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

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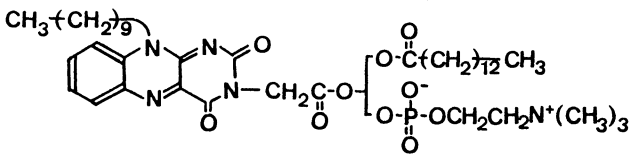
By using a strongly hydrophilic NADH model, BzNAHCOOK in the external electron donor, a transmembrane electron transport to the internal acceptor, $K_3[Fe(CN)_6]$ through the flavolipid (**1**) was observed. BzNAHCOOK was proved to be an appropriate NADH model in the transmembrane electron transport study because of (i) the very low permeability through the bilayer membrane (k=1.0×10^{-3} s^{-1}) and (ii) the slow hydration rate (k=1.1×10^{-5} s^{-1}). The artificial liposome modified with the flavolipid showed a 6—7 times more efficient electron flux conversion from BzNAHCOOK to $K_3[Fe(CN)_6]$ than the liposome modified with the ubiquinone 10, a biologically important electron carrier. In association with the electron transport, electrochemical oxidation and reduction peaks of the flavolipid incorporated into the liposomal membrane were observed clearly by using Hg-electrode.

In the previous communications, we have reported a successful electron transport across the bilayer membrane of liposome functionalized with the flavolipid (**1**) from the external electron source to the internal acceptor.[1] This represents an artificial flavoprotein



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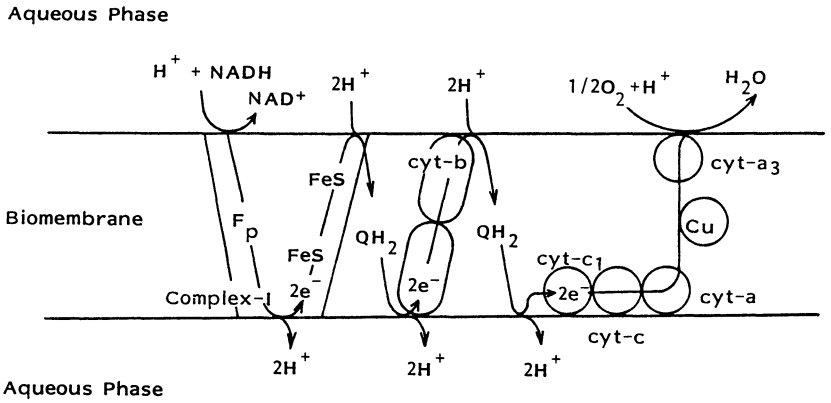
model which accepts electrons from $Na_2S_2O_4$ and donates them to $K_3[Fe(CN)_6]$. In an energetic view point, this means a large transmembrane driving force since $Na_2S_2O_4$ ($E_{1/2}$=−1.13 V vs. NHE) is a powerful reducing agent toward the flavin ($E_{1/2}$=−0.12 V and −0.37 V vs. NHE) and $K_3[Fe(CN)_6]$ ($E_{1/2}$=+0.36 vs. NHE). In native systems, the use of such an excess driving force is carefully avoided by choosing electron donors having appropriate reduction potentials. Dihydronicotinamide ($E_{1/2}$=−0.32 V vs. NHE) is the most important electron donor as seen in various NAD(P)H (hereafter abbreviated generally as RNAH) linked reductases, e.g. the NADH branch of the respiratory chain.[2] Here, RNAH interacts first with the membrane bound flavoprotein to initiate the transmembrane electron transport. RNAH molecular "flux" from the bulk aqueous to the membrane phase is converted to the electron "flux". This process is the first event of the respiratory chain and of primary importance even in the viewpoint of whole bioenergetics.[3]

According to the above considerations, it seems interesting and significant to construct an artificial Complex I of the respiratory chain.[4] The authors wish to report here a successful transmembrane electron transport through the flavoliposome system by using a RNAH model.

Results and Discussion

On the basis of the discussions described in the introductory section, the external electron donor



F_p: flavoprotein
FeS: iron-sulfur cluster
cyt: cytochrome
QH_2: ubiquinone 10

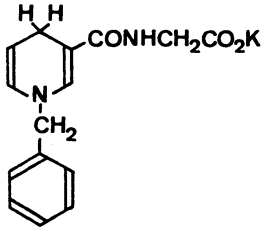
should be changed from a strong reducing agent such as $Na_2S_2O_4$ to RNAH derivatives. NADH itself is not a suitable reducing agent since it gives a too slow electron transport rate in the present flavoliposome system. This is not unusual and it is generally accepted that various NADH models give about 10^2 times faster rates toward flavin reduction than NADH itself in homogeneous solution.[5] This significant decrease of the reactivity of NADH is ascribed to the nature that a bulky and too hydrophilic NAD(P)H cannot get a molecular configuration appropriate for the electron transfer to flavin moiety without an assistance of the enzymatic binding. Therefore, a simple NADH model, 1-methyl-1,4-dihydronicotinamide (MeNAH) was considered as a candidate for the reductant. However the leakage rate of MeNAH through the lecithin bilayer membrane was too large (Table 1) to make practically difficult to estimate the net electron transport rate mediated by flavolipid because the material (MeNAH) transport overshadows the electron transport via flavolipid. MeNAH can not therefore be an appropriate NADH model. Introduction of stronger hydrophilicity is certainly required for the reductant to be used in the present membrane process. Therefore, we tested the potassium salt of *N*-(carboxymethyl)-1-benzyl-1,4-dihydronicotinamide (BzNAHCOOK)[6] as a reductant in the transmembrane electron transport study. The leakage rate of BzNAHCOOK was suppressed by a factor of 60 compared to that of MeNAH.

One of the disadvantages of the use of NADH model in the aqueous system arises often from its hydration.[7] BzNAHCOOK exhibited a superior characteristic in this point of view. The hydration rate of BzNAHCOOK in 5 mmol dm^{-3} Tris·HCl (pH=7.0) (k=1.1×10^{-5} s^{-1}) was 10^2 times slower than that of MeNAH in 5 mmol dm^{-3} Tris·HCl (pH=7.0) (k=1×10^{-3} s^{-1}). As will be illustrated later, this hydration

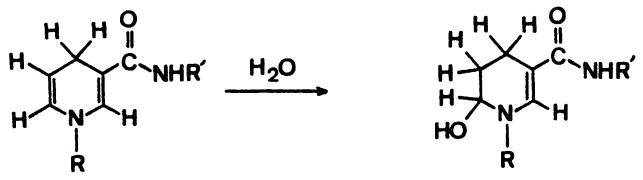
Table 1. Reduction Rate Constants of the Internal $K_3[Fe(CN)_6]$ in the Egg Lecithin Liposome by NADH-Models without the Flavolipid[a)]

NADH-model	k_{obs}/s^{-1} [b)]
MeNAH	60×10^{-3}
BzNAHCOOK	1×10^{-3}
NADH	0.03×10^{-3}

a) ($^{(o)}$NADH-models$|$lecithin$|^{(i)}$[Fe(CN)$_6$]$^{3-}$).
b) $-d[[Fe(CN)_6]^{3-}]/dt=k_{obs}[[Fe(CN)_6]^{3-}]$.



BzNAHCOOK



(RNAH)

Hydration of NADH and NADH-model compound.

rate of BzNAHCOOK is slow compared with rates of the facilitated electron transport across the membrane modified with flavolipid, the latter being 10^{-2}—10^{-1} s^{-1}. In the following, the electron transport through the artificial membrane functionalized with an efficient electron transport catalyst was investigated by using BzNAHCOOK as the external reductant. This electron transport system containing $K_3[Fe(CN)_6]$ in its internal aqueous phase ($^{(o)}$BzNAHCOOK$|$fl-Lip$|^{(i)}$ $K_3[Fe(CN)_6]$) is expected to give a simplified, isotopochemical model of the 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 the external BzNAHCOOK to the flavolipid (fl^{ox}: the oxidized form of the flavin moiety) locating in the membrane phase, was monitored by the reduction of flavolipid, which was incorporated into the liposome without $K_3[Fe(CN)_6]$ in its interior ($^{(o)}|$fl^{ox}-Lip$|^{(i)}$), by the external BzNAHCOOK. In the same way, the electron efflux ($J_{m\to i}$) from the reduced flavolipid (fl^{red}) locating in the bilayer membrane phase to the internal $K_3[Fe(CN)_6]$ was obtained by monitoring the oxidation of fl^{red} in the membrane by $K_3[Fe(CN)_6]$ in the external aqueous phase. The apparent flux constants of $k_{o\to m}$ and $k_{m\to i}$ were obtained from the following equation:

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

where the inner and the outer surface areas, S(i) and S(o) were estimated from the observation that the mean radius and the thickness of the liposome used were 35

Table 2. Oxidation of fl^{red} and Reduction of fl^{ox} in the Membrane

Reductant	Oxidant	$k_{o\to m}$[a,b)]	$k_{m\to i}$[a,b)]
mmol dm^{-3}	mmol dm^{-3}	cm^{-2} s^{-1}	cm^{-2} s^{-1}
BzNAHCOOK	—		—
0.8		0.04×10^{-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}	
—	$K_3[Fe(CN)_6]$	—	
	0.9		1.2×10^{-5}

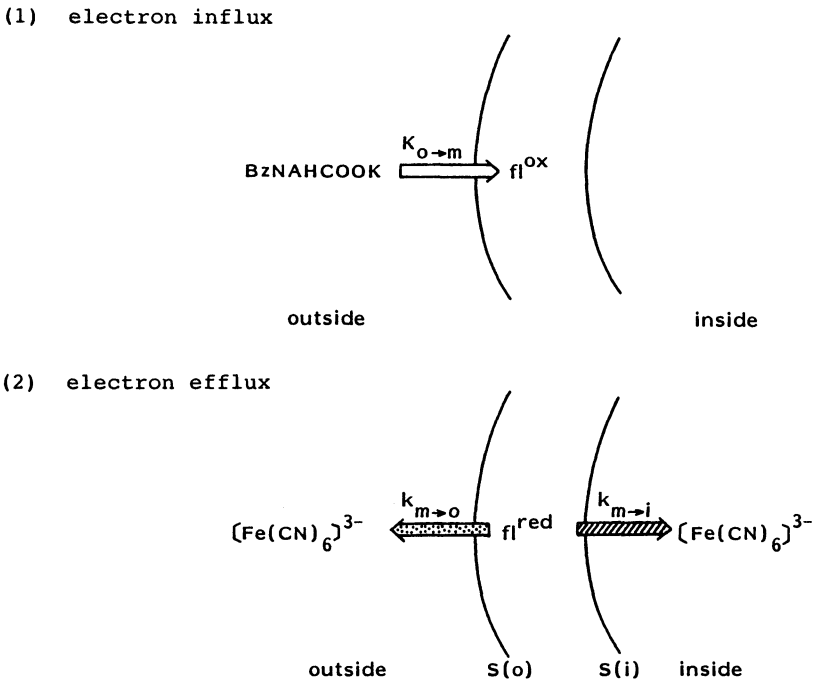
a) [liposome]=1.0×10^{-6} mol dm^{-3}, outer surface area=9×10^4 cm^2. b) $J_{o\to m}=-k_{o\to m}\cdot S(o)$ [fl^{ox}], $J_{m\to i}=-k_{m\to i}\cdot S(i)$[fl^{red}].

and 4 nm, respectively. Since the apparent electron efflux constant ($k_{m\to i}$) can not be determined directly, it was estimated from the following:

$$k_{m\to i}=k_{m\to o}\cdot S(i)/S(o)=2/3\cdot k_{m\to o}$$

where $k_{m\to o}$ is a value determined by the oxidation of the reduced flavolipid by the externally added $K_3[Fe(CN)_6]$ (see Scheme 1). It should be mentioned that the local concentration of $K_3[Fe(CN)_6]$ in the internal aqueous phase was in the order of 10^{-1} mol dm^{-3} in the oxidation of fl^{red} by the internal $K_3[Fe(CN)_6]$ ($J_{m\to i}$), while the concentration of $K_3[Fe(CN)_6]$ in the external aqueous phase was in the order of 10^{-3} mol dm^{-3} in the oxidation of fl^{red} by the external $K_3[Fe(CN)_6]$ ($J_{m\to o}$). It is practically impossible to determine the value of $k_{m\to o}$ at the concentration range of 10^{-1} mol dm^{-3} $K_3[Fe(CN)_6]$. Therefore, $k_{m\to i}$ determined by this way may give an underestimated value. Even so, it is safely concluded that the apparent $J_{m\to i}$ from fl^{red} to Fe^{III} was much larger than the apparent $J_{o\to m}$ from BzNAHCOOK to fl^{ox}.

The apparent electron influx constant ($k_{o\to m}$) from BzNAHCOOK to fl^{ox} was 10^3—10^4 times slower than that from $Na_2S_2O_4$ to fl^{ox}, when compared at a similar reductant concentration (ca. 1.0×10^{-3} mol dm^{-3}). The much slower reduction rate of flavolipid by BzNAHCOOK than by $Na_2S_2O_4$ (−1.13 V vs. NHE)[8c] may reflect the nature of a much weaker electron donor



S(o) : outer surface area of the liposome

S(i) : inner surface area of the liposome

Scheme 1. Measurement of electron influx and electron efflux.

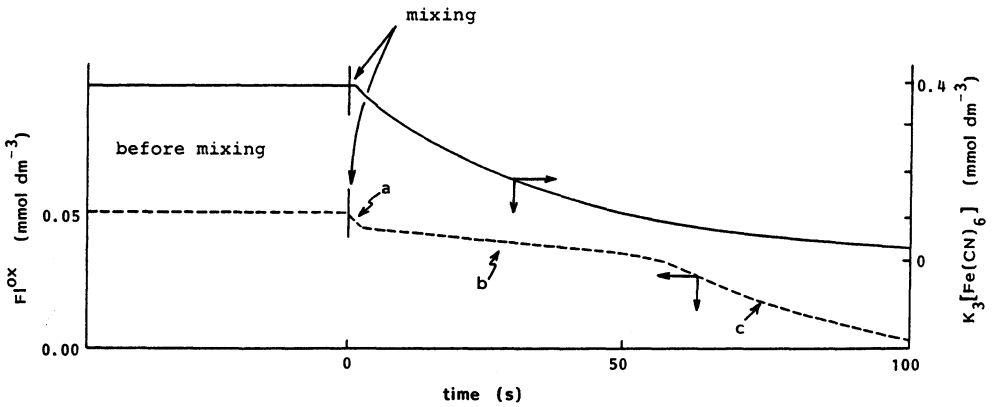


Fig. 1. Change of the concentration of the fl^{ox}-lip in the bilayer membrane (----) and the internal $K_3[Fe(CN)_6]$ (——) during the electron transport reaction by BzNAHCOOK. For a,b, and c, refer the text.

to the flavolipid than the latter.

In the reduction of internal $K_3[Fe(CN)_6]$ by the external BzNAHCOOK across the flavomembrane, a fast reduction of the flavin moiety to the corresponding dihydroflavin was observed at the very initial stage of the reaction (region "a" in Fig. 1). This process was followed by an apparent steady state, where the ratio fl^{red} to fl^{ox} was kept practically constant (region "b", fl^{red}/fl^{ox}=1/9—3/7), while most of the $K_3[Fe(CN)_6]$ was consumed during the period. This fl^{red}/fl^{ox} ratio was much smaller than that in the reduction of internal $K_3[Fe(CN)_6]$ by the external $Na_2S_2O_4$ (6/4=fl^{red}/fl^{ox}) under the same reductant concentration. This reflects the slower electron influx from the outside BzNAHCOOK to the fl^{ox} in the membrane than that from $Na_2S_2O_4$. When most of the $K_3[Fe(CN)_6]$ was reduced, the steady state disappeared 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 the external BzNAHCOOK to the flavolipid in the membrane because the rate was first order with respect to the concentration of the external BzNAHCOOK as seen from Table 3 and Fig. 2. Even at the highest concentration of BzNAHCOOK, the rate was not saturated as observed. As reported previously for the case of $Na_2S_2O_4$, the saturation of the reduction rate of the internal $K_3[Fe(CN)_6]$ indicated that the rate determining step of the overall reaction was shifted from the electron influx process ($J_{o\to m}$) to the flavin-flavin electron transfer one (see Fig. 3).[1] Furthermore the rate observed here (k) was far slower than the $k_{m\to i}\cdot S(i)$. These observations are compatible with the idea of rate-determining influx. The rate constants of the overall electron transport are summarized in Table 3 together with those of liposomes modified with ubiquinone 10 (UQ 10) and dibutylviologen (C_4V^{2+}). The flavolipid is a better catalyst for the electron transport from the external BzNAHCOOK to the internal $K_3[Fe(CN)_6]$ than ubiquinone 10. It is interesting to recall that in the case of $Na_2S_2O_4$, the liposome modified with ubiquinone 10 showed more than four time faster rate for the transmembrane electron

Table 3. Reduction Rate Constants of Internal $K_3[Fe(CN)_6]$ by BzNAHCOOK in the Functionalized Liposomes

BzNAHCOOK	Mediator		k
mmol dm^{-3}	mmol dm^{-3}		s^{-1}
2.6	—		0.1×10^{-2}
0.9	Flavolipid	(0.05)	1.2×10^{-2}
2.6	Flavolipid	(0.05)	6.3×10^{-2}
4.2	Flavolipid	(0.05)	7.1×10^{-2}
9.2	Flavolipid	(0.05)	13×10^{-2}
0.9	UQ 10	(0.05)	0.2×10^{-2}
2.6	UQ 10	(0.05)	0.9×10^{-2}
9.2	UQ 10	(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)	0.04
$Na_2S_2O_4$ (4.0)	UQ 10	(0.07)	1.70
DTT (2.6)	Flavolipid	(0.05)	0.6×10^{-2}
H_2/colloidal Pt	Flavolipid	(0.05)	0.6×10^{-2}

a) $-d[K_3[Fe(CN)_6]]/dt=k[K_3[Fe(CN)_6]]$, at 30 °C, pH 7.0. b) Flavolipid/Lecithin=UQ 10/Lecithin=2.5/97.5 (mol/mol), $[K_3[Fe(CN)_6]]$=0.39 mmol dm^{-3}. Local concentration of $K_3[Fe(CN)_6]$ was 0.75 mmol dm^{-3}. c) DTT: Dithiothreitol.

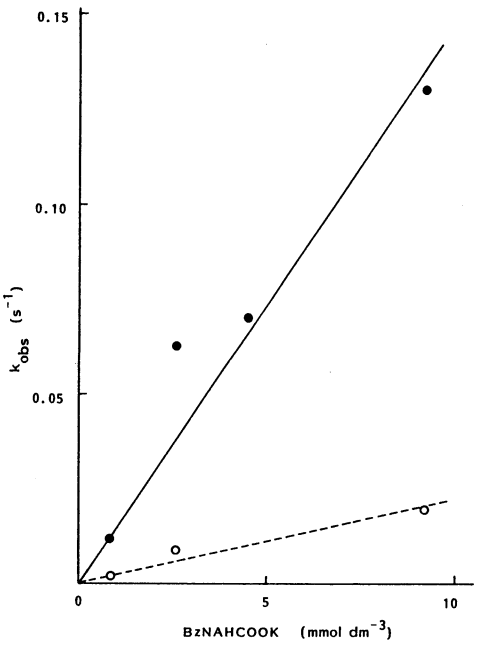


Fig. 2. $K_3[Fe(CN)_6]$ reduction by external BzNAHCOOK across the membrane modified with flavolipid or ubiquinone 10: fl-Lip (—●—), UQ 10 (--○--).

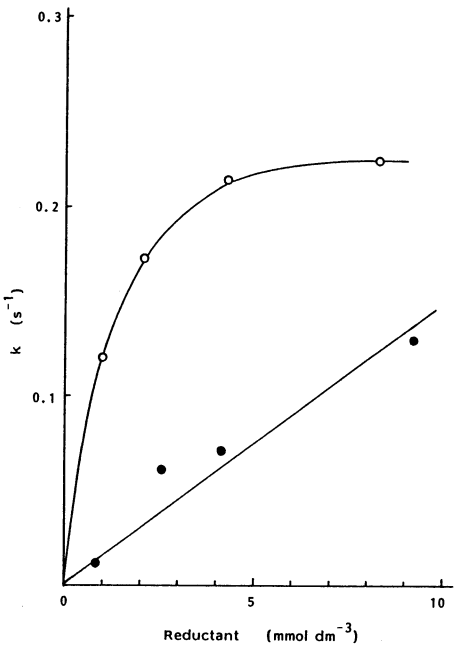


Fig. 3. Dependence of k on the concentration of the external reductant: $Na_2S_2O_4$ (—○—), BzNAHCOOK (—●—).

transport than the present flavoliposome. This rate order is reversed when the NADH model is employed as the reducing agent. In the electron transfer through the flavoliposome, it was demonstrated that the rate determining step was an influx, from the external reductant to the outer fl^{ox} (out) in the membrane in the case of BzNAHCOOK, while it was an efflux, from the inner fl^{red} to the $K_3[Fe(CN)_6]$ in the case of $Na_2S_2O_4$. Although the rate-determining step was determined to be the influx when BzNAHCOOK was employed, there is no information on it for the case of ubiquinone 10 modified liposome by the reductant of $Na_2S_2O_4$.[9] Therefore it is difficult to make a direct comparison of the overall electron-transfer rates for the flavoliposome and the ubiquinone 10 modified liposome in the case of $Na_2S_2O_4$. It is interesting that the flavoliposomes gave faster electron-transfer rates by the combination with a biologically important reducing agent such as NADH model than the ubiquinone 10 modified liposome system did. One of the important roles of the flavoprotein in the Complex I in the respiratory chain is the more efficient flux conversion from NADH in the bulk aqueous phase to the flavin moiety locating in the membrane phase than the direct flux conversion from NADH to ubiquinone 10. The result presented here may suggest a possible operation of such an electron relay system.

Dibutylviologen which is a moderately efficient electron-transport catalyst in the case of $Na_2S_2O_4$, showed only a poor catalytic activity in this case. This may reasonably be accounted for by considering the potential differences. The redox potential of C_4V^{2+} is -0.44 V vs. NHE,[8b] which means the reduction by BzNAHCOOK ($E_{1/2}=-0.36$ V vs. NHE at pH 7, two electron reduction-oxidation potential)[8a] 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 hydrogen-metabolizing system, hydrogen/colloidal platinum was used as a reducing agent in this system.[10] Dithiothreitol (DTT) also reduced this system, suggesting the possibility that the flavoliposome could work as a lipoyl dehydrogenase model.[11] From these various electron transport systems, the artificial liposome modified with the flavolipid was demonstrated to work as an effective model system for the study of biological electron transport.

Electrochemistry of Flavolipid Incorporated into the Bilayer Membrane. In order to know the oxidation states of the flavolipid 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) showed two clear pairs of cathodic and anodic

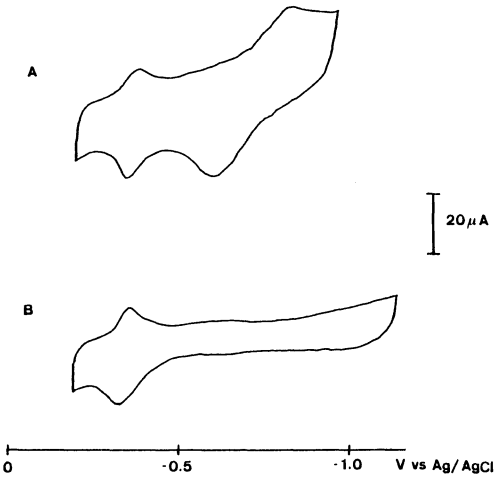


Fig. 4. Cyclic voltammograms on Hg-pool of the flavoliposome in 5 mmol dm^{-3} Tris HCl (pH=7.0). A: ($^{(o)}|$fl-Lip$|^{(i)}$), B: ($^{(o)}|$fl-Lip$|^{(i)}$ $K_3[Fe(CN)_6]$).

waves at -0.35 and -0.70 V vs. Ag/AgCl using a Hg-pool as a working electrode (Fig. 4). It is reported previously[12] that lecithin and other surfactants in water are adsorbed on a Hg electrode surface to inhibit the normal redox reactions of Cd^{2+} on the electrode.[13] In spite of such an inhibitory effect of lecithin, the present flavoliposome (0.025 mmol dm^{-3} flavolipid) afforded clear oxidation-reduction peaks. This may be explained by the idea that the flavin unit (redox site of the liposome) locates in the vicinity of the Hg-lecithin membrane interphase to favor the electron uptake and release from Hg to flavin and vice versa, respectively. In a contrast to the above case, the flavoliposome containing $K_3[Fe(CN)_6]$ in its internal aqueous phase ($^{(o)}|$fl-Lip$|^{(i)}$ $K_3[Fe(CN)_6]$) afforded only one redox couple of cathodic and anodic waves at -0.35 V vs. Ag/AgCl. Assignment of clearly separated two-step redox conversion is now under investigation in relation to the electron transfer reaction from BzNAHCOOK and/or $Na_2S_2O_4$ to $K_3[Fe(CN)_6]$ across the flavomembrane.

Experimental

Instruments. ^{1}H NMR spectra were obtained with a JEOL JNM PMX 60 SI NMR spectrometer, a JEOL JNM FX90Q FT NMR spectrometer or a JEOL JNM GX400 spectrometer. Mass spectra were obtained with a JEOL JMS-DX300 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, 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 otherwise noted, 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 °C under Ar in the dark until its use. BzNAHCOOK was prepared according to the literature method[6] and identified by ^{1}H NMR, IR, electronic spectra and mass spectra.

Preparation of Artificial Single-Wall Bilayer Liposome. The artificial egg lecithin liposome containing $K_3[Fe(CN)_6]$ in its internal aq. phase ($^{(o)}$|lecithin|$^{(i)}$ $K_3[Fe(CN)_6]$), the artificial liposome modified with the flavolipid ($^{(o)}$|fl-Lip|$^{(i)}$) and the liposome modified with the flavolipid containing $K_3[Fe(CN)_6]$ in its internal aqueous phase ($^{(o)}$|fl-Lip|$^{(i)}$ $K_3[Fe(CN)_6]$) were prepared according to the sonication method reported previously by us.[16]

Measurement of Leakage Rate of NADH and its Models through the Bilayer Membrane. Leakage rates of NADH and its model compounds through the lecithin bilayer membrane were measured by observing the reduction of internal $K_3[Fe(CN)_6]$ in the lecithin liposome without the flavolipid ($^{(o)}$|lecithin|$^{(i)}$ $K_3[Fe(CN)_6]$). Into the 2.0 mL of the deaerated liposome solution (5 mmol dm^{-3}. Tris HCl, pH=7.0) was added 0.2 mL of the deaerated NADH model solution freshly prepared using a syringe stopped flow apparatus.[15] Reduction of the internal $K_3[Fe(CN)_6]$ was followed by monitoring the change in the absorbance at 425 nm.

Reduction of Flavolipid (fl^{ox}) Incorporated into the Bilayer Membrane by External BzNAHCOOK. Reduction of the flavolipid incorporated into the bilayer membrane by BzNAHCOOK was performed using the 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 $K_3[Fe(CN)_6]$. A freshly prepared flavoliposome solution ($^{(o)}$|fl-Lip|$^{(i)}$), 2.0 mL) was placed in a 10 mm quartz cell equipped with a three-way stopcock. After the solution was deaerated by the usual method, 0.2 mL of a freshly prepared $Na_2S_2O_4$ (2.0 mmol dm^{-3}) solution was added. The reduction of fl^{ox} was ascertained to be complete within one minute by monitoring the change of absorbance at 444 nm. Then, into the reduced flavoliposome solution ($^{(o)}$ $Na_2S_2O_4$|fl^{red}-Lip|$^{(i)}$) at 25 °C, 0.2 mL of a deaerated $K_3[Fe(CN)_6]$ solution (11.0 mmol dm^{-3}) was added by using a specially designed syringe.[15] The reoxidation 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 $K_3[Fe(CN)_6]$ in the Internal Aqueous Phase of the Flavoliposome by External BzNAHCOOK. Reduction of the internal $K_3[Fe(CN)_6]$ by the 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 reduction.

Cyclic Voltammetry of the Flavoliposome. A Bioanalytical system CV-1B cyclic voltammograph was employed for the cyclic voltammetric determination of the flavolipid incorporated into the bilayer membrane. A freshly prepared flavoliposome solution (5 mL) was degassed by nitrogen bubbling for 0.5 h. As a supporting electrolyte, a KCl solution (0.1 mol dm^{-3}) was used and a Hg-pool electrode was used as a working electrode. Potentials were determined at 25 °C vs. an Ag/AgCl electrode as a reference.

Measurement of Hydration Rate of NADH Models in Tris Buffer Solution. Hydration rate of NADH models was monitored by the first order decrease of the intensity of the absorbance at 360 nm, characteristic of the reduced form of NADH models by using a freshly prepared solution of NADH model compound in 5 mmol dm^{-3} Tris·HCl (pH 7.0).

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