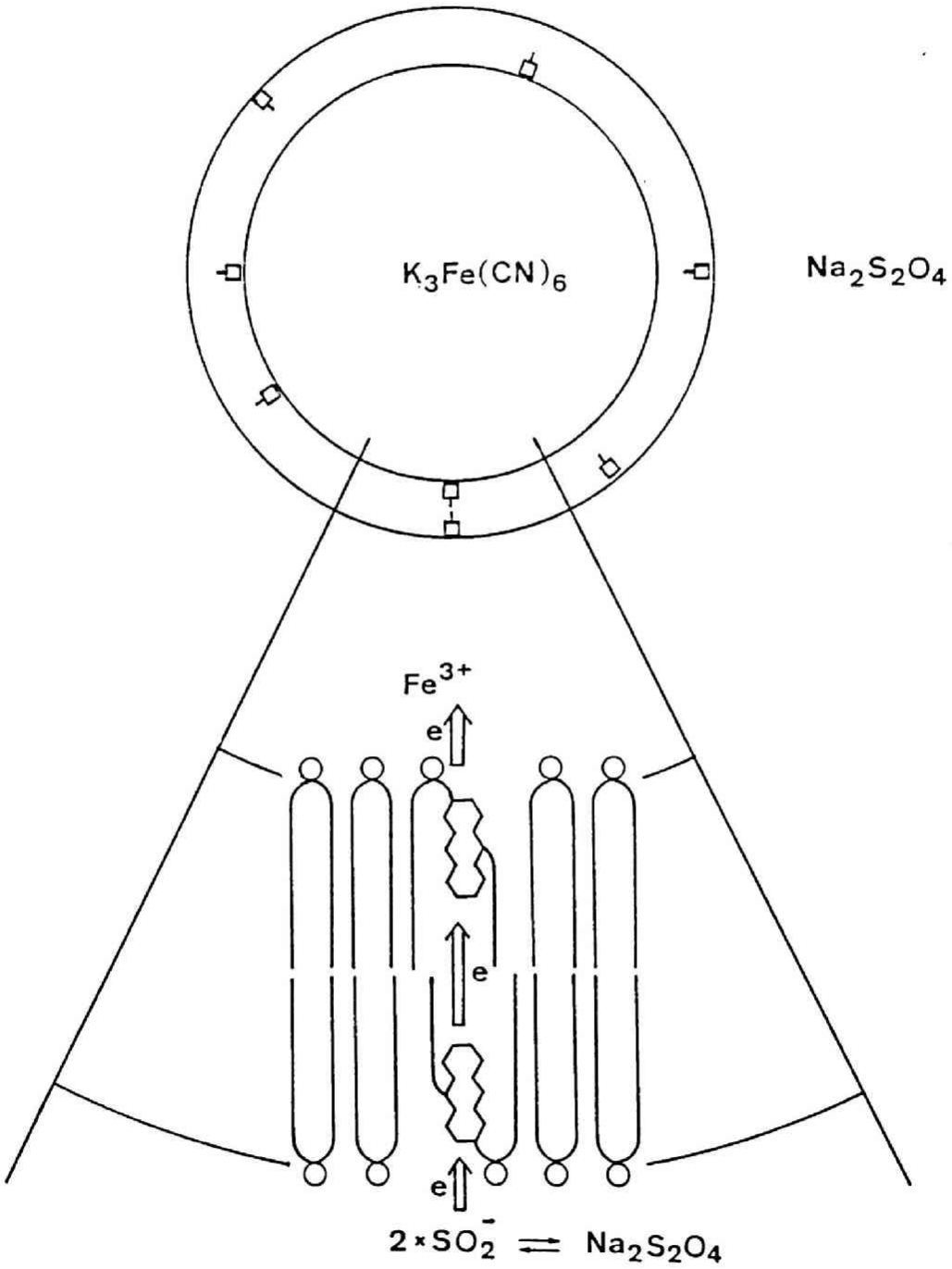
Fig. 3. Dependence of k_{obs} on the Concentration of Flavolipid

the total fl^{OX}. This may suggest that the rate of fl^{red} (out) → fl^{OX} (in) electron transfer step becomes faster than the interphase electron outflux into interior aq. phase ($J_{m \rightarrow in}$). In order to focus on the fl^{red} (out) → fl^{OX} (in) electron transfer step, the reaction condition in the following was chosen so that the concentration of $Na_2S_2O_4$ was 4.2 mM (in the saturated range) and the concentration of flavolipid was changed from 1.5 μM to 14 μM (i.e., less than 70 μM). The observed rates were found to depend on the 2nd powers of the flavolipid concentration (see Fig. 3).

insert Fig 3 here

The second order dependence with respect to the flavolipid concentration strongly supports that electron transfer from fl^{red} (out) to fl^{OX} (in) in the bilayer membrane plays a key role in the transmembrane electron transfer. Here two flavolipid molecules are suggested to associate for at least a short period to form an electron channel, through which electrons are transported. The carrier mechanism by a flip-flop of flavolipids can accounts for neither the observed molecularity nor the transport rate. The flip-flop rate is too slow to be accounted for the rate process of 10^{-1} sec^{-1} here observed as described above.

Scheme. 2.



Experimental Section.

Instruments.

Electronic absorption spectra were measured with either a Union Giken high sensitivity spectrophotometer SM 401 or a Hitachi U-3400 spectrophotometer thermostated at 25 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. Sonication was performed with an ultrasonic disruptor, Mode UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument.

Material.

Buffer solution was made by dissolving tris (hydroxymethyl)-aminomethane and N/10-hydrochloric acid to distilled water to be 5mM Tris concentration. Egg yolk lecithin was carefully purified according to the literature method[22] and stored at -75℃ under Ar in the dark. Flavolipid (1) was prepared by the reported method.[23] Sodium dithionite was purchased from Nakarai Chemicals.

Preparation of Artificial Single Wall Liposome.

Artificial egg lecithin liposomes functionalized with the flavolipid were prepared according to the reported method. Artificial liposomes functionalized with the flavolipid in their

bilayer membrane phase and containing $K_3Fe(CN)_6$ in its interior aqueous phase were prepared by starting from the Tris HCl solution containing 0.75M of $K_3Fe(CN)_6$.

Reduction of Flavolipid Incorporated into the Bilayer Membrane of the Functionalized Liposome by $Na_2S_2O_4$.

A freshly prepared liposome solution (2.0 mL) ($^{(o)}$ |fl-Lip| $^{(i)}$), kept in a 10-mm quartz cell equipped with a three-way stopcock. The solution was deaerated through the careful substitution of air by Ar via evacuation and the introduction of Ar at room temperature. The procedure was repeated 20 times per sample. Into the deaerated liposome solution at 25℃, 0.2 mL of a freshly prepared $Na_2S_2O_4$ (0.052 - 1.04 mM) aq. solution was added using a specially designed syringe. The $Na_2S_2O_4$ solution was freshly prepared before use and its activity was determined by titration with $K_3Fe(CN)_6$ solution. The reduction of the flavolipid present in the bilayer membrane phase was traced by monitoring the change of the absorbance at 444 nm.

Reduction of $K_3Fe(CN)_6$ in the Interior Aqueous Phase of the Functionalized Liposome by $Na_2S_2O_4$.

Reduction of the interior $K_3Fe(CN)_6$ by $Na_2S_2O_4$ was carried out by using the same method as described above .

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Chapter 3

Successful Control of Electron Transport Rate by Thermally Induced Phase Transition of Liposomal Membrane Modified with Flavolipid.

Abstract.

An artificial liposome consisted of dipalmitoylphosphatidylcholine (DPPC) functionalized with a flavolipid was prepared in place of egg lecithin liposome and showed the similar bilayer structure and stability to those of the egg lecithin liposome modified with the flavolipid as demonstrated by dynamic light scattering, gel permeation behavior and electronic absorption spectra. The present liposome has a definite phase transition temperature (Tc) at 38 - 39℃, while the egg lecithin liposome showed a broad phase transition below 0℃. The electron transport rate from outside $Na_2S_2O_4$ to inside $K_3Fe(CN)_6$ in the present liposome above Tc was estimated about 1.5 sec^{-1}, while the rate below Tc was surpressed to about 0.007 sec^{-1}. Thus, the remarkable control (about 10^2 time) of electron transport across the liposomal membrane modified with flavolipid was achieved by thermally induced phase transition.

Introduction.

Electron transporting systems in biological membranes play significant roles in energy metabolisms as demonstrated in the photosynthetic charge separation as well as the oxidative phosphorylation in respiratory systems. [1] These electron transducing devices locate in the membrane as a coupled entity of several elements, making the access to the elucidation of the nature of these biological systems very difficult. Therefore as a simplified model, the development of artificial systems may contribute greatly to the elucidation of the mechanism and to the construction of biomimicking functions. Along this line, we have developed an artificial flavolipid molecule which was demonstrated to facilitate efficiently the transmembrane electron movement. [2]

We now focused on the control of electron transport rate. A successful demonstration of this will also be important for the future development of molecular switching in an ordered matrix. In this communication, we report that the electron transport rate across the bilayer membrane modified with the flavolipid (1) is controlled by the thermally induced phase transition of the flavoliposome.

Results and Discussion.

Preparation and Phase Transition Temperature (Tc) of Dipalmitoylphosphatidyl-choline (DPPC) Liposome Modified with Flavolipid.

Dipalmitoylphosphatidylcholine (DPPC) in place of egg lecithin (having a broad Tc below 0℃) was used to prepare artificial liposomes showing a clear phase transition at a definite temperature (Tc). This liposome was successfully modified with the present flavolipid as shown in the following results : (i) the gel filtration behavior of the DPPC liposome modified with the flavolipid was similar to that of the egg lecithin liposome modified with flavolipid, (ii) a dynamic light scattering study of the DPPC liposome modified with the flavolipid showed that the mean diameter was about 250 Å, and (iii) the electronic spectrum of the flavolipid incorporated into the DPPC liposome (λmax = 336, 443nm) resembled with that in chloroform (λmax = 336, 442nm), which showed the same pattern as that of the flavolipid into the egg lecithin liposome. The phase transition temperature of the flavoliposome ($^{(o)}$ |Fl-Lip (DPPC)| $^{(i)}$) containing the flavolipid in 3 mol % quantity was determined to be 38 - 39℃ by the differential scanning calorimetry (DSC) measurement and there observed also a pretransition peak at 35℃ (See Fig. 1). The DSC measurement was obtained after centrifugation without gel filtration to remove the multilayer

Fig.1 DSC curve of the flavoliposome.
The flavoliposome was purified through centrifugation at 4 °C after sonication.

liposomes, in which, however, less than 5 % of the total lipid molecule are contained. The data are in the range of Tc's (35 - 40℃) reported for the parent DPPC lisposome. [3] No other peak corresponding to the phase transition of a flavolipid domain was detected in the temperature range of 8 - 65℃. The DPPC liposome containing flavolipid in 3 mole % quantity was stable for at least four days at 4℃. This liposome is less stable than the corresponding egg lecithin liposome having 7 days stability. All kinetic studies and the differential scanning calorimetry (DSC) measurements were performed within two days after preparation.

Control of the Rate of Transmembrane Electron Transport by Thermally Induced Phase Transition.

The DPPC-liposome modified with flavolipid containing ferricyanide in its interior ($^{(o)}$ |Fl-Lip (DPPC)| $^{(i)}$ $K_3Fe(CN)_6$) was treated with a reducing agent by the addition of aqueous $Na_2S_2O_4$ solution in its exterior. The decrease of the interior ferricyanide concentration was traced by monitoring the absorbance at 425 nm. A rapid reduction of fl^{ox} to fl^{red} was followed by a slower pseudo first order decrease of ferricyanide. The kinetic behavior was similar to that observed for egg lecithin liposomes. The pseudo first order rate constants thus obtained were summarized in Table I and Fig. 2 in the temperature range of 10℃ to 50℃. As shown in Fig. 2, the

Table I

Reduction Rate Constants of $K_3Fe(CN)_6$ in DPPC-Flavoliposome at Various Temperatures .[a)]

concn of flavolipid (mM)	temperature (℃)	kobs (sec^{-1})
0	10	0.001
0	25	0.001
0	40	0.002
0.02	10	0.007
0.02	20	0.018
0.02	25	0.065
0.02	30	0.19
0.02	35	0.55
0.02	40	1.57
0.02	50	1.62

a) pH 7.0, concentration of $K_3Fe(CN)_6$ = 0.25 mM, concentration of $Na_2S_2O_4$ = 4.2 mM

electron transport rate was very slow below Tc and increased

insert Table I and Fig 2 here

dramatically at the temperature very close to Tc to give the limiting rate which corresponded exactly to that observed with the egg lecithin functionalized liposome. Thus the rate enhancement between below and above Tc amounted around 10^2. The electron transport rate across the DPPC membrane without a modification by the flavolipid was observed to be as negligibly small as 10^{-3} sec^{-1} in the whole temperature range examined. In this case, no abrupt change of the transport rate was observed near Tc. Therefore the dramatic enhancement of the electron transport rate here observed can reasonably be related to the increase of the lateral diffusion rate of the flavolipid in the phospholipid bilayer. The lateral diffusion rate of phospholipids in the bilayer membrane has been reported to increase by a factor of $(1.4 - 5.5) \times 10^2$ above Tc than below Tc by using a technique of fluorescence recovery after photobleaching. [4] The increased rate of lateral diffusion of lipid molecules leads to the increase of collision frequency of two flavolipid molecules, one at the inner half layer and the other at the outer half layer of the membrane (See Fig.3). In other words, the flavolipids provide a "transient channel", through which electrons are transferred across the membrane. It

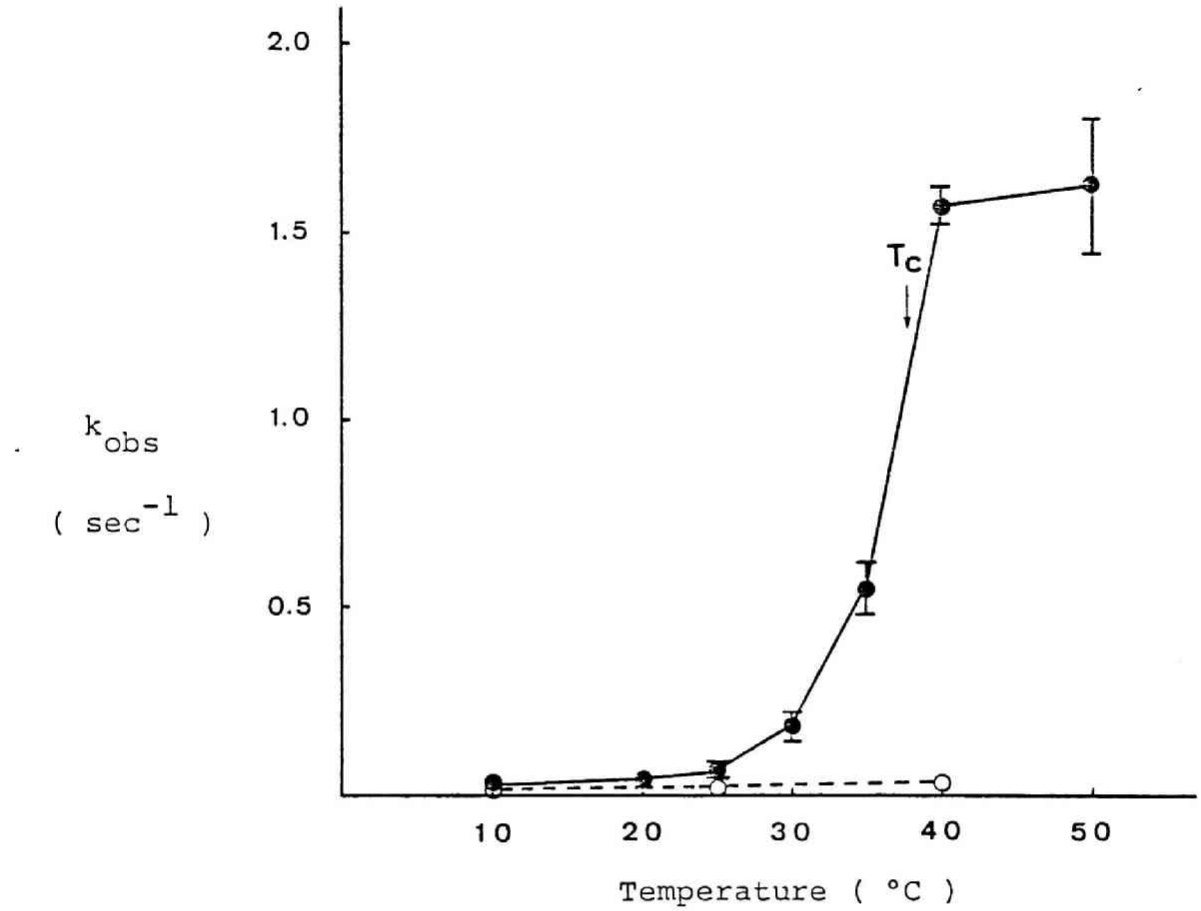
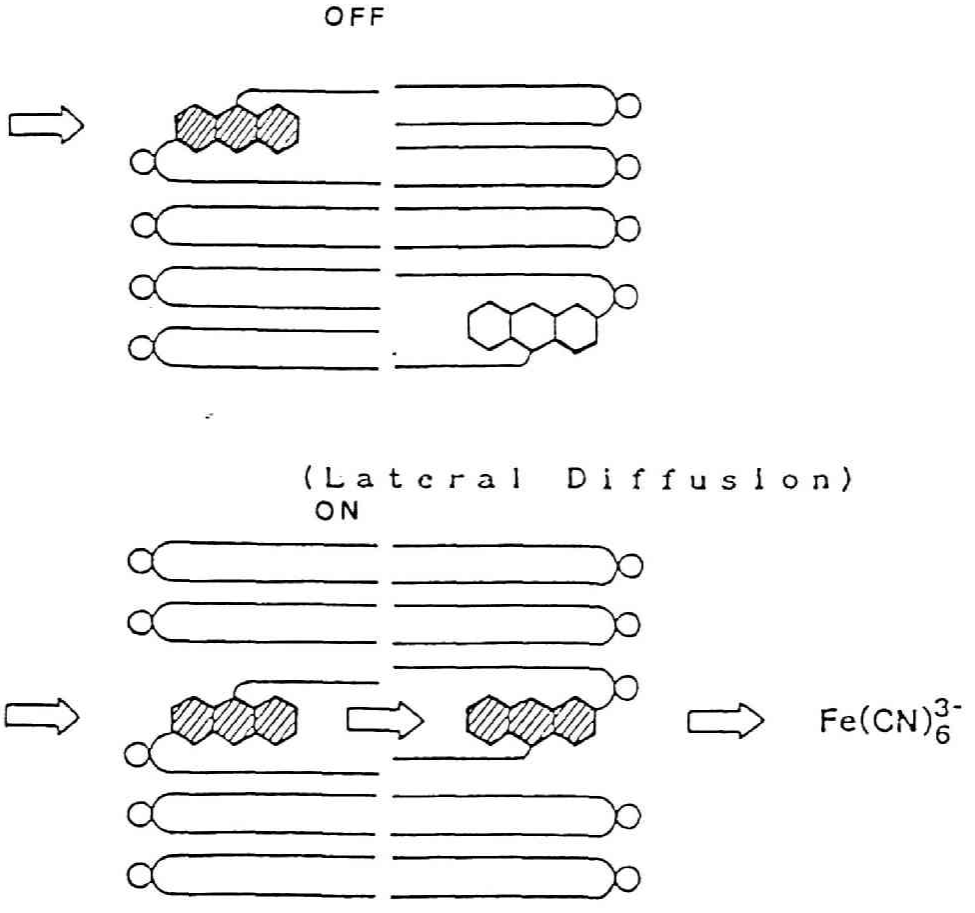


Fig. 2. Dependence of k_{obs} on the Temperature.
DPPC liposome modified with (——) and without (---) flavolipid.

has been reported previously that the permeability of inorganic as well as organic materials through the bilayer membrane is controlled by the temperature. [5] However, all of these permeation controls were interpreted by a simple "leakage" process due to the disorder of the bilayer membrane. In contrast to this mechanism, the present system provides the first demonstration that the electron channel controls the rate of electron transport across the membrane by gel-liquid crystal phase transition.

Fig.3. Schematic ON-OFF Control of Electron Transport Rate



Experimental Section

Instruments.

Electronic absorption spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophotometer thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. Sonication was performed with an ultrasonic disruptor, Mode UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument.

Materials.

Unless stated otherwise, all reagents and chemicals were obtained commercially and used without further purification. Egg yolk lecithin was carefully purified according to the literature method [6] and stored at -75℃ under Ar in the dark. DPPC was purchased from Sigma and stored at -20℃.

Preparation of DPPC Liposome Modified with Flavolipid.

Artificial DPPC liposomes functionalized with the flavolipid were prepared according to the reported procedure of ours except that the suspended solution of the lipid film was sonicated five times with external cooling in an Ar box. [7]

Reduction of $K_3Fe(CN)_6$ in the Interior Aqueous Phase of the DPPC Liposome Functionalized with Flavolipid by $Na_2S_2O_4$.

A freshly prepared liposome solution (2.0 mL), kept at pH 7.0, was deaerated through the twenty times of substitutions of air by Ar via evacuation and the introduction of Ar at room temperature. After the deaerated liposome solution let stand at appropriate temperature for 15 minutes, 0.2 mL of a freshly prepared $Na_2S_2O_4$ solution (by titration with $K_3Fe(CN)_6$ (aq)) was added by using a specially designed syringe. [7] The reduction of $K_3Fe(CN)_6$ and the flavolipid was traced by the intensity change of the visible absorption at 425 nm and 472 nm respectively.

Differential Scanning Calorimetry.

The thermal analysis of the DPPC liposome functionalized with the flavolipid was performed with a Seiko DSC calorimeter (I&E Mode DSC-10 calorimeter combined with a Seiko DSC/580 thermal controller). The flavoliposome employed was obtained through centrifugation after sonication without gel filtration. The sample (50 L, total lipid concentration was about 10 mg/mL) was sealed in aluminium pan with a sealer. The heating rate was 1℃/min. The scanning temperature range was 8 - 65℃.

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Chapter 4

Efficient Electron Flux Conversion from Strongly Hydrophilic NADH Model in the Transmembrane Electron Transfer by Flavolipid.

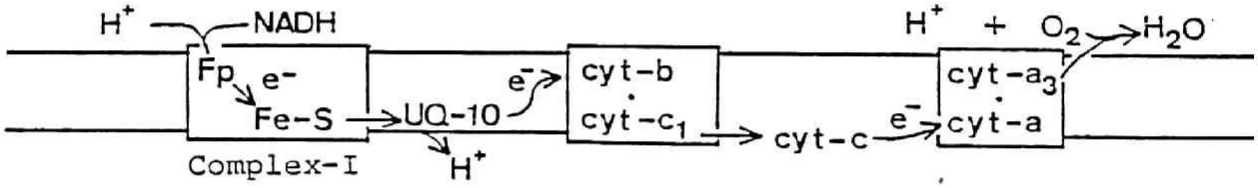
Abstract.

A strongly hydrophilic NADH analog, BzNAHCOOK, was proved to be an appropriate NADH model in the transmembrane electron transport study because of (i) very low permeabilty through a bilayer membrane ($k = 1.0 \times 10^{-3}$ sec^{-1}) and (ii) slow hydration rate ($k = 1.1 \times 10^{-5}$ sec^{-1}). Using BzNAHCOOK as the external electron source, the artificial liposome modified with a flavolipid showed 6-7 times more efficient electron flux conversion from BzNAHCOOK to $K_3Fe(CN)_6$ than the ubiquinone-10 modified liposome. Electrochemical oxidation and reduction of the flavolipid incorporated into the liposomal membrane using Hg-electrode gave its redox potentials at -0.315 V and -0.573 V vs SCE.

Introduction.

Dihydronicotinamides are important electron donors in many biological systems such as the respiratory chain. [1] In order to conduct a transmembrane electron flow from RNAH locating in the liposomal exterior to the electron acceptor in the liposomal interior, dihydronicotinamide first interacts with the membrane bound electron transport substance, frequently via tight binding to a flavoprotein, one of the important oxido-reduction centers of electron transport systems. A NADH molecular "flux" from the bulk exterior phase to the exterior membrane surface is then converted to the electron "flux" at the exterior membrane surface (hereafter, this physicochemical change is called as "flux" conversion from a molecular flux to an electron flux).

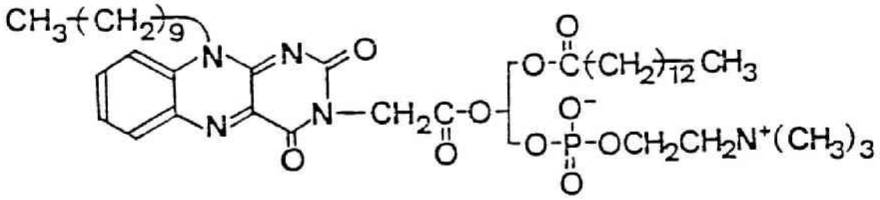
Respiratory Chain



Fp : flavoprotein, **Fe-S** : iron-sulfur cluster, **cyt** : cytochrome

In this respiratory chain, for example, the Complex I containing a membrane-bound flavoprotein and an iron-sulfur cluster [2] is considered to play a key role in the efficient flux conversion. However, little is known about the mechanistic details of the

flux conversion by the flavoprotein in the Complex I. [3] Simplified model systems seem to be useful for the construction of biomimicking function as well as for the elucidation of its molecular mechanisms. Now the author wishes to report the significance of flavin-RNAH couple in the efficient transmembrane electron transport through a flavoliposome system using BzNAHCOOK, a strongly hydrophilic NADH analogue.



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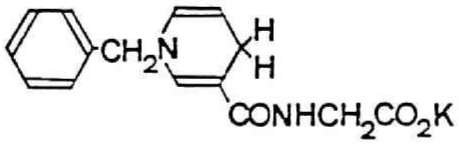
Results and Discussion.

Structure of NADH Models and their Leakage across the Bilayer Membrane.

N-(Carboxymethyl)-1-benzyl-1,4-dihydronicotinamide potassium salt (BzNAHCOOK) [4] was used as a reductant in transmembrane electron transport study because of the following reasons. (i) It is generally accepted that various N-alkyldihydronicotinamide (RNAH) give about 10^2 times faster rates of flavin reduction than NADH itself in homogeneous solutions. [5] The electron transport rate using NADH itself was in fact too slow to be measured in the flavoliposome system described in chapter 1 and 2. (ii) Consequently a simple NADH analogue, MeNAH can be a candidate for the reductant. However the leakage rate of MeNAH through the flavoliposome membrane was too fast (Table I) to make possible to estimate reasonably the rate of the net electron transport mediated by the flavolipid from the reduction which consists of a material (MeNAH) transport and an electron transport via the flavolipid. Thus, MeNAH cannot be an appropriate NADH model for the present purpose. (iii) In contrast to MeNAH, the leakage rate of BzNAHCOOK was surpressed by a factor of 60 compared to that of MeNAH. (iv) One of the disadvantages of the use of RNAH in an aqueous system arises often from its hydration to form (A) (See Scheme 1). [6] BzNAHCOOK exhibited a superior characteristic in



BzNAHCOOH

Table I Reduction Rate Constants of the Interior $K_3Fe(CN)_6$ in the Egg Lecithin Liposome by NADH Models without the Flavolipid [a)]

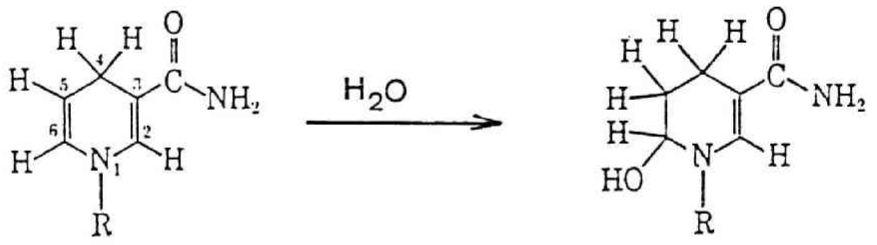
NADH models	k_{obs} (sec^{-1}) [b)]
MeNAH	60×10^{-3}
BzNAHCOOK	1×10^{-3}
NADH	0.03×10^{-3}

a) $(^{(o)}\text{NADH model} \mid \text{Lip} \mid {}^{(i)}Fe(CN)_6^{3-})$

b) $v = -k_{obs}[Fe(CN)_6^{3-}]$

this point of view. The hydration rate of BzNAHCOOK in 5 mM-Tris · HCl (pH = 7.0) ($k = 1.1 \times 10^{-5}$ sec^{-1}) was 10^2 times slower than that of MeNAH in 5 mM-Tris · HCl (pH = 7.0) ($k = 1 \times 10^{-3}$ sec^{-1}). As evidenced later, this hydration rate of BzNAHCOOK is negligibly slow compared with the facilitated electron transport reaction rate across the membrane modified with flavolipid, the latter being $10^{-2} \sim 10^{-1}$ sec^{-1}.

Scheme 1



(A)

Hydration of NADH Model

Next, the electron transport through an artificial membrane functionalized with an efficient electron transport catalyst was investigated by using BzNAHCOOK as an external reductant. This electron transporting system containing $K_3Fe(CN)_6$ in its interior aq. phase ($^{(0)}$ BzNAHCOOK | fl-Lip| $^{(i)}$ $K^3Fe(CN)_6$) may be expected to mimic an NADH dehydrogenase in the complex I of the respiratory chain in the mitochondrial inner membrane.

Effective Flux Conversion of NADH Model by Flavolipid.

Electron influx ($J_{o \to m}$) from external BzNAHCOOK to the flavolipid (fl^{ox} : the oxidized form of the flavin moiety) locating in the membrane phase can be monitored by the reduction of the flavolipid, which is incorporated into the liposome without $K_3Fe(CN)_6$ in its interior ($^{(o)}$ | fl^{ox} -Lip| $^{(i)}$), by external BzNAHCOOK. In the same way, electron outflux ($J_{m \to i}$) from the reduced flavolipid (fl^{red}) locating in the bilayer membrane phase to internal $K_3Fe(CN)_6$ can be obtained by the reoxidation of fl^{red} in the membrane by $k_3Fe(CN)_6$ in external aqueous phase. As shown in Fig. 1. and Table Ⅱ , electron influx ($J_{o \to m}$) from BzNAHCOOK to fl^{ox} was about 10^3 times slower than that from $Na_2S_2O_4$ to fl^{ox} . A much slow reduction rate of the flavolipid by BzNAHCOOK was observed compared with that by $Na_2S_2O_4$ (-1.13 V vs NHE) which acts as a much stronger electron donor to flavolipid than BzNAHCOOK.

In the reduction of internal $K_3Fe(CN)_6$ by external BzNAHCOOK across the flavomembrane, fast reduction of flavin moiety to the corresponding dihydroflavin was observed at the very initial stage of the reaction (region "a" in Figure 2). This process was followed by an apparent steady state, where the ratio of fl^{red} to fl^{ox} was virtually constant (region "b", fl^{red} / fl^{ox} = 1/9～3/7) while most of $K_3Fe(CN)_6$ was consumed during the period. This fl^{red} / fl^{ox} ratio was much smaller than that in the reduction of internal $K_3Fe(CN)_6$ by external $Na_2S_2O_4$

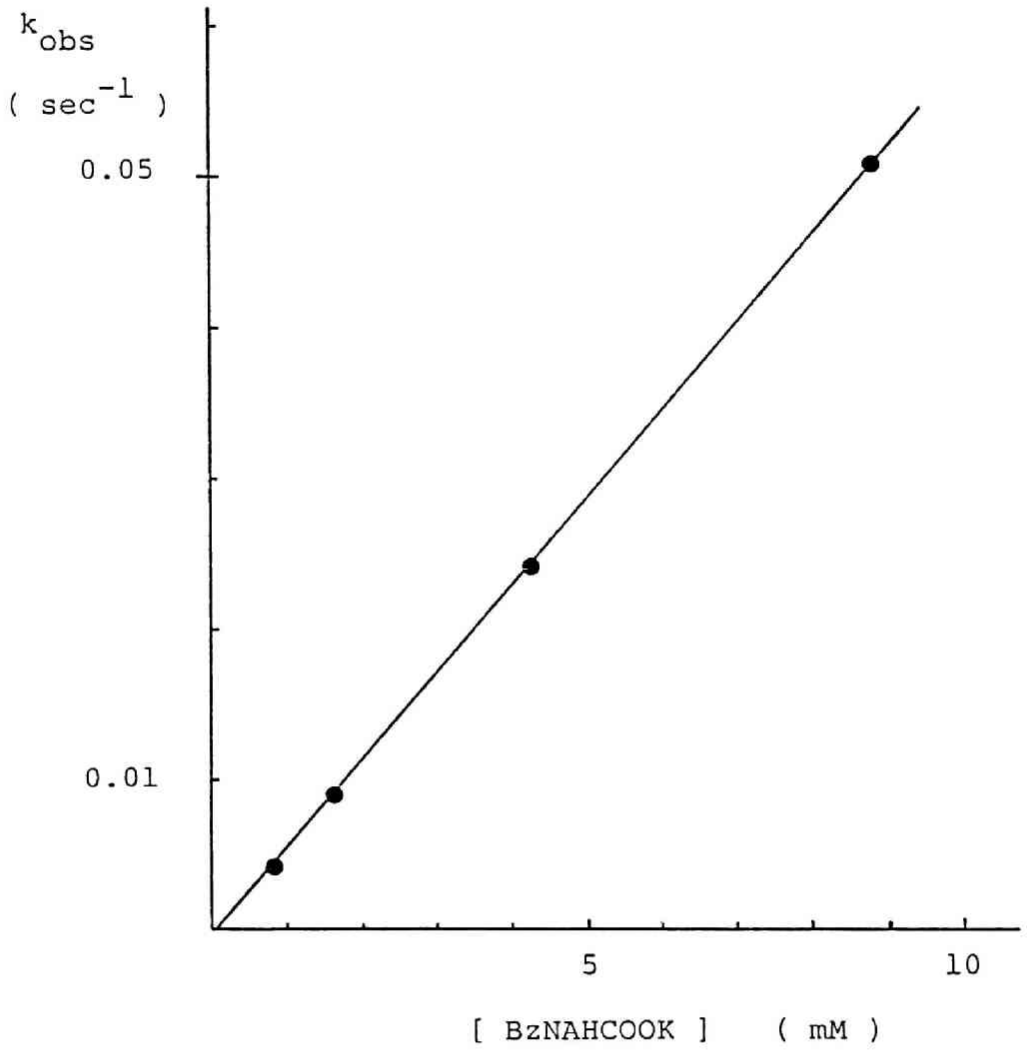


Fig. 1 Fl^{ox}-Lip reduction in the flavo;iposome by BzNAHCOOK : ($^{(o)}$BzNAHCOOK |Fl-Lip| $^{(i)}$)

Table Ⅱ

Oxidation of fl^{red} and Reduction of fl^{ox} in the Membrane

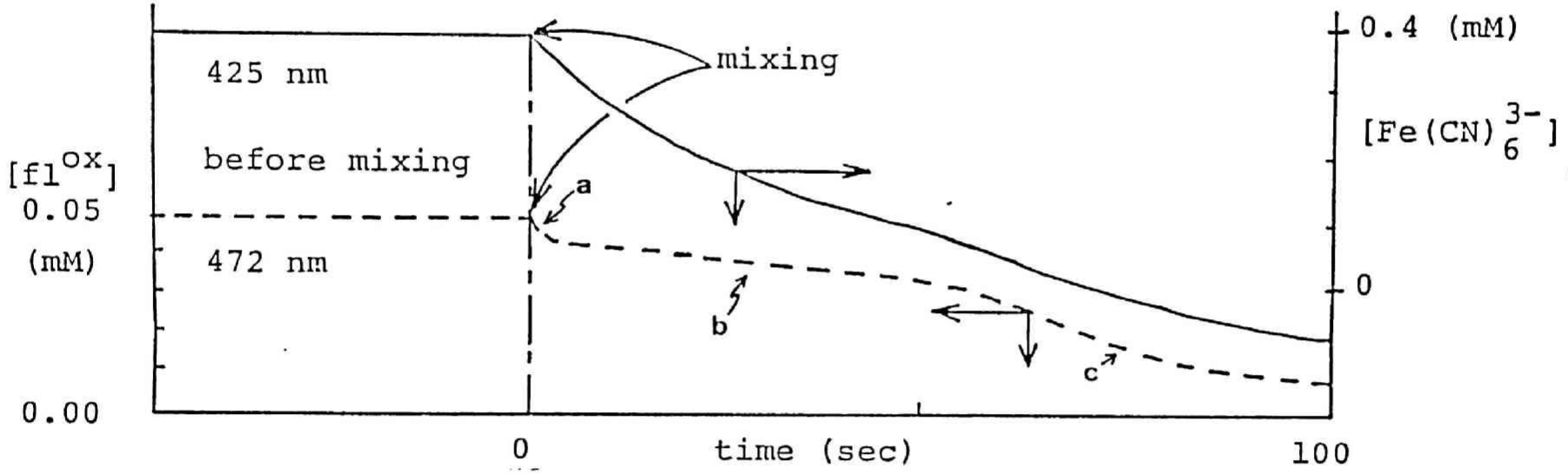
recuctant (mM)	oxidant (mM)	$k_{o \to m}$ ($cm^{-2} \cdot sec^{-1}$)	$k_{m \to i}$ ($cm^{-2} \cdot sec^{-1}$)
BzNAHCOOK	———		———
0.8		0.44×10^{-7}	
1.6		1.0×10^{-7}	
4.3		2.7×10^{-7}	
8.8		5.6×10^{-7}	
$Na_2S_2O_4$	———		
1.0		6.9×10^{-5}	
———	$K_3Fe(CN)_6$	———	
	0.9		1.2×10^{-5}

a) [liposome] = 1.0×10^{-6}M, surface area = 9×10^{4} cm^2

b) $J_{o \to m} = -k_{o \to m} \cdot S(o) \ [fl^{ox}]$

$J_{m \to i} = -k_{m \to i} \cdot S(i) \ [fl^{red}]$

Fig.2 Change in the absorbances at 425nm and 472 nm



(6/4 = fl^{red} / fl^{ox}) under the same reductant concentration. This reflects the slower electron influx from outside BzNAHCOOK to fl^{ox} in the membrane than that from $Na_2S_2O_4$. After the steady state, most of $K_3Fe(CN)_6$ is consumed and a first order decrease of the flavin (fl^{ox}) was followed (region "c"). In the above steady state, the rate determining step of the total electron transport reaction must be the electron influx step from external BzNAHCOOK to the flavolipid in the membrane because the rate was 1st order with respect to the concentration of external BzNAHCOOK (Table Ⅲ or Fig.3). Even at the highest concentration of BzNAHCOOK, the rate did not saturate as observed in the case of $Na_2S_2O_4$ (chapter 2). Furthermore the rate was 20 times slower than the $k_{m\rightarrow i}$ which was estimated from $k_{m\rightarrow o}$ in the reduction of external $K_3Fe(CN)_6$ by reduced flavolipid. (See Table 1)

$$k_{m\rightarrow i} = k_{m\rightarrow o}\, S(i)/S(o) = 2/3\, k_{m\rightarrow o}$$

These observations are compatible with the idea of rate-determining influx. The rate constants of overall electron transport are summarized in Table Ⅲ together with those of liposomes modified with ubiquinone-10 (UQ-10) and di-n-butylviologen (C_4V^{2+}). The flavolipid and ubiquinone-10 are

insert Table Ⅲ here

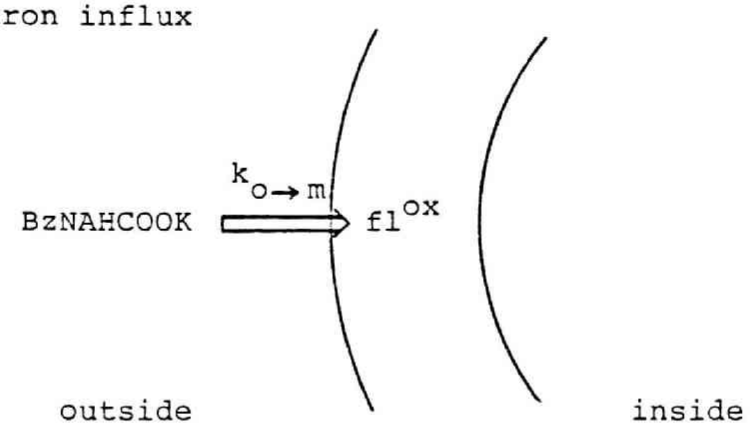
Table Ⅲ Reduction Rate Constants of Internal $K_3Fe(CN)_6$ by BzNAHCOOK in the Functionalized Liposomes [a,b)]

BzNAHCOOK (mM)	mediator (mM)	k (sec^{-1})
2.6	———	0.1×10^{-2}
0.9	flavolipid (0.05)	1.2×10^{-2}
2.6	〃 (0.05)	6.3×10^{-2}
9.2	〃 (0.05)	13×10^{-2}
0.9	UQ-10 (0.05)	0.2×10^{-2}
2.6	〃 (0.05)	0.9×10^{-2}
9.2	〃 (0.05)	2×10^{-2}
2.6	C_4-V^2 (0.05)	0.1×10^{-2}
$Na_2S_2O_4$		
4.0	flavolipid (0.07)	0.44
4.0	UQ-10 (0.07)	1.70
DTT (2.6)[c)]	flavolipid (0.05)	0.6×10^{-2}
H_2/colloidal Pt	〃 (0.05)	0.6×10^{-2}

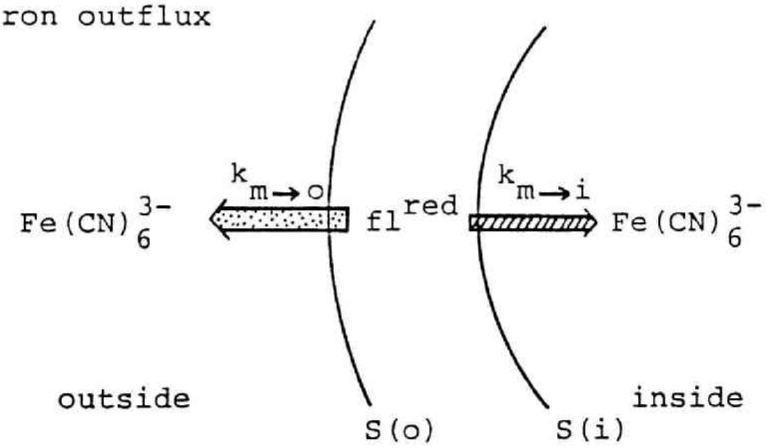
a) At 30℃, pH 7.0 b) Flavolipid/Lecithin = UQ-10/Lecithin = 2.5/97.5 (mol/mol), $[K_3Fe(CN)_6]$ = 0.39 mM. Local Concentration of $K_3Fe(CN)_6$ was 0.75 M. c) DTT: Dithiotheritol

Figure 3. Measurement of electron influx and electron outflux

(i) electron influx



(ii) electron outflux



S(o) : outer surface area of the liposome
S(i) : inner surface area of the liposome

very efficient mediator for the electron transport from external BzNAHCOOK to internal $K_3Fe(CN)_6$. It is intersting to recall, however, that in the case of $Na_2S_2O_4$, the liposome modified with ubiquinone-10 showed more than 4 time faster rate in the transmembrane electron tranport than the present flavoliposome. This order is reverse when RNAH is employed as a reducing agent. In the electron transfer through the flavoliposome, the rate determining step is an influx, from the external reductant to the outer fl^{ox} (out) in the membrane for BzNAHCOOK, while it is shifted to an outflux, the inner fl^{red} to $K_3Fe(CN)_6$ for $Na_2S_2O_4$. Unfortunately, the rate determining step for the ubiquinone-10 modified liposome for the reductant of $Na_2S_2O_4$ has not been identified in the literature [8] although it is determined to be an influx when BzNAHCOOK was employed. Therefore it is difficult to make a direct comparison of the overall electron transfer rates for the flavolipid and ubiquinone-10 modified liposome. Still it may deserve to comment that the flavoliposomes gave faster electron transfer rates by the combination with an important reducing agent such as BzNAHCOOK than the ubiquinone-10 modified liposome system. This suggests an interesting possibility that one of the important roles of the flavoprotein in the complex I of the respiratory chain is the more efficient flux conversion from NADH in the bulk agueous phase to a flavin moiety locating in the membrane than the direct flux conversion from NADH to ubiquinone-10.

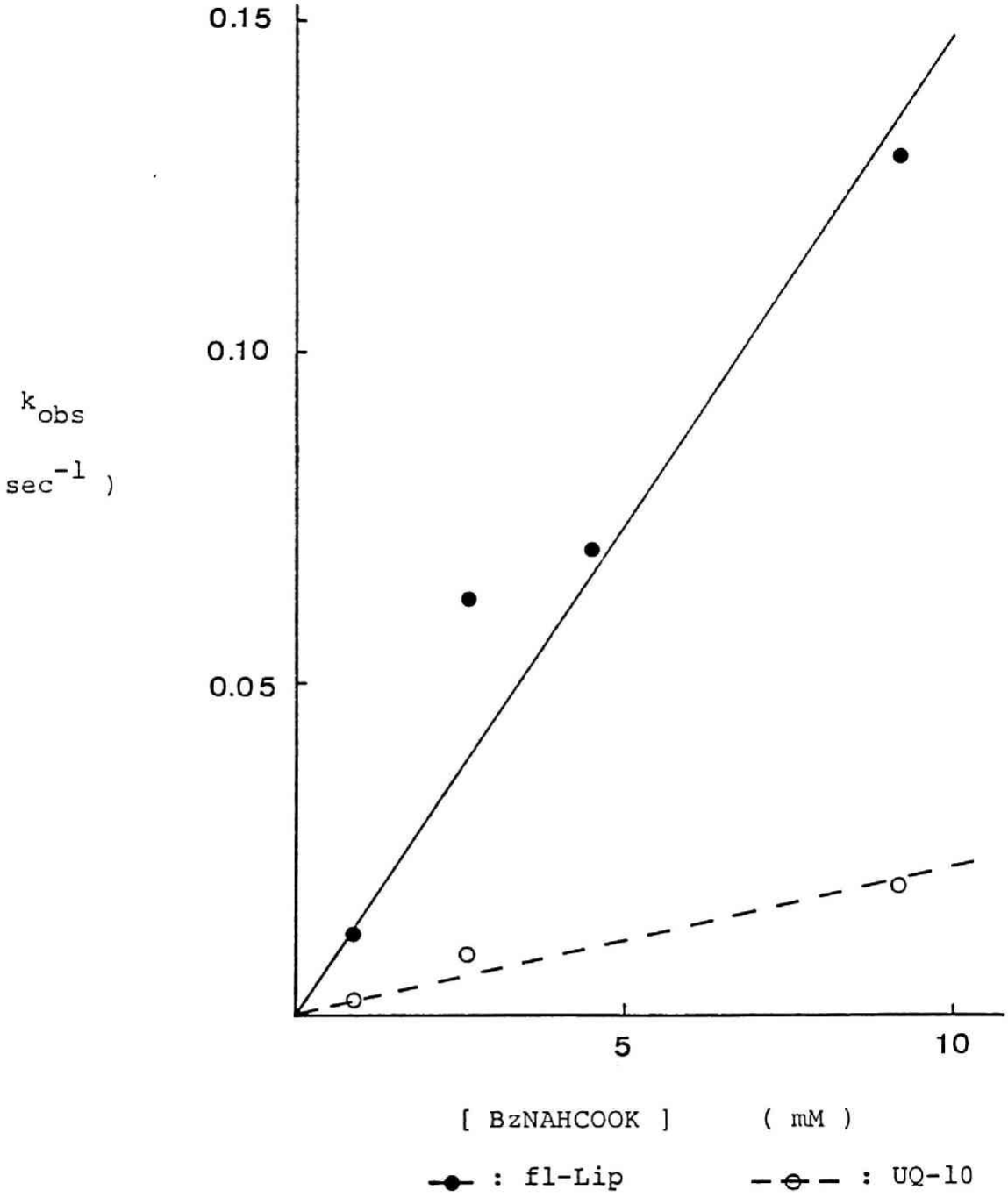


Fig. 4. $K_3Fe(CN)_6$ Reduction by Exterior BzNAHCOOK across the Membrane Modified with Flavolipid or Ubiquinone-10.

Di-n-butylviologen which is moderately efficient electron transport mediator in the case of $Na_2S_2O_4$, showed very poor activity in this case. This may reasonably be accounted for by considering potential differences. The redox potential of C_4V^{2+} is -0.44 vs NHE, [7] which means the reduction by BzNAHCOOK ($E_{1/2}$ = -0.36 V vs NHE) is a thermally unfavorable reaction. Thus, the very slow electron transport rate of C_4V^{2+} using BzNAHCOOK can be ascribed to the slow reduction rate of C_4V^{2+} by BzNAHCOOK.

The present flavoliposome can be reduced also by other weak electron donors. As a model for hydrogen metabolizing systems, hydrogen / colloidal platinum was used as a reducing agent for this system. [9] Dithiothreitol (DTT) also reduced this systems, which suggests the possibility that the flavoliposome could work as a lipoyl dehydrogenase model. [10] For these various electron tranport systems, the artificial liposome modified with the flavolipid was demonstrated to serve as an effective model in the study of biological electron transports.

Electrochemistry of Flavolipid Incorporated into the Bilayer Membrane.

In order to know whether the flavolipid participates in the electron transport process across the lipid membrane, electrochemical redox reactions of the flavolipid were examined. The cyclic voltammogram (CV) of the flavolipid incorporated into

the lecithin bilayer membrane in water ($^{(o)}$ |fl-Lip| $^{(i)}$) (pH 7.0) clearly showed two pairs of cathodic and anodic waves at -0.35 V vs SCE and at -0.70 V vs SCE using a Hg-pool as a working electrode (Fig. 5.) It is reported previously [11] that lecithin and other surfactants in water, are adsorbed on a Hg electrode surface to inhibit normal redox reactions of Cd^{2+} on the electrode. [12] In spite of the report on such an inhibitory effect of lecithin, the present flavoliposome (0.025 mM flavolipid) afforded clear oxidation-reduction peaks. This may be explained by the idea that the flavin unit (redox site of the liposome) locates at the vicinity of the Hg-lecithin membrane interphase to favor the electron uptake and the electron release from Hg to flavin and vice versa, respectively. Clearly separated two-step redox conversions may reasonably correspond to the formation of an intermediate semiquinone (flH$\cdot$) and a reduced flavin flH_2. The redox potentials of flavodoxin are separated at -0.315V and -0.573V vs SCE ($E_{1/2}$) which are ascribed to flH$\cdot$ / fl^{ox} and flH_2 / flH$\cdot$, respectively. It is interesting to note that a relatively large splitting of 1st and 2nd reduction steps is observed for flavins locating in a hydrophobic enviroment. In contrast to the above case , the flavoliposome containing $K_3Fe(CN)_6$ in its interior aqueous phase ($^{(o)}$ | fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$) afforded only one redox couple of cathodic and anodic waves at -0.35 v vs SCE ($E_{1/2}$). Here, the redox reaction was observed only for the

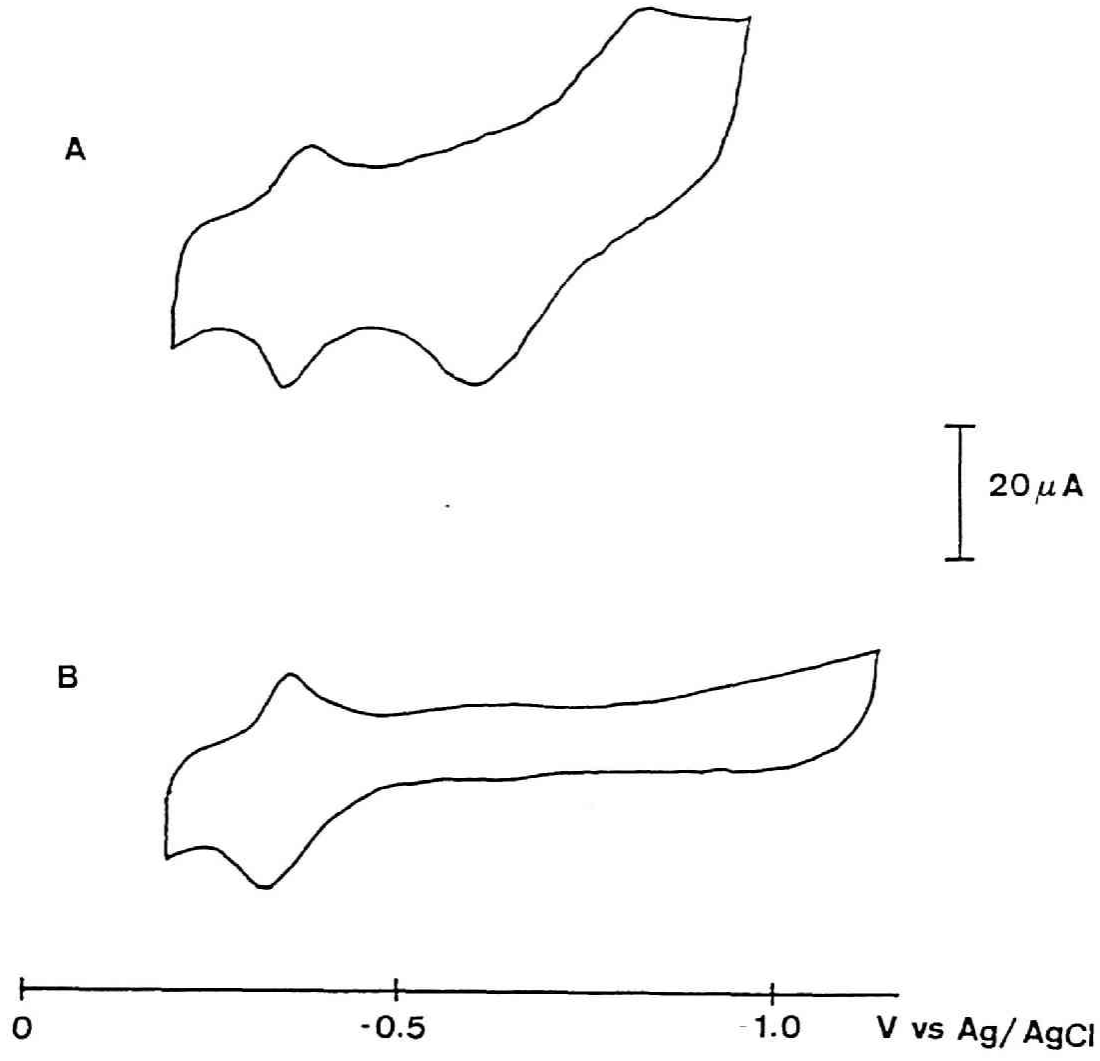


Fig. 5. Cyclic Voltammograms on Hg-Pool of the Flavoliposome in 5 mM-Tris HCl (pH=7.0)

(A) ; ($^{(o)}$|fl-Lip|$^{(i)}$)

(B) ; ($^{(o)}$|fl-Lip|$^{(i)}$ $K_3Fe(CN)_6$)

fl^{ox} / flH· redox couple but not for the formation of flH_2 by the presence of Fe(Ⅲ) which may well quench the intermediate flH_2. This may provide an additional evidence of the incorporation of the flavolipid in the electron transport process.

Experimental Section

Instruments.

^{1}H-NMR spectra were obtained with a JEOL JNM PMX 60SI NMR spectrometer, a JEOL JNM FX90Q FT NMR spectrometer or JEOL JNM GX400 spectrometer. Mass spectra were obtained with a JEOL JMS-DX 300 mass spectrometer. IR spectra were recorded on a Hitachi model 260-50 spectrophotometer. Fluorescence spectra were measured with a Shimadzu difference spectrofluorophotometer RF-503A. Electronic spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophotometer. Sonication was performed with an ultrasonic disruptor, Mode UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument.

Materials.

Unless otherwise, all reagents and chemicals were obtained commercially and used without further purification. Egg yolk lecithin was carefully purified according to the literature method [14] and stored at -75℃ under Ar in the dark until its use. BzNAHCOOK was prepared according to the literature method [4] and identified by ^{1}H-NMR, IR, electronic spectra and mass spectra.

Preparation of Artificial Single-Wall Bilayer Liposome.

Artificial egg lecithin liposome containing $K_3Fe(CN)_6$ in its interior aq. phase ($^{(o)}$ |lecithin| $^{(i)}$ $K_3Fe(CN)_6$), arfificial liposome modified with the flavolipid ($^{(o)}$ |fl-Lip| $^{(i)}$) and the liposome modified with the flavolipid containing $K_3Fe(CN)_6$ in its interior aq. phase ($^{(o)}$ |fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$) were prepared according to the sonication method reported proviously by us [15].

Measurement of Leakage Rate of NADH and its Models through the Bilayer Membrane.

Leakage rates of NADH and its models through the lecithin bilayer membrane were measured by the reduction rate of internal $K_3Fe(CN)_6$ contained in the liposome without a functionalization by the flavolipid ($^{(o)}$ |lecithin| $^{(i)}$ $K_3Fe(CN)_6$). Into the 2.0 mL of the deaerated liposome solution (5 mM-Tris HCl, pH = 7.0) was added 0.2 mL of the deaerated NADH model solution freshly prepared using a syringe stopped flow apparatus. Reduction of the internal $K_3Fe(CN)_6$ was followed by monitoring the change of absorbance at 425 nm.

Reduction of Flavolipid (fl^{ox}) Incorporated into the Bilayer Membrane by External BzNAHCOOK.

Reduction of flavolipid incorporated into the bilayer membrane by BzNAHCOOK was performed using a freshly prepared flavoliposome ($^{(o)}$ |fl-Lip| $^{(i)}$) in a way similar to the

reduction by $Na_2S_2O_4$ described previously.[15]

Oxidation of Flavolipid (fl^{red}) Incorporated into the Bilayer Membrane by External $K_3Fe(CN)_6$.

A freshly prepared flavoliposome solution ($^{(o)}|fl\text{-}Lip|^{(i)}$, 2.0 mL) was placed in a 10mm - quartz cell equipped with a three-way stopcock. After the solution was deaerated with the usual manner, 0.2 mL of a freshly prepared $Na_2S_2O_4$ solution (2.0 mM) was added. The reduction of fl^{ox} was completed within 1 minute, which was ascertained by monitoring the change of absorbance at 444 nm. Then, into the reduced flavoliposome solution ($^{(o)}Na_2S_2O_4 \mid fl^{red}\text{-}Lip|^{(i)}$) at 25℃, 0.2 mL of a deaerated $K_3Fe(CN)_6$ solution (11.0 mM) was added by using a specially designed syringe.[15] The re-oxidation of fl^{red} in the bilayer membrane was followed by monitoring the change of the fluorescence intensity at 510 nm excited at 340 nm.

Reduction of $K_3Fe(CN)_6$ in the Interior Aq. Phase of the Flavoliposome by External BzNAHCOOK.

Reduction of internal $K_3Fe(CN)_6$ by external BzNAHCOOK was carried out by using the method similar to the reduction by $Na_2S_2O_4$, except that the ratio of fl^{red} to fl^{ox} was 1/9～3/7 in the steady state of the reduction.

Cyclic Voltammetry of the Flavoliposome.

Bioanalytical system CV-1B cyclic voltammograph was employed for cyclic voltammetric determination of the flavolipid incorporated into the bilayer membrane. A freshly prepared flavoliposome solution (5 mL) was degassed by nitrogen bubbling for 1/2 hour. As a supporting electrolyte KCl(0.1M) was used and Hg-pool electrode was used as a working electrode. Potentials were determined at 25℃ vs an Ag/AgCl electrode as a reference.

Hydration Rate Measurement of RNAH in Tris buffer Solution.

Hydration rate of RNAH was monitored by the 1st order decrease of the intensity in absorbance at 360 nm, characteristic of the reduced form of NADH models, by using freshly prepared NADH model solution of 5 mM Tris · HCl.

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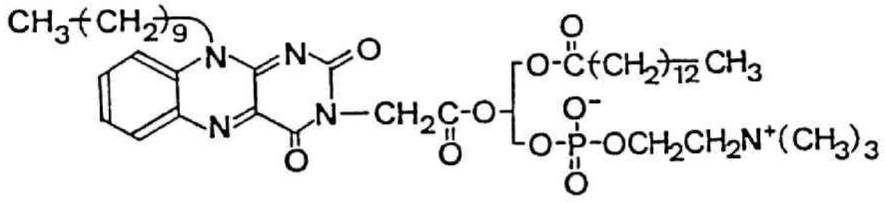
Chapter 5

Electron/Proton Flux Coupling and pH Gradient Generation across the Bilayer Membrane with a Flavolipid.

Abstract.

Pyranine, a hydrophilic pH indicator, was incorporated into the interior aqueous phase of an artificial liposome which transduces electron through the flavolipid (1). Proton influx coupled with the facilitated electron transport across the lipid bilayer membrane was observed by monitoring the fluorescence of the pyranine incorporated. The total proton influx by this proton/electron flux coupling served to generate a pH gradient as high as 3.9 which was large enough for the ATP synthesis. The proton transport was facilitated by the electron transport by a factor of 10^2 compared with the passive proton transport across the bilayer membrane without such an electron transducing unit.



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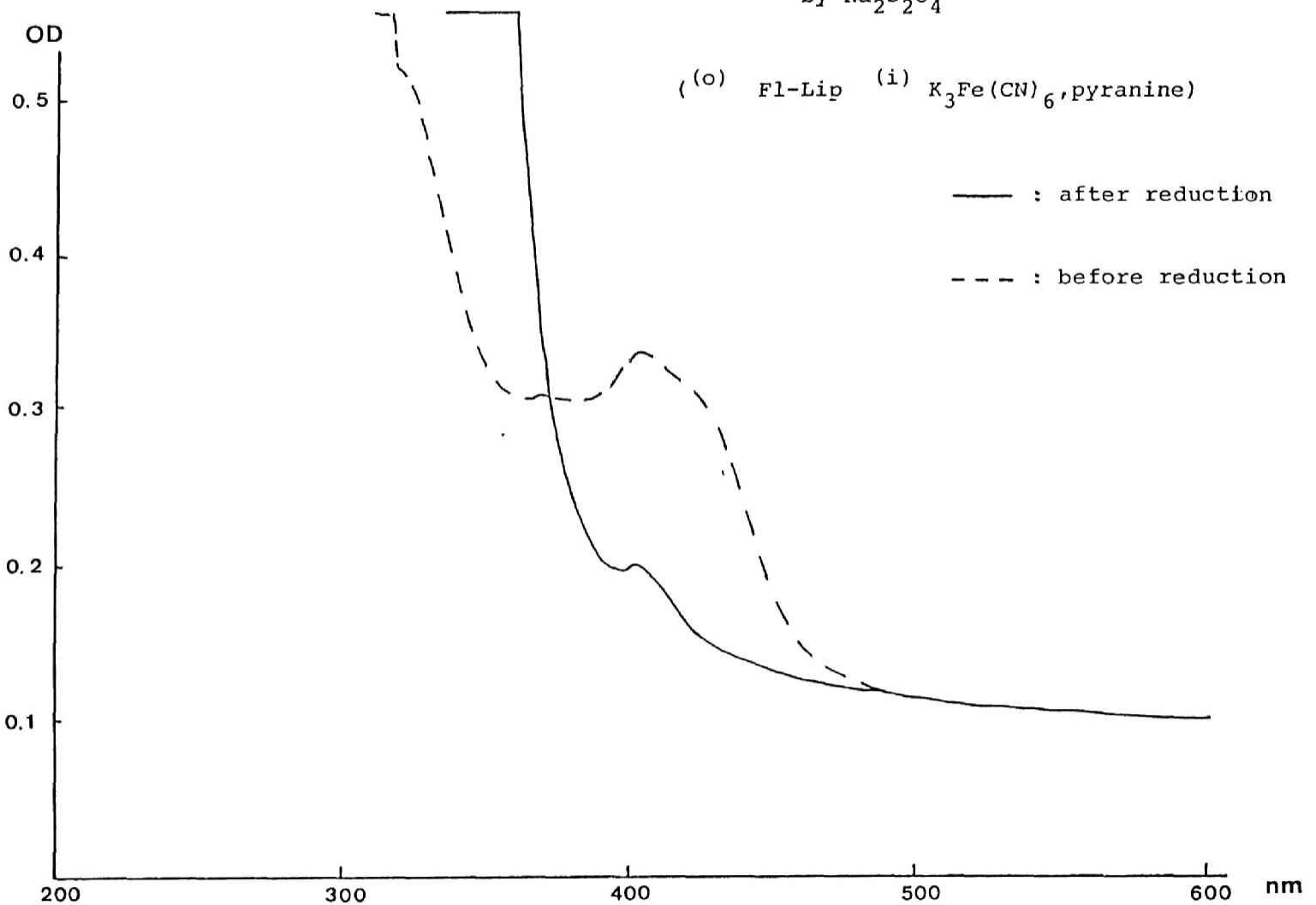
Introduction.

The respiratory chain of mitochondria is known to carry out the efficient electron and proton transports in a coupled manner across a biological membrane to generate a pH gradient. [1] The chemiosmotic hypothesis originally proposed by P. Mitchell is now established as that the pH gradient thus generated was effectively used for the ATP synthesis by ATPase located at the same coupling membrane. [2] In the miltienzyme system of mitochondria, the flavoprotein in the Complex I plays a key-role in the electron transport and the proton translocation at the initial event of the oxidative phosphorylation. [3] It is of our central interest to construct a simplified artifitial system which mimics such a biologically important function and to give the molecular mechanism of the flux coupling. As the simplest flavoenzyme model, the artificial flavolipid (1) was synthesized and demonstrated to transduce electrons through an artificial liposomal membrane. [4] In this chapter, we wish to report that a pH gradient is generated by the H^+ transport coupled with the effective electron transport enough to give rise to the synthesis of ATP .

Fig. 1. Electronic Spectra of the Flavoliposome : before and after reduction by $Na_2S_2O_4$



Results and Discussion.

Preparation of a pH Sensitive Artificial Liposome.

As a pH indicator of the liposomal interior, trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate, pyranine, was chosen because it satisfies fundamental requirements to monitor the pH change of the interior aqueous phase of the artificial cell. (i) Pyranine is reported to have pKa at 7.22 ± 0.04 and should be sensitive to the pH change around 7. [5] (ii) Pyranine is incorporated stably into the interior aqueous phase and its leakage was determined to be less than 1% per day. This rate is far slow compared with rate processes of electron and / or proton movement which are in the order of 10^{0} sec^{-1}. (iii) Furthermore, pyranine is highly stable to reductants such as $Na_2S_2O_4$, H_2 / colloidal Pt, dithiothreitol and NADH model compounds and to oxidants such as oxygen and $K_3Fe(CN)_6$. There was observed no change of the electronic and fluorescence maxima of pyranine incorporated into the liposomal interior before and after the sonication in the presence of $K_3Fe(CN)_6$ and the reduction with various reductants ($Na_2S_2O_4$, NADH analogues) as shown in Fig. 1. (iv) In spite of the presence of iron (III) (0.2 mM), logarithms of ratios of the emission intensities at 510 nm upon exciting pyranine at 450 nm and 400 nm, log [I_{450} / I_{400}], which correspond to exitations of dissociated and undissociated forms of pyranine, respectively, showed a good linear

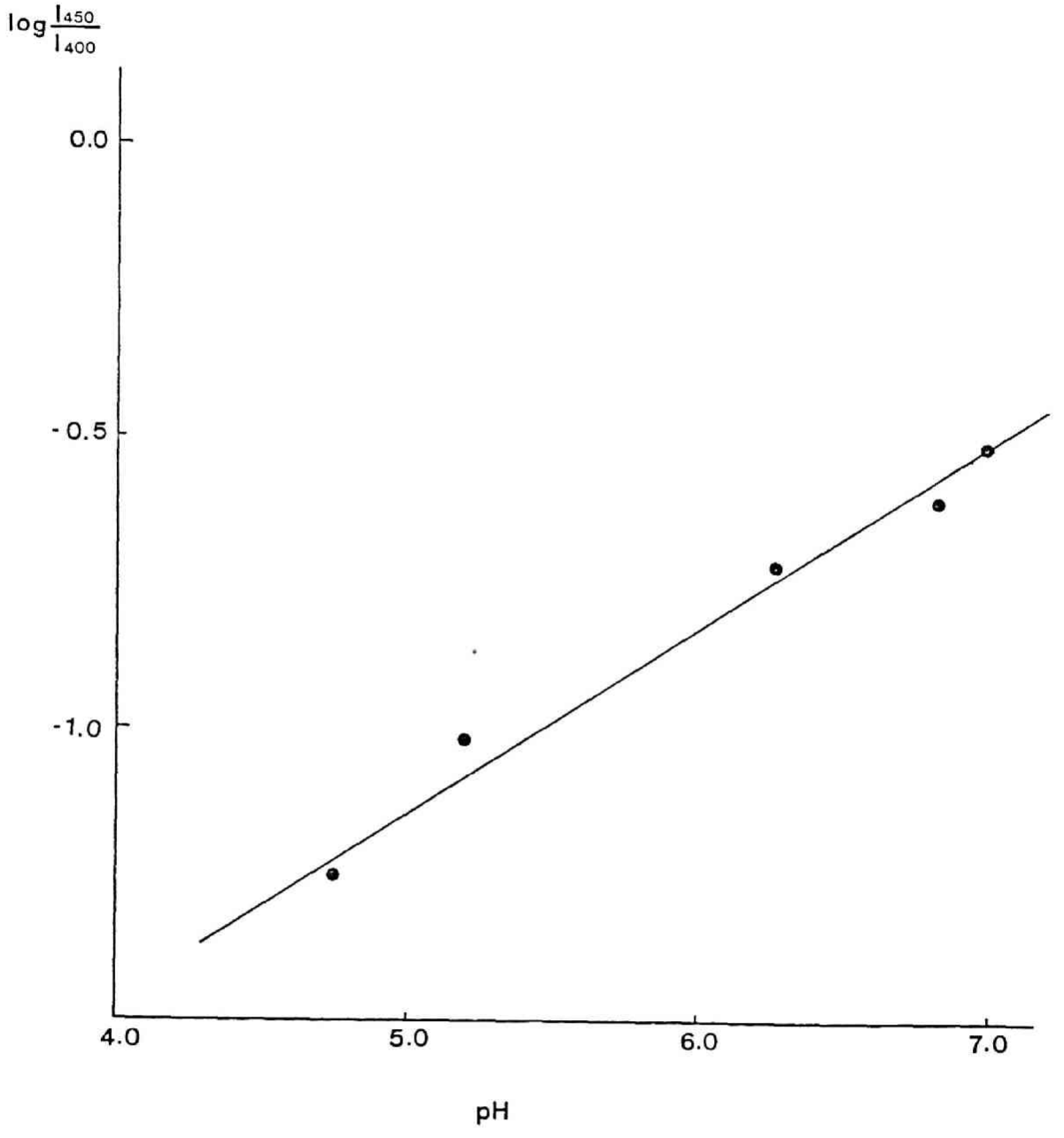
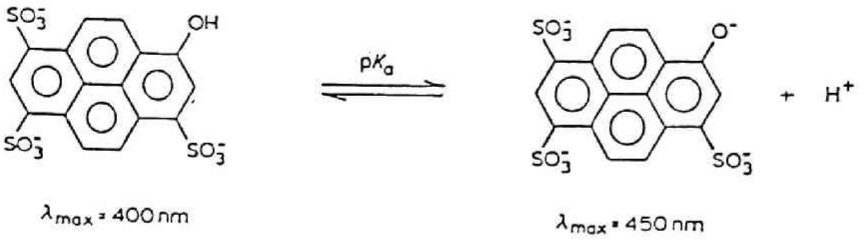


Fig. 2. Ratio of relative intensities in the excitation spectra at 450 nm over 400 nm, observed at an emission at 510 nm - vs pH.

relationship to the pH of the lecithin liposome solution in the pH range of 4.1-7.0. (see Fig. 2.) Therefore, the fluosescence intensity ratio, log [I_{450} / I_{400}], can be a measure of the pH of the liposomal interior .



The artificial liposome, incorporating pyranine to its interior, functionalized with the flavolipid showed normal behaviors with respect to gel permeation, dynamic light scattering (mean diameter = 250 Å), electronic spectroscopy except an additional peak at 400 nm characteristic of pyranine, and good stability (no precipitate was observed after 7 days). These observations demonstrate that pyranine does not disturb significantly the bilayer membrane structure and that it is located stably into the liposomal interior aqueous phase.

Electron / Proton Flux Coupling.

Accompanied with a reduction of ferri- to ferrocyanide, an electromotive force is generated to drive an influx of cationic, or an outflux of anionic species, respectively. Here the proton influx and / or the hydroxide outflux, (hereafter these are called simply as the proton influx), can be followed by

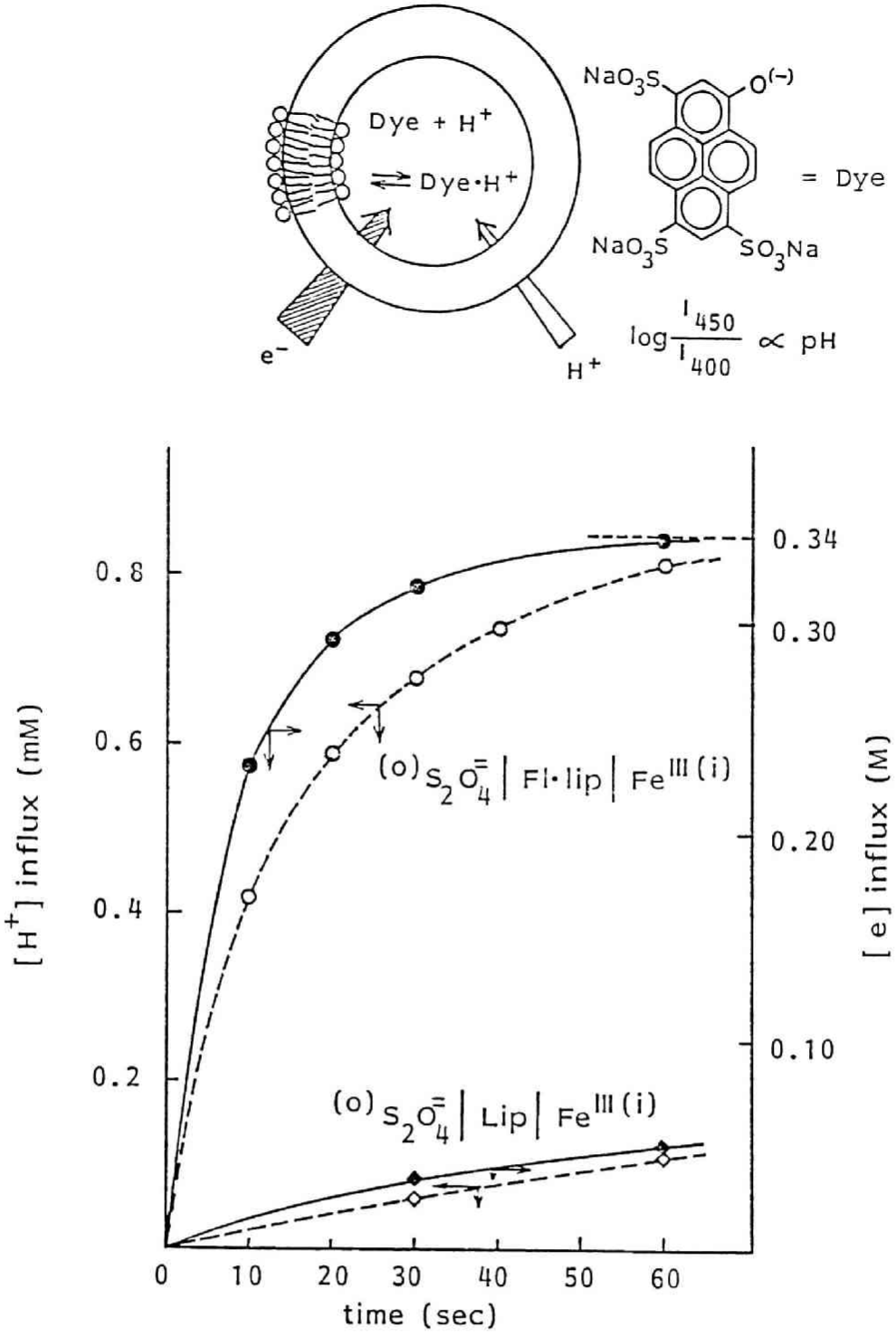
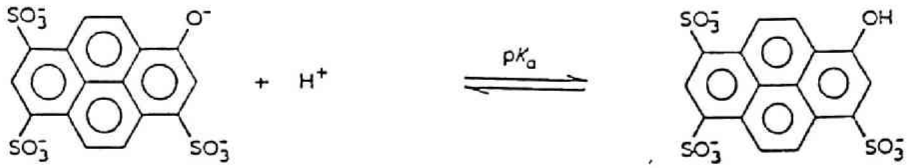


Fig. 3. Proton/Electron Flux Coupling

◆, ● : proton influx

◇, o : electron influx

monitoring the fluorescence of pyranine. Most of the proton transported were consumed to shift the equilibria of the indicator and the buffer :



$$H_2NC(CH_2OH)_3 + H^+ \overset{pKa'}{\rightleftharpoons} H_3\overset{+}{N}C(CH_2OH)_3$$

The amount of protons transported, $\triangle [H^+]$, was calculated from the observed pH difference from the initial, $\triangle pH(obs)$, and plotted along with the amount of electrons transported as a function of time in Fig. 3. The proton influx showed a similar behavior to that of the electron influx into the flavoliposome system. About 90 % of ferricyanide initially present in the interior aqueous phase was converted to ferrocyanide after 60 sec, when the proton influx reached almost its apparent equilibrium state, $\triangle [H^+]$ being 0.8 mM at the equilibrium. The similarity of the influx behavior strongly suggests that the active proton transport from the exterior to the interior is coupled with the facilitated electron transport via the flavolipid. [7)]

The pKa of the oxidized form of a flavin locates near zero. In contract, both half reduced and reduced flavins are known to

have pKa's around pH 7.0, namely pKa's of the semiquinone of flavin and of the reduced flavin being about 6.5 and 6.2 respectively (Fig. 4). [8] Then, the reduced flavolipid can easily uptake and release protons from the exterior and to the interior aqueous phases. The proton leakage through the membrane containing the flavolipid in an oxidized form was obtained separately as 10^{-8} sec^{-1}. cm . Compared to this, the rate of proton flow coupled with an electron flow (active transport of protons through the membrane with the reduced and / or semiquinone form of the flavolipid), P_{H^+} , (10^{-6} sec^{-1}. cm) is enhanced by a factor of 10^2. This enhanced permeability may be explained by the idea that the pKa's of the flavolipids are shifted into an appropriate region to lower the potential barriers of proton movement. The net amount of the transport of protons here observed must be a result of the true facilitated influx of $[H^+]$,"active transport by the use of the energy of electroneutrality principle" minus a passive leakage of H due to the proton gradient generated by the above process. Therefore the true $[H^+]$ influx seems to be higher than the apparent $[H^+]$ influx here observed. Still the equilibrium coupling ratio of a proton to an electron flux, expressed by, $\triangle [H^+] / \triangle [e^-] = 1/400$ after 60 sec, is certainly low. It is clear that flavin itself is acting as an efficient electron-transducer with a little capacity of proton mediator, but cannot be an efficient coupler. It is highly probable that the

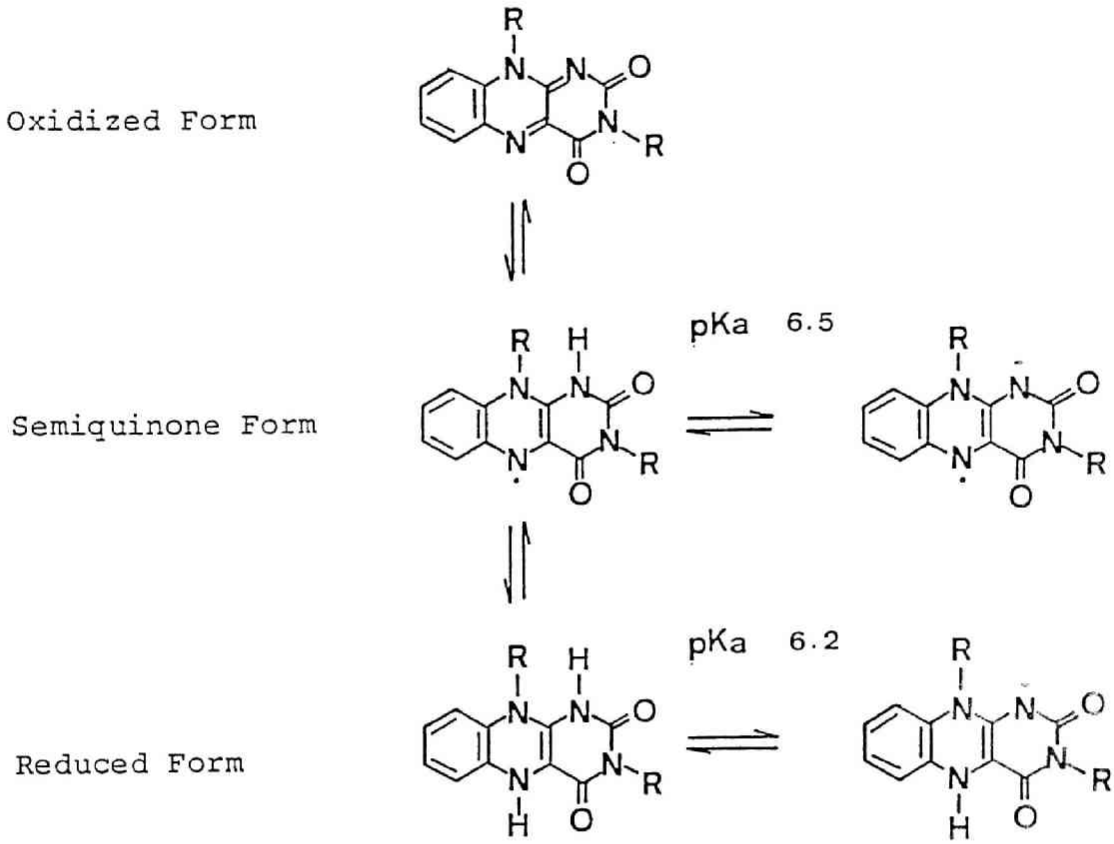


Fig. 4. Redox states and pKa' of of flavin

electromotive force here generated could be coupled with various other endoergonic processes. The magnitude of this pH gradient is large enough for the energy needed for ATP synthesis. [6)]

Experimental Section

Instruments.

Electronic spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophoto-meter thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. Fluorescence spectra were measured with a Shimadzu difference spectrofluorophotometer RF-503A thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. Sonication was performed with an ultrasonic disruptor, Model UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. Measurement of pH was performed on a Toa pH meter, Model HM 5-ES instrument.

Materials

Unless stated otherwise, all reagents and chemicals were obtained commercially and used without further purification. Egg yolk lecithin was carefully purified according to the literature method [9] and stored at -75℃ under Ar in the dark until the use. Flavolipid was prepared according to the method reported previously by us. [10]

Preparation of Single-Wall Bilayer Liposomes.

The artificial liposome functionalized with the flavolipid (1)

containing pyranine and $K_3Fe(CN)_6$ in its interior aqueous phase ($^{(o)}$ |fl-Lip| $^{(i)}$ $K_3Fe(CN)_6$ pyranine) was prepared according to the slightly modified procedure reported previously by us [10]. A solution of 78 mg of carefully purified egg yolk lecithin and 2.4 mg of the flavolipid (1) in 15 mL of $CHCl_3$ was evaporated gently under reduced pressure under Ar. The resulting thin film was dried in vacuo for 8 h in the dark at room temperature. To the dried thin film was added 5 mL of a 1 mM Tris HCl (pH 7.0) solution containing 0.34 M of $K_3Fe(CN)_6$ and 0.004 M of pyranine and the lipid film was suspended in the solution. The suspended mixture chilled in an ice bath was sonicated in a box filled with Ar for 5 min. The sonication was repeated twice at an interval of 10 min. The sonicated mixture was centrifuged (1.9×10^3 rpm) for 15 min at 4℃ and the yellow supernatant was applied onto a Sepharose 4B column (1.0×40 cm) by using a 1 mM aqueous Tris HCl buffer (pH 7.0) as an eluent, at 4℃. Single-wall liposomes were collected form eluates between 34 to 45 mL (± 2 mL). The retention volume was similar to that of the egg lecithin liposome without an incorporation of pyranine ($^{(o)}$ |licithin| $^{(i)}$ $K_3Fe(CN)_6$).

Fluorescence Spectra - pH Titration of Pyranine Incorporated into the Aqueous Phase of the liposome.

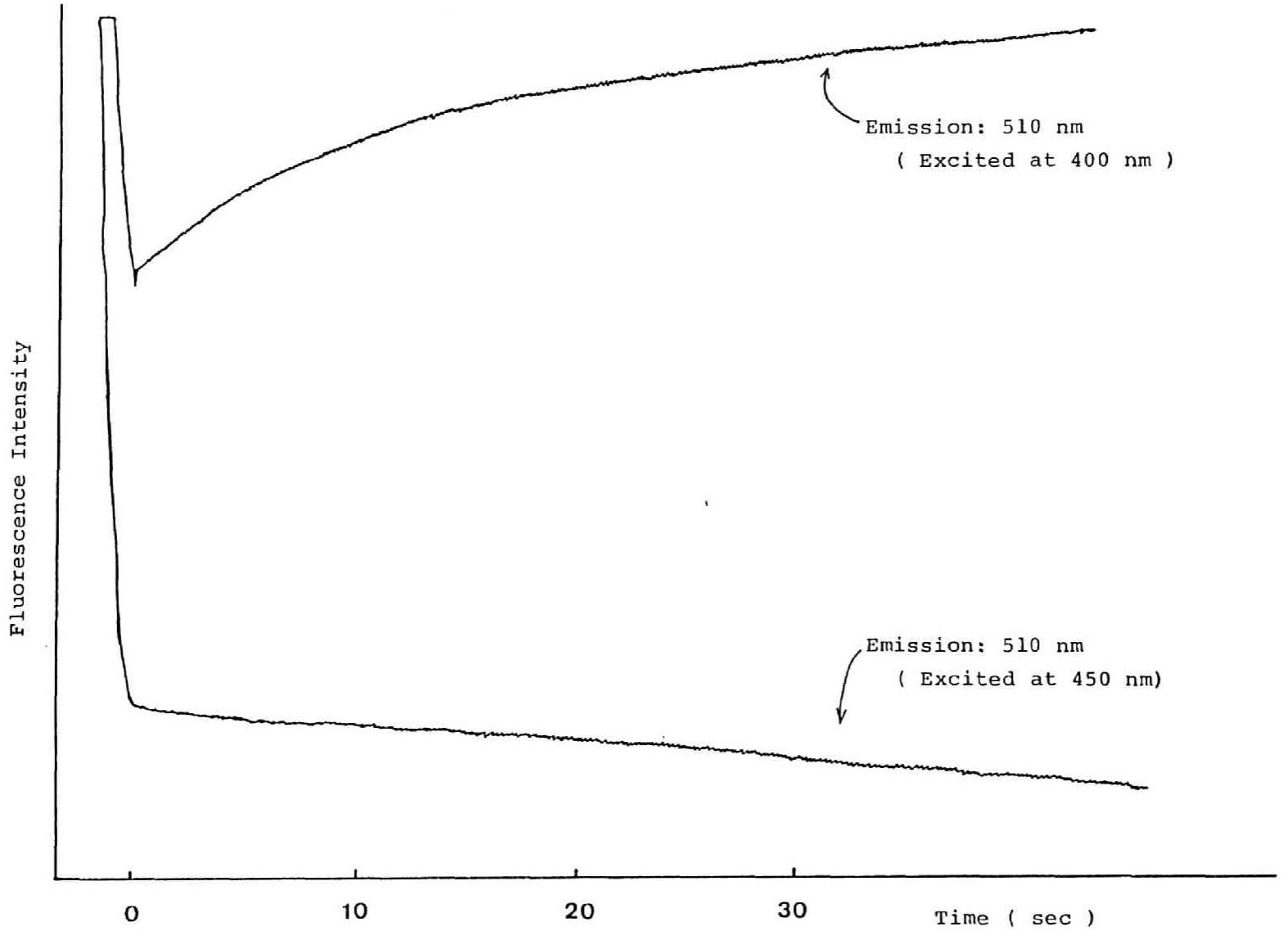
To 2 mL each of the liposome containing pyranine (2 mM) and $K_3Fe(CN)_6$ (0.17 M) in its interior aqueous phase,

($^{(o)}$ |lechthin| $^{(i)}$ $K_3Fe(CN)_6$ pyranine), was added 0.5 mL of an appropriate concentration ($10^{-4} \sim 10^{-3}$ M) of hydrochloric acid. The solutions were allowed to stand at 4℃ for 24 h to make the internal pH equal to the external pH. Emission intensities of pyranine at 510 nm exciting at 450 nm and 400 nm of each solution was measured at 25℃ and pH of the solution was measured by using a pH electrode.

Reduction of $K_3Fe(CN)_6$ Incorporated into the Interior aq. Phase by $Na_2S_2O_4$ and Proton Influx.

Reduction of the interior $K_3Fe(CN)_6$ by the exterior $Na_2S_2O_4$ was carried out by using the method described by us. [4] The change in the $K_3Fe(CN)_6$ concentration was estimated by following the change of absorptions at 425 nm and 472 nm. The change of the hydrogen ion concentration in the interior aq. phase was monitored by the change of emission intensities at 510 nm exciting at 450 nm and 400 nm separately (I_{450} / I_{400}) as shown in Fig. 5. All kinetic measurements were performed at 25 ± 0.5℃.

Fig. 5. Change of Emission Intensity at 510 nm Excited at 400 nm and 450 nm.



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Chapter 6

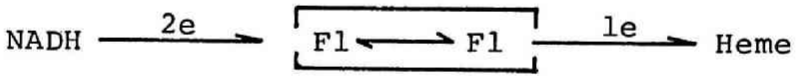
Synthesis of Novel Bis-flavosurfactants as a Transmembrane Electron Channel in Bilayer Membrane

Abstract

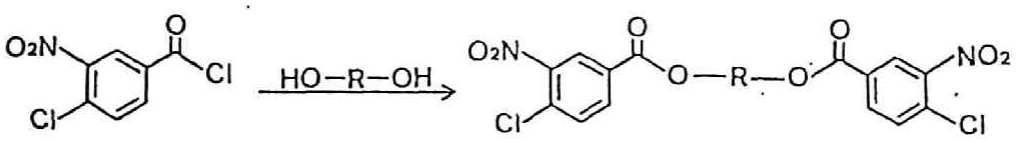
The artificial bis-flavosurfactants (1a,b), which have two flavins connected with dodecamethylene or cholesteryl skeleton and have two ammonium groups as a polar head, were synthesized according to the synthetic scheme 1. The bis-flavosurfactant was proved to be incorporated stably into the lecithin bilayer membrane based on behaviors observed for gel filtration, dynamic light scattering and electronic absorption spectrum of the liposome solution. Electron transport via the bis-flavosurfactant (k_{obs} = 0.64 sec^{-1}) across the bilayer membrane showed a 25 times faster rate than that for the artificial flavolipid of a half channel type under the low concentration conditions (6 molecules of the electron mediator per 1 liposome). The result may suggest that the bis-flavosurfactant acts as an efficient transmembrane electron channel.

Introduction.

In the action of cytochrome P-450 reductase containing two flavins, two electrons uptaken from NAD(P)H are released one by one to the heme part of cytochrome P-450. [1] Iyanagi and coworkers suggested that these two flavins play a key role in such a flux control of cytochrome P-450 reductase. [2] Detailed mechanisms of the electron transfer between two flavins and of flux control still seem unclear.

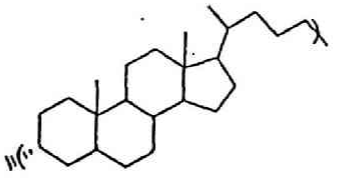


Synthesis of a simplified model system having two flavins may be expected to give insights into the electron transfer occurring in cytochrome P-450. We wish to report here the successful synthesis of the bis-flavin surfactant. This is a logical extension of the model reaction described in the previous chapters, [3] where the electron transport was claimed to depend on the formation of "transient" channel. The electron transfer enhanced by a factor of 25 compared to that with the flavolipid was observed and the result suggests the occurrence of the transmembrane electron channel.

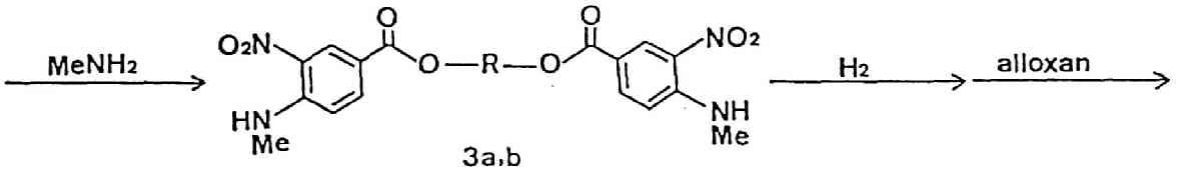


2a,b

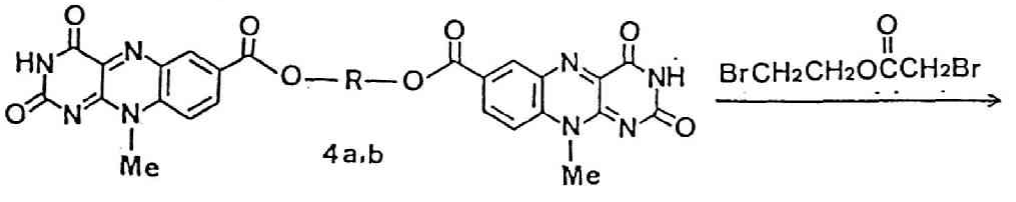
R : $-(CH_2)_{12}-$ a



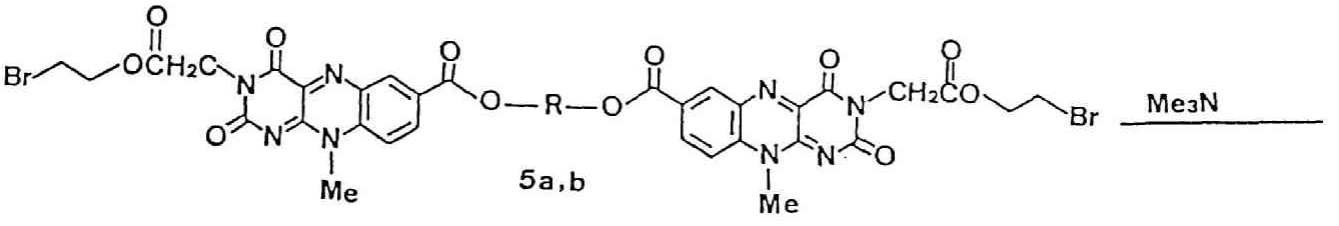
b



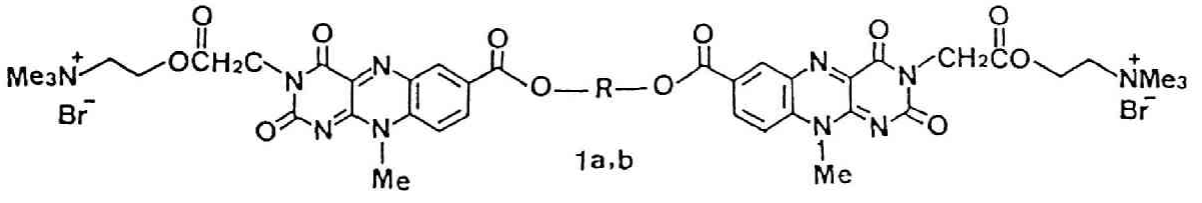
3a,b



4a,b



5a,b



1a,b

Results and Discussion.

Prepartion of Bis-flavosurfactant and Its Incorporation into the Bilayer Membrane.

Bis-flavosurfactants (1a,b) were synthesized according to Scheme 1. Preparation of two flavin rings was carried out by the reduction of bis-(nitro-aminobenzoate) (2a,b) with hydrogen / Pt followed by the treatment with alloxan monohydrate in the presence of HCl. [4] The dimmeric flavin thus obtained was successfully alkylated at the 3-position of the flavin rings and quaternalized with trimethylamine. Both flavin rings are connected with dodecamethylene or cholesteryl skeleton and the edge-edge distance between two flavins was estimated to be about 18Å by CPK model. Both ends of the surfactants are fitted with ammonium as a hydrophilic head group. Total molecular lenghth of the bis-flavosurfactant was adjusted to the width of the bilayer membrane composed of egg lecithin (about 40Å).

The artificial bis-flavosurfactant (1a) was incorporated into the lecithin liposome according to the method previously described. [5] The stable incorporation (0.15 mol% with respect to lecithin) was ascertained by the following observations. (i) The gel permeation behavior of the sonicated mixture of surfactant and egg lecithin in Tris buffer was similar to that of the liposome from lecithin itself (See Fig.1). (ii) Dynamic

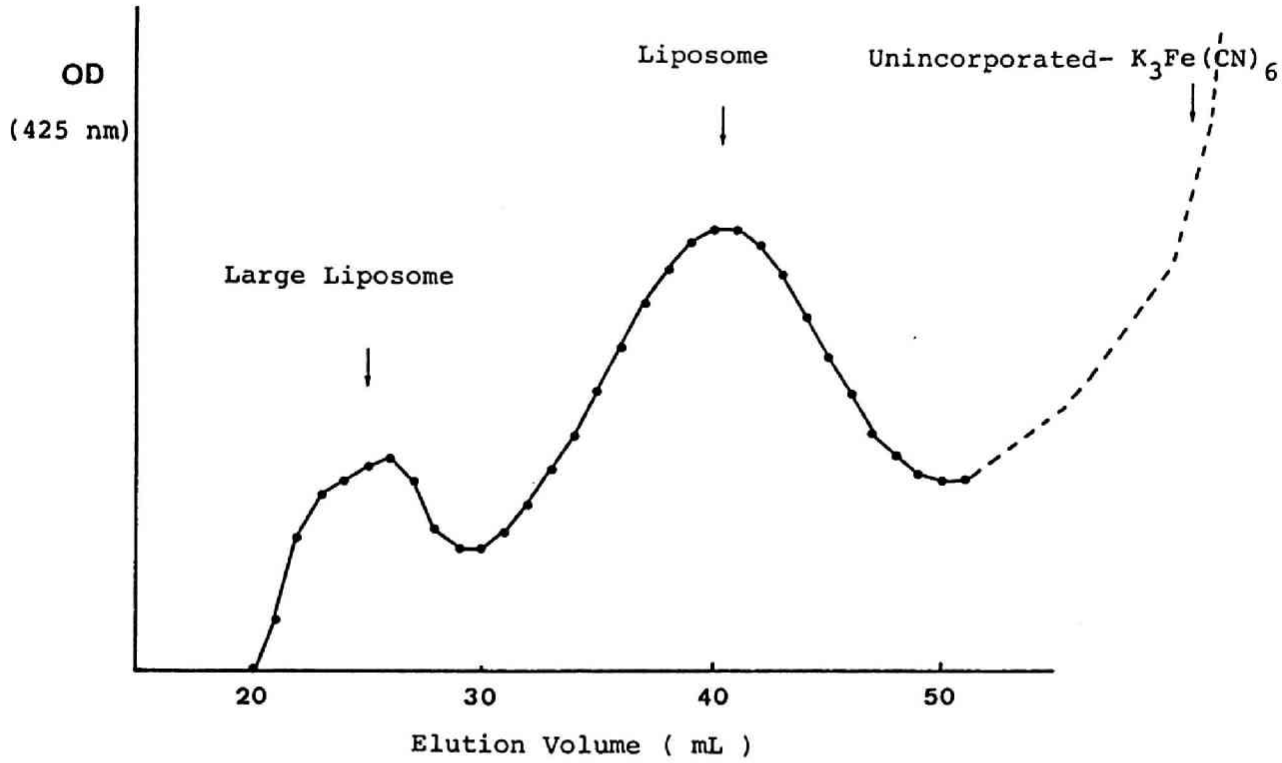


Fig. 1. Elution Curve of the Liposome Modified with Bis-flavosurfactant (1a).

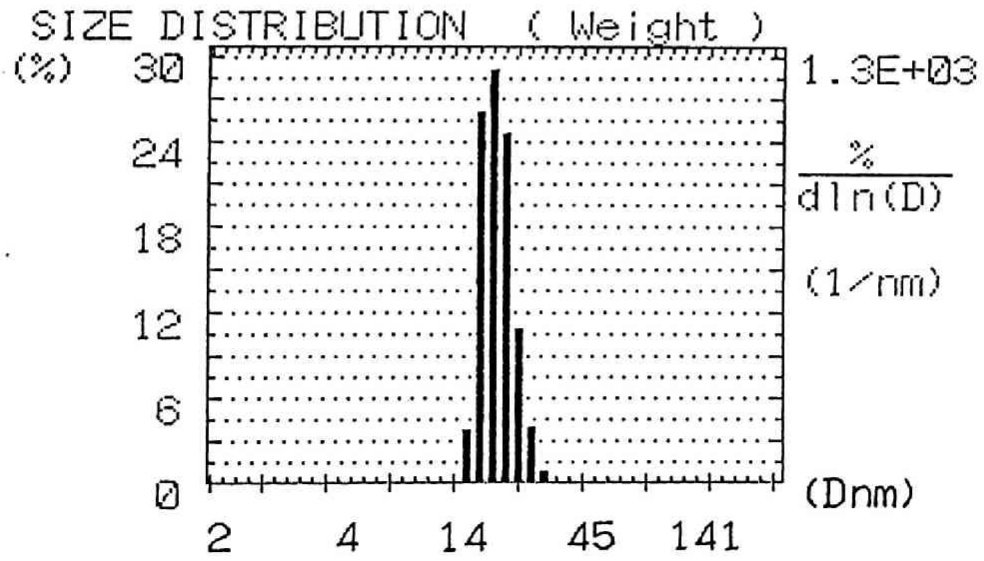


Fig. 2. Dynamic Light Scattering of the Liposome Modified with Bis-flavosurfactant (1a).

light scattering studies of the liposome solution ($^{(o)}$ |bis-fl|$^{(i)}$, $^{(o)}$ |bis-fl|$^{(i)}$ $K_3Fe(CN)_6$) indicated that the diameter of liposome particles was distributed from 160Å to 300Å with a maximum at ca 210Å (Fig.2). (iii) The present liposome modified with the bis-flavosurfactant containing $K_3Fe(CN)_6$ in its interior aq. phase ($^{(o)}$ |bis-fl|$^{(i)}$ $K_3Fe(CN)_6$) showed absorptions at 438 nm with a shoulder at 464 nm, characteristic of the flavin chromophore and 425 nm characteristic of the interior $K_3Fe(CN)_6$. (iv) No precipitates were observed in the liposome solution ($^{(o)}$ |bis-fl| $^{(i)}$) within one week at 4℃. This indicates almost the same stability as the egg lecithin liposome.

Facilitated Electron Transport via the Bis-flavosurfactant Incorporated into the Bilayer Membrane.

Interior $K_3Fe(CN)_6$ of the artificial liposome modified with the bis-flavosurfactant (1a) ($^{(o)}$ |bis-fl| $^{(i)}$ $K_3Fe(CN)_6$) was reduced with $Na_2S_2O_4$ added to the external aqueous phase. Similarly to the previously cases, a pseudo first order decrease of interior $K_3Fe(CN)_6$ was observed :

$$- d[Fe(CN)^{3-}_6] / dt = k_{obs}[Fe(CN)^{3-}_6].$$

The results are summarized in Table I. The pseudo first order rate constant (k_{obs}) was found to be 1/2 order dependent of

Table I Reduction Rate Constants of Interior $K_3Fe(CN)_6$ by $Na_2S_2O_4$ via Bis-flavosurfactant.

concn.of $K_3Fe(CN)_6$ (mM)	concn.of bis-fl (mM)	concn.of $Na_2S_2O_4$ (mM)	k_{obs} (sec^{-1})
0.41	0	4.30	0.001
0.41	0.006	0.57	0.23
0.41	0.006	2.10	0.45
0.41	0.006	4.30	0.64
	fl-Lip (mM)		
0.41	0.007	4.30	0.025

a) at 25℃, pH = 7.0. (5mM-Tris · HCl).

$Na_2S_2O_4$ concentrations.

$$k_{obs} = k_1 [Na_2S_2O_4]^{1/2}.$$

The electron transport rate of the bis-flavosurfactant was remarkably enhanced by a factor of 640, compared with the unmodified lecithin liposome. Furthermore, compared with the artificial flavolipid (a half-channel), the electron transport rate was about 25 times faster than that of the flavolipid at a very low concentration of the electron transport mediator (ca 6 molecule per 1 liposome). This shows that the bis-flavosurfactant may function as an effective transmembrane electron channel in the bilayer membrane.

Experimental Section

Instruments.

^{1}H-NMR spectra were obtained with a JEOL JNM PMX 60SI NMR spectrometer, a JEOL JNM FX90Q FT NMR spectrometer or a JEOL JNM GX400 spectrometer. Mass spectra were obtained with a JEOL JMS-DX 300 mass spectrometer. IR spectra were recorded on a Hitachi model 260-50 spectophotometer. Microanalyses were performed at the Microanalytical Center of Kyoto University. Electronic spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophotometer. Sonication was performed with an ultrasonic disruptor, Model UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument. Dynamic light scattering studies were performed with an Otsuka Electronics DLS-700 spectrophotometer.

Materials.

Unless stated otherwise, all reagents and chemicals were obtained commercially and used without further purification. Egg yolk lecithin was carefully purified according to the literature method [6] and stored at -75℃ under Ar in the dark until the use. Dry dimethylformamide was obtained by stirring on BaO at room temperature overnight, followed by distillation

under reduced pressure under Ar. Tetrahydrofuran was purified by refluxing first over Na for 12 h then on $LiAlH_4$ for 1 h, followed by distillation under N_2. Dry triethylamine was obtained by stirring with KOH overnight, refluxing with CaH_2 for 5h, followed by distillation.

Preparation.

2-Bromoethyl bromoacetate

Into a solution of 25.0 g (0.12 mole) of bromoacetyl bromide in 100mL of dry THF was added dropwise a mixture of 16.0 g (0.13 mole) of ethylene bromohydrin and 11.8 g (0.15 mole) of dry pyridine with ice cooling in a period of one hour. The reaction mixture was allowed to warm up to room temperature and stirred for two hours. A white precipitate formed was filtered off and washed with 10 mL of THF. Combined filtrates were concentrated and the pale yellow oily residue was dissolved in 100 mL of chloroform. The chloroform layer was washed successively with two portions of 20 mL of N/10-hydrochloric acid and two portions of 20 mL of a saturated $NaHCO_3$ aqueous solution and dried over Na_2SO_4. After chloroform was evaporated, the pale yellow residue was distilled under reduced pressure (75-80 ℃ (4mmHg)) to yield 20.0 g (0.081 mole, 68%) of a clear oil. ^{1}H-NMR ($CDCl_3$), δ : 3.50 (t, 2H, $-CH_2-Br$, J = 6.0 Hz), 3.86 (s, 2H, $CO-CH_2Br$), 4.45 (t, 2H, $-OCH-_2$, J = 6.0 Hz).

(2a) : 1,12-Bis-(4'-chloro-3'-nitrobenzoyloxy) dodecane

To a solution of 10.0 g (49.6 mmole) of 4-chloro-3-nitrobenzoyl chloride in 50 mL of dry tetrahydrofuran (THF) was added dropwise a solution of 5.0 g (24.8 mmole) of 1,12-dodecandiol and 5.1 g (50.2 mmole) of dry triethylamine in 60 mL of dry THF in a period of 1 hour at room temperature and the mixture was stirred for further 30 hours. Precipitated triethylamine hydrochloride was filtered off and the precipitate was washed with 10 mL of THF. Combined filtrates were concentrated to dryness and the residual pale yellow solid was dissolved in 200 mL of chloroform. Chloroform solution was washed with two portions of 50 mL of H_2O and dried over anhydrous Na_2SO_4. Chloroform was evaporated to yield 13.4 g (95%) of colorless crystals. The analytical sample was recrystallized from ethanol. ^{1}H-NMR($CDCl_3$), δ : 1.62 - 2.08 (broad - s, 20H, $-(CH_2)_{-10}$), 4.62 (t, 4H, $-CH_2O$, J = 6.0 Hz), 7.83 (d, 2H, benzene-H(5), J = 8 Hz), 8.37 (dd, 2H, benzene-H(6), J = 2, 8 Hz), 8.68 (d, 2H, benzene-H(2), J = 2 Hz). IR(kBr), ν : 1710cm^{-1}. mass: 568 (M^+). mp : 100 -102℃. Anal. Cald. for $C_{26}H_{30}N_2O_8Cl_2$, C, 54.84 ; H, 4.92 ; N, 4.92 ; Cl, 12.45. Found, C, 55.08 ; H, 5.24 ; N ,4.84; Cl, 12.30.

(3a) : 1,12-Bis-(4'-methylamino-3'-nitrobenzoyloxy) dodecane

To a solution of 12.68 g (22.3 mmole) of 2a in 410 mL of benzene was added 28 mL of 40% - methylamine in methanol and the

mixture was stirred at room temperature for 24 hr. Orange precipitates formed were collected by filtration and dissolved in 250 mL of chloroform. The chloroform solution was washed with 50 mL of H_2O and dried over Na_2SO_4. After chloroform was removed, 5.83 g (47%) of a crude orange solid was obtained. The crude product was purified by recrystallization from THF. ^{1}H-NMR ($CDCl_3$), δ : 1.30 - 1.83 (broad - s, 20H, $-(CH_2)_{-10}-$), 3.07 (d, 6H, $-NCH_3$, J = 5.0 Hz), 4.30 (t, 4H, $-CH_2O-$, J = 6.0 Hz), 6.85 (d, 2H, benzene-H(5), J = 9.0 Hz), 8.07 (dd, 2H, benzene-H(6), J = 2,9 Hz), 8.33 (broad-s, 2H, -NH), 8.87 (d, 2H, benzene-H(2), J = 2 Hz). IR (KBr), ν_{CO}: 1710 cm^{-1}, ν_{NH} : 3390 cm^{-1}. mass : 558 (M^+). mp :128-131℃ . Anal. Cald. for $C_{28}H_{38}N_4O_8$, C, 60.20 ; H, 6.86 ; N, 10.03. Found, C, 60.14 ; H, 6.82 ; N, 10.07.

(4a) : 1,12-Bis-(10'-methyl-7'-isoalloxazinoyloxy)dodecane

In 100mL of dry THF, 563 mg (1.0 mmole) of 3a was dissolved and 30 mg of platinum oxide was added to this yellow solution. The catalytic reduction was conducted at room temperature at atmospheric H_2 pressure in the dark with vigorous stirring for 24 hr. After 150 mL (6.0 mmole) of hydrogen gas was absorbed at room temperature, platinum powder was filtered off. To the filtrate was added 352 mg (2.2 mmole) of alloxan monohydrate and 2 mL of 1N hydrochloric acid dissolved in 10 mL of methanol and the mixture was refluxed for 1.5 hours under Ar atmosphere in

the dark. After cooling in a refrigerator at 4℃ overnight, dark yellow precipitates formed were collected by filtration and washed successively with 5 mL of ethanol and 5 mL of ether to give 179 mg (25%) of a yellow green solid. The crude product was purified further by recrystallization from dimethylsulfoxide-ethanol. ^{1}H-NMR (DMSO-d_6), δ : 1.30-1.80 (broad-s, 20H, $-(CH_2)_{10}-$), 3.97 (s, 6H, N-CH_3), 4.35 (t, 4H, $-CH_2O-$), 8.01 (d, 2H, isoalloxazin-ring(H_9), J = 9.0 Hz), 8.34 (dd, 2H, isoalloxazin-ring(H_8), J = 2.0, 9.0 Hz), 8.52 (d, 2H, isoalloxazin-ring(H_6), J = 2.0 Hz). IR (KBr), ν_{CO} : 1710, 1670 cm^{-1}. mass : 710 (M^+). mp : >300 ℃. UV-VIS spectrum ($CHCl_3$) : λ_{max} = 331nm (ϵ = 1.5 × 10^4), 438 nm (ϵ = 2.6 × 10^4). Anal. Cald. for $C_{36}H_{38}N_8O_8 \cdot H_2O$, C, 59.33 ; H, 5.53 ; N, 15.37. Found, C, 59.49 ; H, 5.24 ; N, 15.07.

(5a) : 1,12-Bis-[3'-(2-bromoethoxy)carbonylmethyl-10'-methyl-7'-isoalloxazinoyloxy]decane [4)]

In 250 mL of dry dimethylformamide (DMF) , 500 mg (0.70 mmole) of 4a was dissolved and then 395 mg (1.61 mmole) of bromoethyl bromoacetate and 1.00 g (7.23 mmole) of finely powdered anhydrous potassium carbonate were added to the solution. The mixture was stirred at 40℃ in the dard for 24 hr and the precipitated material was filtered off. DMF and excess bromoethly bromoacetate were distilled off under reduced pressure below 40℃. A brown residual solid was dissolved in

350 mL of chloroform and the chloroform solution was washed with 100 mL of H_2O and dried over anhydrous Na_2SO_4. After chloroform was evaporated, 615 mg of a yellow brown solid remained was purified by reprecipitation from chloroform-hexane. Further purification was performed by silica-gel column chromatography (eluent = ethyl acetate / methanol = 40 / 1(V/V)) followed by recrystallization from ethanol. Yellow crystals thus obtained were dried in vacuo at room temperature for one day to give 136 mg (0.13 mmole) of the product (Yield = 19%). ^{1}H-NMR ($CDCl_3$), δ : 1.30-1.90 (broad-s, 20H, $-(CH_2)_{10}-$), 3.52 (t, 4H, $Br-CH_2-$, J = 6.0 Hz), 4.17 (s, 6H, $N-CH_3$), 4.40 (t, 4H, $-OCH_2-$, J = 6.0 Hz), 4.48 (t, 4H, $-OCH_2-$, J = 6.0 Hz), 4.90 (s, 4H, $-N-CH_2COO-$), 7.72 (d, 2H, isoalloxazin-ring (H_9), J = 9.0 Hz), 8.53 (dd, 2H, isoalloxazin-ring (H_8), J = 2.0 , 9.0 Hz), 8.98 (d, 2H, isoalloxazin-ring (H_6), J = 2.0 Hz). IR (KBr), ν_{CO} : 1710, 1655 cm^{-1}. FAB-mass : 1039 ($(M+H)^+$), 1041 ($(M+2+H)^+$), 1043 ($(M+4+H)^+$). mp: 209-211 ℃.

(1a): 1,12-Bis-[3'-(2-trimethylammonio)ethoxycarbonylmethyl-10'-methyl-7'-isoalloxazinoyloxy]dodecane dibromide.

In 90 mL of dry THF, 98 mg (94 mole) of 5a was dissolved and 3 mL of trimethylamine was added to the solution at 0℃. The resultant yellow solution was stirred at room temperature in the dark for 4 days. Yellow precipitates formed were collected by filtration and washed with 10 mL of ether. The crude

ammonium salt was purified by reprecipitation from methanol - ethyl acetate and dried in vacuo at room temperature for 1 day to give 58 mg of the ammonium salt (yield = 53%). ^{1}H-NMR (DMSO-d_6), δ : 1.17-1.90 (broad-s, 20H, $-(CH_2)_{-10}$), 3.16 (s, 18H, $-N(CH_3)_3$), 3.77 (m, 4H, $-CH_2N$), 4.05 (s, 6H, $N-CH_3$), 4.36 (t, 4H, $-OCH_2-$, J = 6.0 Hz), 4.57 (m, 4H, $-OCH_2-$), 4.77 (s, 4H, $N-CH_2COO-$), 8.13 (d, 2H, isoalloxazin-ring (H_9), J = 9.0 Hz), 8.42 (dd, 2H, isoalloxazin-ring (H_8), J = 2.0, 9.0 Hz), 8.61 (d, 2H, isoalloxazin-ring (H_6), J = 2.0 Hz). IR (KBr), ν_{CO} : 1715, 1660 cm^{-1}. UV-VIS spectrum (1%-methanol in chloroform) :λ_{max} = 334 nm (ϵ = 1.6 × 10^4), 438 nm (ϵ = 2.8 × 10^4). Anal. Cald. for $C_{50}H_{66}N_{10}O_{18}Br_2 \cdot 2H_2O$, C, 50.26 ; H, 5.90 ; N, 11.72, Found, C, 49.99 ; H, 5.84 ; N, 11.17.

(2b) : 3α,24-Bis-(4'-chloro-3'-nitrobenzoyloxy)cholane

4-Chloro-3-nitrobenzoylchloride was prepared by refluxing in thionyl chloride overnight.

To a solution of 4.16 g (20.2 mmole) of 4-chloro-3-nitrobenzoylchloride in 200 mL of dry tetrahydrofuran (THF) was added 3.66 g (10.1 mmole) of 3α,24-dihydroxycholane in one portion and 2.92 g (28.9 mmole) of dry triethylamine under external cooling with ice-water. The reaction mixture was stirred at room temperature for 40 hours. Precipitated triethylamine hydrochloride was filtered off and the precipitate was washed with 10 mL of THF. Combined filtrates were

concentrated to dryness and the residual pale yellow solid was dissolved in 200 mL of chloroform. The chloroform solution was washed with two portions of 50 mL of H_2O and dried over Na_2SO_4. Chloroform was distilled off at room temperature to obtain a crude white solid. The white solid was purified by reprecipitation from THF-methanol to yield 6.27 g (85%) of a colorless solid. ^{1}H-NMR ($CDCl_3$) δ : 0.58-2.15 (m, 37H, Cholanyl-CH_3,-CH_2 and - CH), 4.36 (t, 2H, $-CH_2O-$, J = 6.0 Hz), 5.03 (q, 1H, CH-O-, J = 5.4 Hz), 7.64 (d, 1H, benzene-H(5), J = 8.3 Hz), 7.65 (d, 1H, benzene-H(5'), J = 8.3 Hz), 8.17 (dd, 1H, benzene-H(6), J = 2.0, 8.3 Hz), 8.18 (dd, 1H, benzene-H(6'), J = 2.0, 8.3 Hz), 8.50 (d, 2H, benzene-H(2,2'), J = 2.0 Hz). IR (KBr), ν_{NH} : 2930, 2860 cm^{-1}, ν_{CO} : 1702 cm^{-1}, ν_{NO_2} : 1530, 1299 cm^{-1}. mass : 728 (M^+). mp : 199-201 ℃. Anal. Cald. for $C_{38}H_{46}N_2O_8Cl_2$, C, 62.55 ; H, 6.35 ; N, 3.84 ; Cl, 9.72. Found, C, 62.54; H, 6.32; N, 3.71; Cl, 9.50.

(3b) : 3α,24-Bis-(4'-methylamino-3'-nitrobenzoyloxy)cholane

To a solution of 10.5 g (14.4 mmole) of 2b in 600 mL of THF was added 12.0 mL of 40% methylamine in methanol and the mixture was stirred at room tempertature for 24 hr. After the reaction was completed (monitored by TLC eluted by chloroform), precipitated methylamine hydrochloride was filtered off and the precipitate was washed with 20 mL of THF. Combined filtrates were concentrated to dryness and the residual orange solid was

dissolved in 200 mL of chloroform. The chloroform solution was washed twice with 30 mL of H_2O and dried over Na_2SO_4. After chloroform was evaporated, a crude orange solid was obtained. The orange solid was further purified with silica-gel column chromatography (eluent = chloroform). An orange pure solid thus obtained was dried in vacuo at room temperature overnight to give 8.71 g (12.1 mmole) of the product. (Yield = 85 %) ^{1}H-NMR ($CDCl_3$), δ : 0.60-2.23 (m, 37H, Cholanyl-CH_3,CH_2 and CH), 3.10 (d, 6H, -N-CH_3, J = 5.3 Hz), 4.28 (t, 2H, -CH_2O-, J= 6.0 Hz), 4.90 (q, 1H, CH-O, J= 6.0 Hz), 6.86 (d, 1H, benzene-H(5), J = 9.0 Hz), 6.87 (d, 1H, benzene-H(5'), J = 9.0 Hz), 8.09 (dd, 1H, benzene-H(6), J = 2.3, 9.0 Hz), 8.10 (dd, 1H, benzene-H(6'), J = 2.3, 9.0 Hz), 8.35 (q, 2H, -NH, J = 5.3 Hz), 8.88 (d, 2H, benzene-H(2,2'),J = 2.3 Hz). IR (KBr) , ν_{NH} : 3390 cm^{-1}, ν_{CH} : 2930, 2870 cm^{-1}. ν_{NO_2} : 1529,1325 cm^{-1}. mass: 718 (M^+). mp: 115-116 ℃. Anal. Cald. for $C_{40}H_{54}N_4O_8$, C, 66.83 ; H, 7.57 ; N, 7.79. Found, C, 66.38 ; H, 7.64 ; N, 7.77.

(4b) : 3α,24-Bis-(10'-methyl-7'-isoalloxazinoyloxy)cholane

In a solution of 20mL of ethanol and 40 mL of THF was dissolved 1.01g (1.41 mmole) of 3b and 50 mg of platinum oxide was added to this yellow solution. The catalytic hydrogenation was conducted at room temperature at atmospheric hydrogen pressure in the dark with vigorous stirring for 24 hr. After a stoichiometric amount (200 mL) of hydrogen gas was absorbed at

room temperature, platinum powder was filtered off. To the filtrate was added 514 mg (3.21 mmole) of alloxan monohydrate and 4 mL of 1N hydrochloric acid and the mixture was refluxed for 1.5 hours under Ar atmosphere in the dark. Yellow green precipitates thus formed were collected by filtration and washed successively with 10 mL of ether to give 311 mg (25%) of a yellow green solid. The crude product was purified further by reprecipitation from DMF-ether. ^{1}H-NMR (DMSO-d_6) δ : 0.36 - 2.16 (m, 37H, Cholanyl-CH_3, CH_2 and CH), 3.98 (s, 6H, N-CH_3), 4.06-4.50 (broad-s, 2H, -CH_2-O), 4.70-5.17 (broad-s, 1H, CH-O), 8.03 (d, 2H, isoalloxazin-ring-H(9), J = 9.4Hz), 8.36 (dd, 2H, isoalloxazin-ring-H(8), J = 9.4, 1.5 Hz), 8.54 (d, 1H, isoalloxazin-ring-H(6), J = 1.5 Hz), 8.58 (d, 1H, isoallxazin-ring-H(6'), J = 1.5 Hz). IR (KBr), ν_{CO} : 1720, 1690 cm^{-1}.

(5b) : 3α,24-Bis-[3'-(2-bromoethoxy)carbonylmethyl-10'-methyl-7'-iso-alloxazinoyloxy]cholane

In 100 mL of dry DMF was dissolved 103 mg (0.12 mmole) 4b and then 64 mg (0.26 mmole) of 2-bromoethyl bromoacetate and 145 mg (1.05 mmole) of finely powdered anhydrous potassium carbonate were added to the solution. The mixture was stirred at 40℃ in the dark for 6 hours and the precipitated materials were filtered off. DMF and excess of bromoethyl bromoacetate were removed by distillation under reduced pressure below 40℃. A brown residual solid was washed with 10 mL of H_2O and dried over

anhydrous Na_2SO_4. After chloroform was evaporated at room temperature, a brown residue was applied onto a silica-gel column (3.0 × 30cm). Fractions containing the main product (Rf = 0.73, ethyl acetate : methanol = 9:1 (v/v)) were collected. Removal of solvent under reduced pressure followed by drying in vacuo (0.1 mmHg) for 24 h. at room temperature afforded 36 mg (25%) of yellow solid. ^{1}H-NMR ($CDCl_3$), δ : 0.38-2.35 (m, 37H, Cholanyl-CH_3, CH_2 and CH), 3.52 (t, 4H, -CH_2Br, J = 6.3 Hz), 4.06-4.50 (broad-s, 2H, -CH_2-O), 4.18 (s, 6H, N-CH_3), 4.49 (t, 4H, -CH_2-O-, J = 6.3 Hz), 4.70-5.17 (broad-s, 1H, CH-O), 4.92 (s, 4H, N-CH_2COO), 7.70 (d, 1H, isoalloxazin-ring- H(9), J = 6.8 Hz), 7.72 (d, 1H, isoalloxazin-ring-H(9'), J = 6.8 Hz), 8.53 (dd, 2H, isoalloxazin-ring-H(8), J = 6.8, 1.5 Hz), 8.99 (d, 1H, isoalloxazin-ring-H(6), J = 1.5 Hz), 9.01 (d, 1H, isoalloxazin-ring-H(6'), J = 1.5 Hz). IR (KBr), ν_{CH} : 2920, 2840 cm^{-1} C=C-C=O : 1710, 1660 cm^{-1}. UV-VIS spectrum ($CHCl_3$) : λmax = 333 nm (ϵ = 1.6 × 10^4), 438 nm (ϵ = 1.7 × 10^4).

(1b) : 3α,24-Bis-[3'-(2-trimethylammonio)ethoxycarbonylmethyl-10'-methyl-7'-isoalloxazinoyloxyl-cholane dibromide.

In 5 mL of dry THF was dissolved 33 mg (0.028 mmole) of 5b and then ca 2 mL of trimethylamine was added to the solution at 0℃. The yellow mixture was stirred at room temperature in the dark for 4 days. The yellow precipitates formed were collected by filtration and washed successively with 5 mL of THF. The

crude ammonium ammonium salt (27 mg (75%)) was purified further by cellulose column chromatography (eluent = chloroform / methanol = 20/1 (v/v)) to yield a yellow solid. ^{1}H-NMR (DMSO-d_6), δ : 0.30-2.20 (m, 37H, Cholanyl-CH_3, CH_2 and CH), 3.13 (s, 18H, $-N(CH_3)_3$), 3.61 (t, 4H, $-CH_2-N$, J = 7.5 Hz), 4.4 (s, 6H, N-CH_3), 4.10-4.67 (broad-s, 2H, $-CH_2-O-$), 4.43-4.73 (broad-s, 4H, -CH_2-O-), 4.60-5.23 (broad-s, 1H, CH-O), 4,76 (s, 4H, $-NCH_2COO-$), 8.11 (d, 1H, isoalloxazin-ring- H(9), J = 9.4 Hz). 8.13 (d, 1H, isoalloxazin-ring-H(9'), J = 9.4 Hz), 8.43 (dd, 2H, isoalloxazin-ring-H(8), J = 1.9, 9.4 Hz), 8.61 (d, 1H, isoalloxazin-ring-H(6), J = 1.9 Hz, 8.65 (d, 1H, isoalloxazin-ring-H(6), J = 1.9 Hz). IR (KBr), ν_{CO} :1703, 1649 cm^{-1}. FAB-mass : 1159 ((M-Br× 2+H)$^+$).

UV-VIS-spectrum

($CHCl_3$): λ max = 334 nm (ϵ = 1.3 × 10^4), 438 nm (ϵ = 1.0 × 10^4).

Preparation of Artificial Single-Wall Bilayer Liposome.

Artificial egg lecithin liposomes functionalized with the bis-flavosurfactant in its membrane phase and containing $K_3Fe(CN)_6$ in its interior aqueous phase were prepared according to a slightly modified procedure of ours. [5] A solution of 75 - 80 mg of carefully purified egg yolk lecithin and an appropriate amount of the bis-flavosurfactant in 14 mL of $CHCl_3$ and 1 mL of methanol was gently evaporated under reduced pressure under Ar. The resulting thin film was dried in vacuo for 8 h in the dark at room temperature. Into the dried lipid film was added 5 mL of 5 mM aqueous Tris HCl buffer (pH 7.0) solution containing 0.75 M of $K_3Fe(CN)_6$ and the lipid film was suspended in the solution. The suspended mixture chilled in an ice bath was sonicated in a box filled with Ar for 5 min. The sonication was repeated three times at intervals of 10 min. The resulting mixture was centrifuged (1.9×10^3 rpm) for 15 min at 4℃ and the yellow supernatant was applied on a Sepharose 4B column (1.0 cm$\times$ 40 cm) and eluted with 5 mM aqueous Tris HCl buffer (pH 7.0) at 4℃. Single-wall liposomes were obtained from eluents between 34 to 45 mL ($\pm$ 2 mL).

Dynamic Light Scattering.

Size distribution of the artificial liposome functionalized with the bis-flavosurfactant was measured with an Otsuka Electronics DLS-700 spectrophoto-meter thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. The flavoliposome employed was freshly purified by gel-filtration using a Sepharose-4B (Pharmacia). The single-wall liposome fractions were collected by monitoring the electronic absorption spectrum of every 1 mL fraction and diluted about 5 times with 5 mM-Tris・HCl.

Reduction of $K_3Fe(CN)_6$ in the Interior Aqueous Phase of the Functionalized Liposome by $Na_2S_2O_4$.

Reduction of the inteior $K_3Fe(CN)_6$ by $Na_2S_2O_4$ was carried out by using the same method as described previously. The reduction of the interior $K_3Fe(CN)_6$ was traced by the intensity change of the visible absorption at 425 nm. The absorption of flavolipid at 425 nm was ascertained not to interfare seriously the above measurement based on the following facts: (i) The reduction rate of flavolipid was independently estimated by monitoring the intensity change of its absorpiton at 472 nm, characteristic of the flavin chromophore. Thus the net decrease of $K_3Fe(CN)_6$ was easily substracted from the overall change.

(ii) The change in the $K_3Fe(CN)_6$ concentration was measured in the region where the concentrations of the oxidized and the reduced form of flavolipid were practically constant in the steady state.

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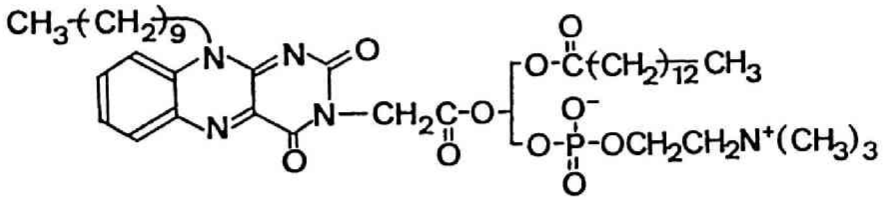
Chapter 7

Facilitated Electron Transfer from NADH to a Flavolipid Bound to the DODAC Liposome.

Abstract

As a new electron transfer system from NADH to flavin, the artificial flavolipid (1) stably bound in an ammonium bilayer efficiently accelerated the oxidation of NADH. A kinetic study of this system showed the Michaelis-Menten type kinetics, from which k_{cat} of 1.2 s^{-1}, Km of 6×10^{-4} M and k_{cat}/Km of 2000 M^{-1} s^{-1} were determined. The k_{cat}/Km value is comparable to that of the old yellow enzyme (k_{cat}/Km = 610～3200 M^{-1} s^{-1}). Rate enhancement of the present system was remarkably suppressed by increasing the ionic strength in this reaction (k_2 = 20 M^{-1} s^{-1} at μ = 0.1). In contrast with 1 in the ammonium bilayer, 1 in lecithin bilayer showed less efficiency for NADH oxidation by a factor of 1 / 46000. From the fluorescence titration of NADH by the DODAC liposome, the oxidation of NADH was concluded to be accelerated by the favorable conformational change of NADH as well as the proximity of NADH to the flavolipid on the cationic surface of DODAC by the electrostatic binding.



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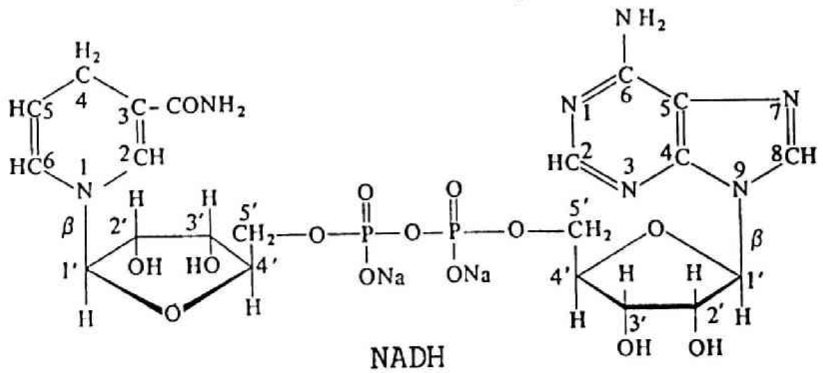
Introduction

NADH (Dihydronicotinamide adenine dinucleotide) is one of the main electron donors to flavoproteins in many biological oxido-reduction processes such as respiratory chain, cytochrome P-450 reductase and flavoenzyme monooxygenases. A number of investigations have been reported for the nonenzymatic oxidation of NADH model compounds by various flavins, [1] but there are few examples which utilize NADH itself as the electron donor. This is attributable mainly to the fact that the electron transfer from native NADH to flavins is about 10 - 10^2 times slower than that from NADH model compounds.

In this chapter, we wish to report that the artificial flavolipid (1) bound in a cationic liposome showed significant acceleration for the rate of NADH oxidation, compared with 1 located in a zwitterionic liposome, representing a high catalytic efficiency as a simplified NADH dehydrogenase model.



NADH

Results and Discussion

Formation of Stable Bilayer Membrane from Flavolipid and Ammonium Amphiphile

In order to obtain an assembled cationic surface of the bilayer membrane, dioctadecyldimethylammonium chloride (DODAC) was used and the artificial flavolipid (1) was incorporated into the bilayer membrane of DODAC by the sonication procedure in a similar manner to the incorporation into the egg lecithin liposome to give a stable bilayer membrane. After purification by centrifugalization followed by a gel filtration through a Sephadex G-50 column, the dynamic light scattering measurement of the flavoliposome indicated that the diameter of liposome particles was distributed from 1100 Å to 1900 Å with a maximum at ca 1300 Å, which were similar to the values of DODAC liposomes without the incorporation of the flavolipid. [2] The flavin chromophore in the DODAC liposome showed an absorption maximum at 444 nm with distinct shoulders at 418 nm and 472 nm. The maximum appeared at almost the same position in chloroform solution with characteristic shoulders in the above region. On the other hand, the absorption maximum of flavin in water and Tris buffer appeared at a significantly lower wavelength of 433 nm without any shoulders. These experiments confirm us the view that the flavin unit in the DODAC liposome is tightly bound in its hydrophobic region.

Table I Reduction Rate Constants of Flavolipid Incorporated into the Bilayer Membrane [a,b].

NADH (mM)	k_{obs} (sec^{-1}) egg lecithin [c]	k_{obs} (sec^{-1}) DODAC [c]
0.095	0.4×10^{-5}	0.43×10^{-1}
0.143	———	0.64×10^{-1}
0.194	0.9×10^{-5}	0.84×10^{-1}
0.285	———	1.23×10^{-1}
0.393	1.7×10^{-5}	1.56×10^{-1}

a). at 30℃, pH 7.0

b). [flavolipid] = 3.0×10^{-5}M

c). fl-Lip / lecithin = fl-Lip / DODAC = 2 / 98

NADH Oxidation by Flavolipid in the Bilayer Membrane.

The oxidation of NADH by the flavolipid in the bilayer membrane was examined under a pseudo 1st order condition with respect to the flavolipid at 30 ℃, pH 7.0 (5 mM Tris · HCl) under argon. The reduction of flavolipid locating at the membrane was monitored by the absorption at 444 nm.[3] In the DODAC liposome system, the observed first-order rate constants (k_{obs}) vs NADH concentrations showed saturation at higher concentrations of NADH (Table I). Therefore, 1 : 1 complex formation between the flavolipid and NADH is assumed to give the Michaeles-Menten scheme. The double reciprocal plots of k_{obs} vs the NADH concentration (Lineweaver-Burk plot) gave k_{cat} = 1.2

$$\text{Fl-Lip} + \text{NADH} \underset{\text{Km}}{\rightleftharpoons} \text{Fl-Lip} \cdot \text{NADH} \xrightarrow{k_{cat}} \text{Fl}H_2\text{-Lip} + \text{NAD}$$

sec^{-1} and Km = 6×10^{-4}M, therefore, the overall catalytic efficiency (k_{cat}/Km) as 2×10^3 $M^{-1}.sec^{-1}$. (See Fig. 1).

In contrast to the kinetic behavior of the flavolipid (1) in a DODAC liposome, the oxidation of NADH by 1 in an egg lecithin liposome did not show no saturation kinetics with regard to the NADH concentration. A simple second order rate constant (k_2) was obtained as 0.043 M^{-1} sec^{-1}.

The oxidation rate of NADH by 1 in the DODAC liposome (cationinc = ammonium) is therefore remarkably enhanced by a factor of 4.6×10^4, compared with the NADH oxidation by 1 in

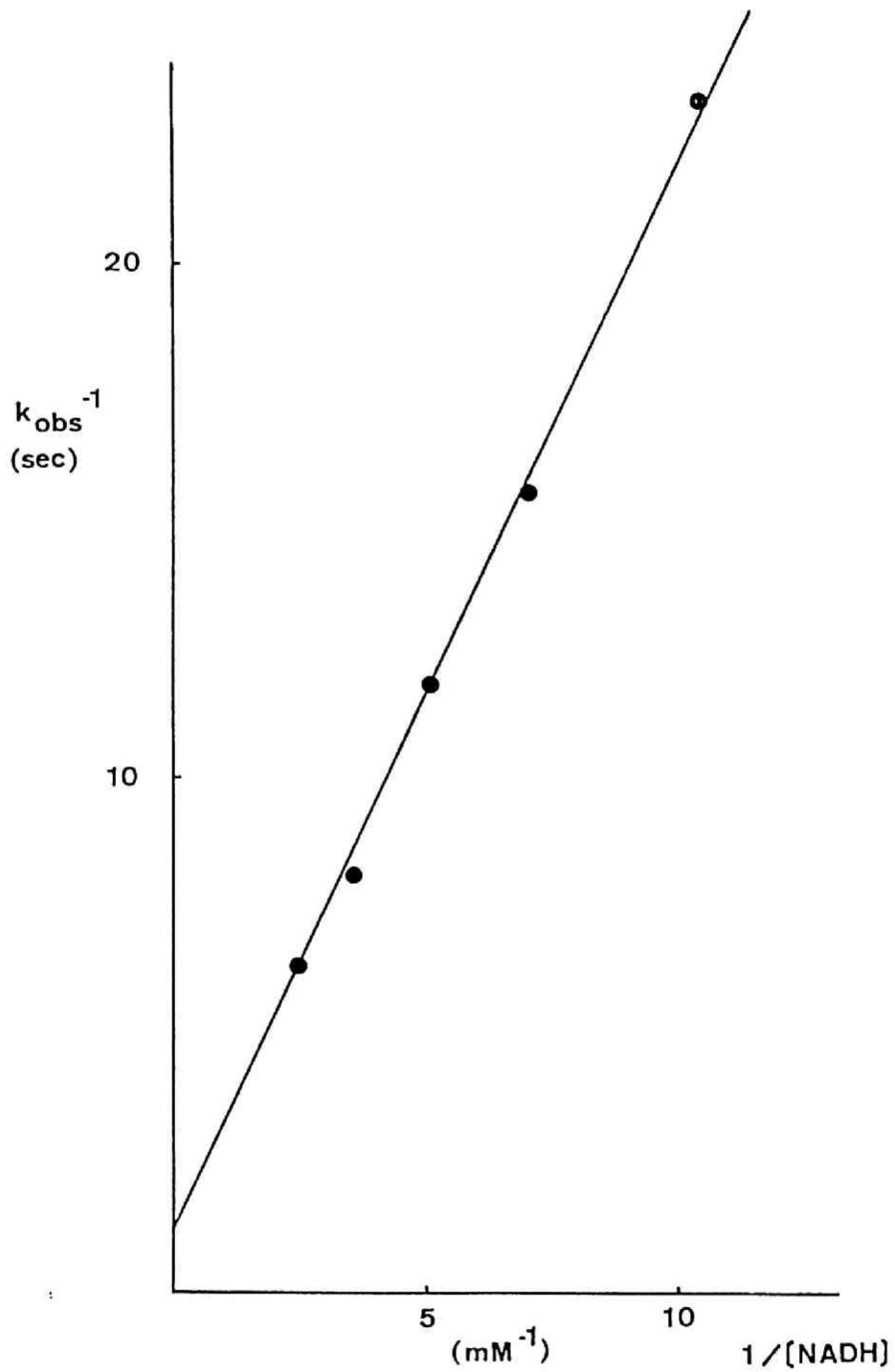
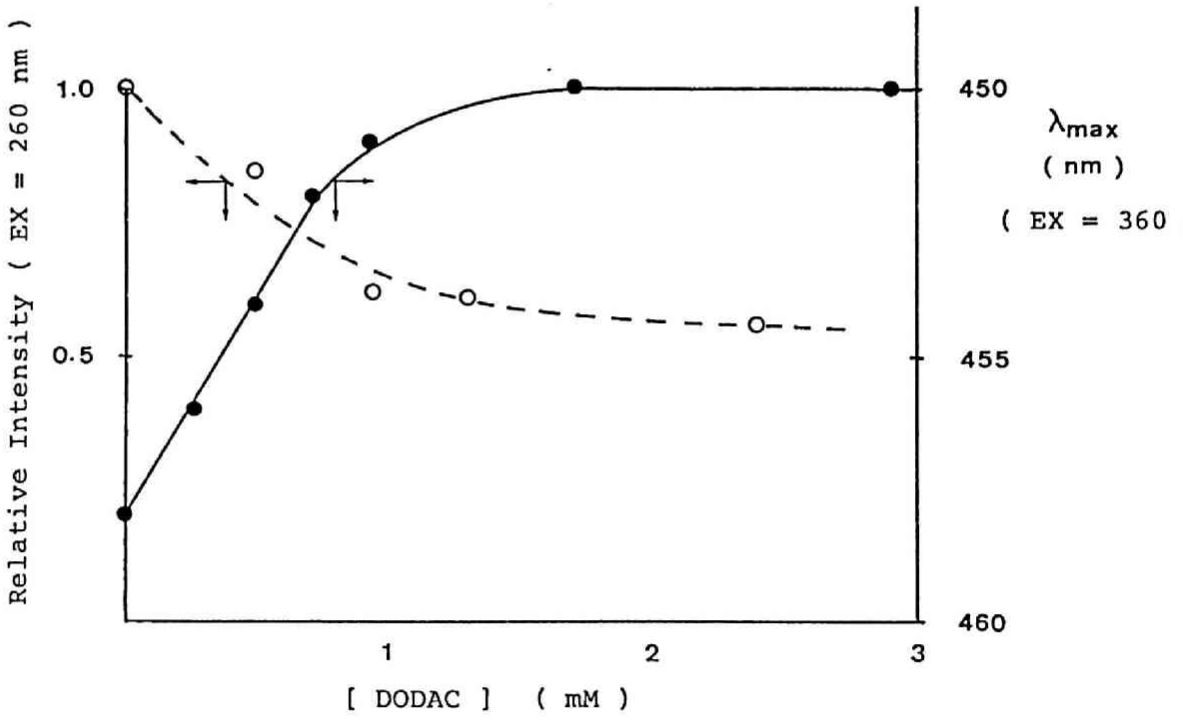


Fig. 1. Lineweaver-Burk plot of the oxidation of NADH by flavolipid in the DODAC liposome.

an egg lecithin liposome (zwitterionic = phosphatidylcholine). This enhancement represents that a hydrophilic, bulky NADH molecule cannot approach the flavin moiety in the membrane without an assistance of binding by the positively charged membrane surface. In order to know how the electrostatic binding influences the electron transfer rate, the effect of the ionic strength (μ) on the NADH oxidation rate was examined. The increase of value from 0.005 to 0.1 (k_2 = 20 $M^{-1} \cdot sec^{-1}$ at μ = 0.1) suppressed the rate by 100 fold. This supports the idea that NADH is bound at the cationic surface of the DODAC liposome through an electrostatic interaction. Binding of NADH onto the surface of the DODAC liposome was also elucidated from the fluorescence titration of NADH by the addition of the DODAC liposome. As shown inf Fig. 2, the emission maximum of NADH (EX = 360 nm) showed a blue shift from 458 nm in the bulk aqueous phase to a saturated value of 450 nm at the DODAC concentration of more than 1 mM. Furthermore, the fluorescence arising from the energy transfer from the adenyl ring of NADH to the dihydropyridne ring observable in a closed conformation of NADH (EX = 260 nm) was quenched to about 55 % ralative to the fluorescence strength in the bulk solution.[4] The effective quenching indicates that the conformation of NADH bound to the DODAC liposome is altered to an open form by binding from a closed form which is shown to be predominant in the aqueous buffer.[4] Interestingly, the blue shift in the DODAC

Figure 2.

Change of fluorescence spectrum of NADH by the addition of the DODAC liposome at pH 7.0, 30 °C in 5 mM Tris buffer: emission of 450 nm excited at 260 nm (-O-) ; shift of emission maximum excited at 360 nm (—●—)



liposome indicates that the dihydropyridine ring of NADH is located in the membrane (hydrophobic) phase rather than exposed to the aqueous phase. Therefore, the accelerated oxidation of NADH by the flavolipid in the DODAC liposome may be explained by the favorable conformational change of NADH as well as the proximity of NADH to the flavolipid on the cationic surface of DODAC by the electrostatic binding. The oxidation rates of NADH by native flavoenzymes as well as their models are listed in Table Ⅱ. The present flavolipid in the DODAC liposome shows rate acceleration of about 2200 - fold, compard with the homogeneous NADH oxidation by 3,10-dimethylisoalloxazine (k_2 = 0.9 $M^{-1} \cdot sec^{-1}$).[5]

In a nonenzymatic oxidation of NADH by flavins, there is only one raport by Shinkai, [6] in which the cationic polymer-immobilized flavin showed rate acceleration of 460 - 4650 fold. This acceleration suggested that the electrostatic interaction was important in the oxidation of NADH by flavin.

In a semienzymatic system, flavopapain produces only 4 - fold rate enhancement when NADH is employed as a substrate, but very large enhancement of about 620 is observable when N^1 - hexyldihydronicotinamide (Hex-NAH) is employed as a substrate (k_{cat}/Km = 5.7 × $10^6 M^{-1} \cdot sec^{-1}$). [7] This is explained by the idea that the flavopapain has only a hydrophobic binding site and favors 1 - alkyl-dihydronicotinamide over the native NADH having too bulky and hydrophilic groups.

Table II . NADH Oxidation by Various Flavin Systems

Flavin System	k_2 ($M^{-1}.s^{-1}$)	k_{cat} / Km ($M^{-1}.s^{-1}$)	Ref
3,10-dimethylisoalloxazine (homogeneous)	0.9	———	5
3-methylriboflavin (homogeneous)	5.2	———	6
acetylflavin (homogeneous)	5.1	———	7
flavine immobilized in polymer	———	2.4×10^3-2.4×10^4	6
flavopapain	———	21	7
bovin heart NADH dehydrogenase	———	10^8	8
old yellow enzyme	———	610～3227	9
flavoliposome (DODAC)	———	2000	this
(lecithin)	———	0.043	work

The largest enzymatic efficiency (k_{cat} /Km) of 10^8 $M^{-1} \cdot s^{-1}$ is reported for bovin heart NADH dehydrogenase, [8] which is assumed to be the upper limit for NADH oxidation. Although the value of k_{cat} /Km of 2×10^3 $M^{-1} \cdot s^{-1}$ for the present system is inferior to this limiting value but comparable to that for old yellow enzyme. [9]

In conclusion, it was clearly demonstrated that a change of the nature of the surface incorporated with the flavolipid caused a great enhancement of the oxidation rate of the native NADH not only by binding the redox counterpart near the flavolipid, but by inducing the conformational change of NADH to favor the electron transfer from the nicotinamide moiety to falvolipid on the DODAC membrane.

Experimental Section.

Instruments.

Electronic absorption spectra were measured with either a Union Giken high sensitivity spectrophotometer SM 401 or a Hitachi U-3400 spectrophotometer thermostated at 25 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. Sonication was performed with an ultrasonic disruptor, Model UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20Ⅲ. pH measurement was performed on a Toa pH meter, Model HM 5-ES instrument.

Material.

Buffer solutions were made by dissolving tris(hydroxymethyl)-aminomethane and N/10-hydrochloric acid in distilled water to be 5mM Tris concentration. Egg yolk lecithin was carefully purified according to the literature method [10] and stored at -75℃ under Ar in the dark. Flavolipid (1) was prepared by the reported method [11]. NADH was purchased from Oriental Yeast Co., LTD.

Preparation of Artificial Single Wall Liposome.

Artificial ammonium (DODAC) liposomes functionalized with the flavolipid were prepared according to a slightly modified procedure reported previously [12]. A solution of 30 mg of DODAC

and 1.1 mg of the flavolipid in 15 mL of $CHCl_3$ was gently evaporated under reduced pressure under Ar. The resulting thin film was dried in vacuo for 8 h in the dark at room temperature. Into the dried lipid film was added 5 mL of 5 mM aqueous Tris・HCl (pH 7.0) solution and the suspended mixture was sonicated at 35 -40℃ in a box filled with Ar for 5 min. The sonication was repeated twice at interval of 10 min. The resulting mixture was centrifuged (1.9×10^3 rpm) for 15 min at 20℃ and the yellow supernatant was applied on a Sephadex G-50 column (1.0×40 cm) and eluted with 5 mM aqueous Tris・HCl buffer (pH 7.0). Liposome fractions were obtained from eluents between 19 to 26 mL (± 2 mL).

Reduction of Flavolipid incorporated into the Bilayer Membrane of the Functionalized Liposome by NADH.

A freshly prepared liposome solution (2.0 mL, $^{(o)}$|fl-Lip|$^{(i)}$), kept at 10 mm-quartz cell equipped with a three-way stopcock. The solution was deaerated through the careful substitution of air by Ar via evacuation and the introduction of Ar at room temperature. The procedure was repeated 20 times per sample. Into the deaerated liposome solution at 25℃, 0.2 mL of a freshly prepared NADH (0.094 - 0.39 mM) was added using a specially designed syringe [12]. The reduction of the flavolipid present in the bilayer membrane phase was traced by monitoring the change of the absorbance at 444 nm.

Dynamic Light Scattering.

Size distribution of the artificial liposome functionalized with the flavolipid was measured with an Otsuka Electronics DLS-700 spectrophotometer thermostated at 25.0 ± 0.1℃ with a circulation system, Type Handy Cooler TRL-108. The flavoliposome employed was freshly purified by gel-filtration using a Sephadex-G 50 (Pharmacia) . The single-wall liposome fractions were collected by monitoring electronic absorption spectra of every 1 mL fraction and diluted about 5 times with 5 mM-Tris・HCl.

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   I. Tabushi; I. Hamachi, The 54th Annual Meeting of Chemical Society of Japan, Tokyo, Japan, April, 1987.
3. "Molecular / Electron Flux Conversion by Using Flavolipid and Strongly Hydrophilic NADH-model in Artificial Liposome System",
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