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CATALYSIS OF ELECTRON TRANSFER ACROSS PHOSPHOLIPID BILAYERS BY IRON-PORPHYRIN COMPLEXES

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Phospholipid vesicles containing $K_3Fe(CN)_6$ were prepared from egg yolk phosphatidylcholine. Hemin dimethyl ester was incorporated into these vesicles during preparation in ratios of phospholipid to hemin dimethyl ester that varied from 200 : 1 to 45 000 : 1. Electron transfer across the bilayer was measured anaerobically after injecting the vesicles into a solution containing reduced indigotetrasulfonic acid. Vesicles containing hemin dimethyl ester exhibited high rates of electron transfer (240 electrons/molecule hemin dimethyl ester per min). Conditions could be selected where the rate-limiting step for catalysis was either the bimolecular reaction between ferric hemin dimethyl ester and reduced indigotetrasulfonic acid or the movement of hemin dimethyl ester from interface to interface. The hemin dimethyl ester-catalyzed electron transfer went to completion within a few seconds, completely oxidizing the reduced indigotetrasulfonic acid. Valinomycin (in the presence of potassium) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were without effect on catalyzed electron transport. Thus, the electron transport is not electrogenic but is a coupled, neutral system. By specific assay, neither phosphate nor cyanide was significantly transported during electron transfer but evidence is provided to suggest that a coordinated hydroxide accompanies movement of Fe(III) hemin dimethyl ester from the inside surface to the outside surface of the bilayer. It was also demonstrated in a bulk phase transport system that hemin dimethyl ester readily catalyzes transfer of $S^{14}CN^-$ through a chloroform layer separating two aqueous phases. Another more hydrophobic iron-porphyrin complex, Fe(III) tetraphenylporphyrin, was found to be twice as effective as hemin dimethyl ester. Other porphyrin complexes were also tested as control systems. No significant catalysis was found for metal-free protoporphyrin IX dimethyl ester or Ni(II) tetraphenylporphyrin. The results are discussed in comparison with in vivo electron transport and the future usefulness of this model system.

Introduction

Electron transport across membranes plays a central role in both oxidative metabolism and in photosynthesis. Not only does over 90% of the energy release in this kind of metabolism occur in association with such membranes, but its coupling to ATP formation and ion and metabolite transport is also intimately associated with the same membrane. Although most of the electron-transport components are known for some of these systems, the actual mechanism of electron transfer is not understood in detail for even one of the many redox reactions that occur. The most knowledge is available for the interaction of cytochrome *c* with cytochrome oxidase [1–4], but the latter component is a large, poorly defined multi-redox-centered complex. In bacterial photosynthesis, the reaction between the oxidized primary electron donor and ferrous cytochrome c_2 is also a well defined reaction [5–11], but neither is it yet delineated at a mechanistic level.

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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A difficulty, of course, has been the isolation of individual integral membrane electron-transport components in pure form with retention of native activity. Also lacking is a general understanding of oxidation-reduction reactions which occur at phospholipid bilayer interfaces and within the apolar region of such bilayers. Because so many redox centers in electron-transport systems are heme proteins, we felt it would be helpful at this juncture to study systematically a series of model iron-porphyrin complexes and determine their oxidation-reduction behavior in liposomes and in apolar environments. We expected that iron porphyrins would readily associate with phospholipid bilayers, possibly being catalytic within them because the molecule in the ferrous oxidation state has no net charge (assuming there are no charged side chains). In the ferric oxidation state such iron porphyrins may behave as permeant cations [12–14]. In preliminary studies we have reported [15–17] that hemin dimethyl ester is indeed catalytic in carrying electrons across the phospholipid bilayer of liposomes prepared from either soybean phospholipids or egg yolk phosphatidylcholine.

The catalytic electron transfer by ubiquinone, ferrocene, or dyes across phospholipid bilayers has been reported previously by others [18–22]. In some of these systems, electron transfer was accompanied by proton transfer [18,20,22–24]. Electron transfer catalyzed by iron porphyrins such as hemin dimethyl ester should be coupled to anion or cation transport, or result in the formation of a transbilayer potential. Therefore, these model systems also offer the opportunity to probe systematically ion transport which may accompany electron transport or the generation of membrane potentials.

Methods and Materials

Materials. L-α-Dipalmitoylphosphatidylcholine, L-α-phosphatidylcholine from egg yolk (type III-E), cholesterol, bovine heart cardiolipin, L-α-phosphatidylethanolamine (type V from *Escherichia coli*), valinomycin, FCCP, G-25-80 Sephadex, hemin type III, and protoporphyrin IX dimethyl ester were purchased from Sigma Chemical Co., St. Louis, MO. Iron(III) tetraphenylporphyrin chloride and Ni(II) tetraphenylporphyrin were purchased from Strem Chemicals, Newburyport, MA. Indigotetrasulfonic acid was obtained from City Chemical Corp., New York, NY, and indigodisulfonic acid from ICN Life Sciences Group, K & K Laboratories, Plainview, NY. $KS^{14}CN$ was purchased from Amersham Corp., Arlington Heights, IL, ^{33}P as the monopotassium phosphate from New England Nuclear, Boston, MA, and [^{14}C]sucrose from ICN, Irvine, CA. For synthesis of hemin dimethyl ester, 203 mg of equine hemin type III were stirred for 20 h at 0°C with 5% H_2SO_4 in methanol. Hemin dimethyl ester was recrystallized from chloroform/methanol [25].

Preparation of porphyrin-containing phosphatidylcholine vesicles. 100 mg of egg yolk phosphatidylcholine were dissolved in chloroform in a 100 ml round-bottom flask. Hemin dimethyl ester, usually between 0.2 and 0.6 mg, was then added from a more concentrated stock solution. The chloroform was removed at reduced pressure using a rotary evaporator for 20 min at room temperature. The film of hemin dimethyl ester/phosphatidylcholine was mixed with 2.5 ml of water containing 0.1 M ferricyanide and either 0.4 M imidazole chloride or 0.2 M potassium phosphate at pH 7, and the solution was hand shaken until all phospholipid and hemin dimethyl ester were removed from the sides of the vessel. The mixture was then sonicated for two 5-min intervals at 0°C under nitrogen using a 100 W power setting on a Braunsonic 1410 Sonicator, and passed over a G-25 column in order to remove external ferricyanide. Dilution of the vesicles during this gel filtration was less than 10%. Buffers used in the chromatography and later for assay contained 0.175 M NaCl, 0.025 M KCl, and 0.4 M imidazole, pH 7, or 0.4 M potassium phosphate. Following gel filtration, the sample was centrifuged at 140 000 × g for $\frac{1}{2}$ h. When the sonication had been efficient, only a very small pellet formed (5% of the total material). Immediately after centrifugation, air was removed from the approx. 2 ml vesicle preparation by passing argon gas over and through the vesicles for $\frac{1}{2}$–1 h. Usually the vesicles were used the same day they were prepared.

The concentration of hemin dimethyl ester incorporated into the vesicles was determined by measuring the absorbance spectra in the presence of imidazole (see Fig. 1) and using the reduced-minus-oxidized extinction coefficient of $20.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 557 nm, the extinction coefficient difference for the pyridine hemochrome of iron protoporphyrin IX

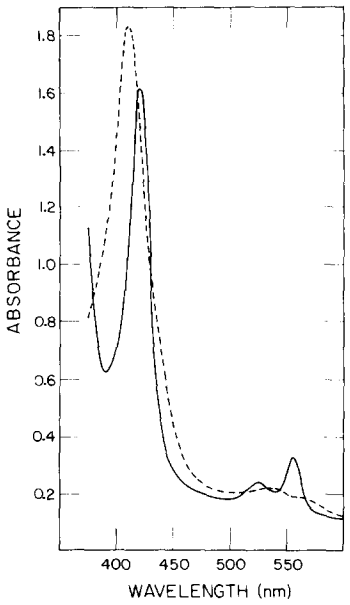


Fig. 1. Vesicle absorption spectrum of hemin dimethyl ester in egg phosphatidylcholine vesicles containing internally trapped ferricyanide: oxidized (· · · · ·) and dithionite reduced (———). The sample contained 20 nmol of hemin dimethyl ester and approx. 10 mg of egg phosphatidylcholine in 2.85 ml of buffer which had 0.175 M NaCl, 0.025 M KCl and 0.4 M imidazole, pH 7. The reference cuvette contained buffer only. Although sample scattering makes a contribution to the absorbance (increasing at lower wavelengths) the hemin dimethyl ester has sufficiently narrow bands that its spin state and concentration are readily evaluated. Room temperature.

[25]. We found that the extinction coefficients for the pyridine and imidazole hemochrome spectra were nearly the same in chloroform. Imidazole is known to be rapidly taken up by membranes and diffuse readily across them [26,27]. In all cases our spectra indicated quantitative hemochrome formation with imidazole present. Spectra were recorded with an Acta CIII Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The average of a number of similar spectra gave an oxidized Soret peak maximum at 411 ± 1 nm, a reduced Soret maximum at 422 ± 2 nm and the α peak maximum at 557 ± 1 nm.

In order to determine the efficiency of hemin dimethyl ester incorporation into the vesicles, an aliquot of the original hemin dimethyl ester was dissolved in a 1 : 1 chloroform/pyridine solution and the oxidized spectrum of this solution was recorded. The hemin dimethyl ester was reduced by adding small amounts of solid $SnCl_2 \cdot 2H_2O$ with subsequent shaking and the spectra recorded until they no longer changed upon further reductant additions. The difference at 557 nm for this reduced-minus-oxidized spectrum was then considered to represent what should be observed for a 100% hemin dimethyl ester incorporation into vesicles. When the egg yolk phosphatidylcholine vesicles were made quickly in air with 0.3 mg hemin dimethyl ester/100 mg phospholipid, the percent incorporation was 81 ± 15%. However, if smaller amounts of hemin dimethyl ester were added initially, or if the vesicle preparation was slow, then lower percent incorporations were observed (e.g., 25–50%) unless additional precautions were taken to remove air during lipid solubilization and sonication.

For vesicles containing iron tetraphenylporphyrin, nickel tetraphenylporphyrin or protoporphyrin IX dimethyl ester, the same procedure as above was followed. For hemin-containing vesicles, however, since hemin was not readily soluble in chloroform, it was dissolved in chloroform/methanol (1 : 1). An aliquot of the latter solution was mixed with the phospholipid dissolved in chloroform, bringing the methanol concentration to 10%. Subsequent steps were identical to those already described.

The electron-transfer assay. In this assay hemin dimethyl ester catalyzed the transfer of an electron from a reduced dye across the lipid bilayer to the oxidant ferricyanide which was trapped within the vesicles. The dye used was indigotetrasulfonic acid, although indigodisulfonic acid gave similar results. Indigotetrasulfonic acid has an intense absorption band at 600 nm in the oxidized form which disappears upon reduction [28] (extinction coefficient at 600 nm was $2.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ as determined by ferricyanide titration). In the assay, then, we monitored the electron-transfer reaction by following the increasing absorbance at 600 nm.

For the electron-transfer assay, a 2.6 ml aliquot of dye solution (absorbance = 1.2 at 600 nm) was placed in a cuvette capped by a rubber septum and deaerated for 20 min with argon which was introduced at the bottom of the cuvette by inserting a Hamilton stainless-steel needle through the septum. The dye solution was then reduced about 85–90% by addition of less than 10 μl of sodium dithionite solution and the

cuvette assembly was placed in the spectrophotometer. While recording the absorbance at 600 nm, 0.25 ml of degassed vesicles was anaerobically transferred and quickly injected with constant argon flow to provide continuous mixing. Two or three duplicate experiments were conducted for each vesicle preparation and the electron-transfer rate in control vesicles (ferricyanide but no hemin dimethyl ester) was also determined several times. The reverse of this experiment was also occasionally used: 2.35 ml of buffer and 0.25 ml of vesicles plus any other desired chemical such as valinomycin or FCCP were added to the cuvette and degassed, and then 0.25 ml of degassed reduced indigotetrasulfonic acid ($A_{600nm} = 12$ when oxidized) was injected. We found that our mixing time was about 1 s. Consequently, the initial rate for transbilayer electron transfer was determined from the dye oxidation rate profile measured after the first second.

Background electron-transfer rates. The amount of ferricyanide trapped within the vesicles depended upon the concentration of ferricyanide used in the buffer for forming the vesicles. We have varied this concentration from 0.1 to 0.8 M. The largest amount of ferricyanide trapped was 360 nmol per mg of phospholipid. The breakage of vesicles or the leakage of ferricyanide out of the vesicles was slow as 80% of the initially trapped ferricyanide was still trapped after storage overnight at 5°C under argon (as determined after gel filtration).

It was found that the background rate of electron transfer was dependent upon both the trapped ferricyanide concentration and the external dye concentration. Consequently, in most experiments we chose to limit the amount of ferricyanide trapped to about 28.5 nmol per mg of phospholipid and the dye to $5 \cdot 10^{-5}$ M in order to keep the background rate low. To eliminate the possibility that a sulfur species (e.g., SO_2^-) might be a transmembrane electron carrier, which was causing the background electron-transport rate, KBH_4 was used to reduce the dye rather than dithionite. The background rate was unchanged. Furthermore, when reduced riboflavin monophosphate was used in place of reduced indigotetrasulfonic acid, it was oxidized at a similar rate. Thus, the reductant added does not appear to be directly responsible for the background rate. Because chemically synthesized dipalmitoylphosphatidylcholine substantially reduced the background rate (but did not eliminate it), some of this electron transport is perhaps due to a very small quantity of an electron-transport contaminant (e.g., ubiquinone) in the egg yolk phosphatidylcholine. Whatever the cause, the background rate is so low that we felt it could be ignored.

[^{14}C]Sucrose and ^{33}P experiments. Hemin dimethyl ester vesicles were made in the usual fashion except that $5 \cdot 10^6$ cpm [^{14}C]sucrose were added to the 2.5 ml of preparation. A typical sample of the vesicles so prepared contained 2000 cpm per 100 μl and retained all this radioactivity (to within 5%) when submitted to a second G-25 gel filtration column. Furthermore, no sucrose was lost from the vesicles during the electron-transfer reaction and therefore vesicle rupture was not occurring.

The experiment with radioactive phosphate was similarly performed. For vesicle formation $5.4 \cdot 10^6$ cpm of monopotassium [^{33}P]phosphate were included with the 2.5 ml of 0.1 M potassium ferricyanide, 0.1 M potassium citrate, and 0.04 M potassium phosphate, pH 6.8. After gel filtration typically 2000–4000 cpm/100 μl remained with the vesicle fraction. These counts were not diminished upon further chromatography of vesicles, regardless of whether or not they contained hemin dimethyl ester.

Cyanide determination. 1 ml of anaerobic hemin dimethyl ester containing vesicles was mixed with either 1 ml of indigotetrasulfonic acid ($A_{600nm} = 10$), 1 ml of reduced indigotetrasulfonic acid (oxidized $A_{600nm} = 10$), or 1 ml of indigotetrasulfonic acid ($A_{600nm} = 10$) containing between $1 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M KCN. Argon was bubbled through the solution and exited into a quartz cuvette containing $5 \cdot 10^{-3}$ M $AgNO_3$. At 5-min intervals after the experiment was initiated, the cuvette was monitored at 350 nm for a precipitate of AgCN. The assay gave a linear relationship for HCN concentrations between $5 \cdot 10^{-5}$ and $1 \cdot 10^{-3}$ M with a limit for detection of about $5 \cdot 10^{-5}$ M. In these experiments we were typically assaying for $5 \cdot 10^{-4}$ M.

pH measurements. 6 ml of a solution containing 0.45 M KCl, $2 \cdot 10^{-4}$ M K_2HPO_4, and indigotetrasulfonic acid ($A_{600} = 1$) were degassed for $\frac{1}{2}$ h with argon in a vessel capped with a rubber septum. A Radiometer combination pH electrode was inserted through the septum before degassing commenced.

The usually basic solution was brought to pH 7.00 with small quantities of $1.4 \cdot 10^{-2}$ M HCl. When reduced dye was desired a small aliquot of $3 \cdot 10^{-2}$ M $Na_2S_2O_4$ in $3 \cdot 10^{-2}$ M KOH was added. Then 0.5 ml of anaerobic vesicles was added and the external pH recorded at 5-s intervals. The resulting pH change was compared to that obtained by addition of 0.25 ml of $2.4 \cdot 10^{-3}$ M ferricyanide. The pH meter used was a Beckman Digital pH Meter, model 76.

Thiocyanate exchange. 0.6 ml of chloroform containing hemin dimethyl ester was placed in a small U-tube [29]. On each side of the U-tube above the chloroform was placed 0.6 ml of water containing 1 mM KSCN and 1 mM phosphate, pH 7. $KS^{14}CN$ was then added to only one of these aqueous solutions. The chloroform layer was agitated for many hours with a stir bar, after which 25- or 100-μl aliquots were withdrawn from the aqueous phase which had not received the $KS^{14}CN$ addition and the ^{14}C content determined.

Results

Catalysis of electron transfer

The amount of hemin dimethyl ester incorporated into the vesicles varied from 0.285 to 57 nmol per 10 mg of phospholipid. This is a heme-to-lipid ratio between about 1 : 45 000 and 1 : 200, assuming that all the lipid initially added was recovered in the vesicle preparation. As Fig. 2 (top) shows, hemin dimethyl ester catalyzed transbilayer electron transfer many-fold over the background rate. The rate of electron transfer showed a first-order dependence on the reduced indigotetrasulfonic acid concentration over a 30-fold range of hemin dimethyl ester concentrations. These reactions proceeded to completion, i.e., until all the reduced indigotetrasulfonic acid was oxidized, since the $K_3Fe(CN)_6$ present was chosen to be slightly in excess. At low hemin dimethyl ester concentrations, the rate of electron flow was 240 electrons/min per molecule of hemin dimethyl ester. By plotting the first-order rate constant of dye oxidation vs. the hemin dimethyl ester concentration, the rate was also shown to be first order in the concentration of hemin dimethyl ester (Fig. 2, bottom).

Thus, at the relatively low concentration of reduced indigotetrasulfonic acid employed in the experiment of Fig. 2, the rate-limiting step of catalysis is a bimolecular reaction between the hemin dimethyl ester and the reduced dye. Separate experiments showed that the rate of catalysis was independent of the $K_3Fe(CN)_6$ concentration under these conditions. However, at about 5-fold higher concentrations of reduced indigotetrasulfonic acid (and $K_3Fe(CN)_6$) and low concentrations of hemin

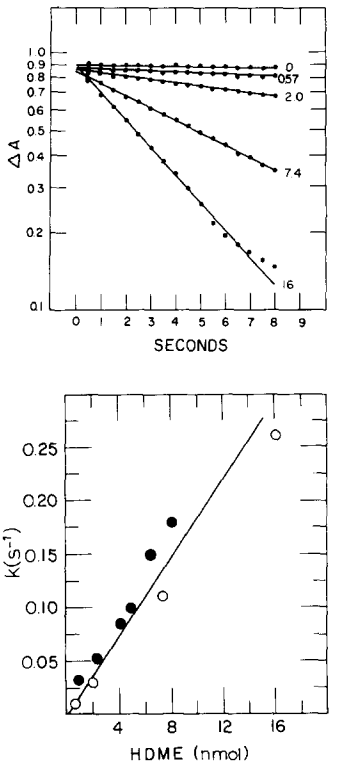


Fig. 2. (Top) The log of the change in absorbance at 600 nm due to electron transfer catalyzed by hemin dimethyl ester. ΔA is proportional to the reduced indigotetrasulfonic acid concentration. The content of hemin dimethyl ester in nmol per 10 mg phospholipids is indicated on the curves. The buffer solution was as given in Fig. 1 and contained about 285 nequiv. of reduced indigotetrasulfonic acid ($5 \cdot 10^{-5}$ M, A = 1.0 when oxidized), 10 mg of egg phosphatidylcholine and 310 nmol of trapped ferricyanide. See Methods and Materials for further details of the assay. (Bottom) Rate constant for reduced indigotetrasulfonic acid oxidation with increasing concentration of hemin dimethyl ester (HDME) in the egg phosphatidylcholine bilayer. Solution conditions were as described above. The data points were taken from two sets of experiments as noted by the open and closed circles, the open circles representing the rate constants for dye oxidation as shown by the data above.

dimethyl ester, the rate-limiting step was not dependent on the concentration of either reduced indigotetrasulfonic acid or $K_3Fe(CN)_6$, but showed only a first-order dependence on the hemin dimethyl ester concentration. Thus, by selecting appropriate concentrations, the rate-limiting step could be chosen to be the redox reaction at the bilayer interface or the presumed movement of the hemin dimethyl ester from interface to interface.

Effect of varying the pH buffer

As an alternate to the 0.4 M imidazole chloride buffer, vesicles were also prepared using 0.2 M potassium phosphate, pH 7. The amount of hemin dimethyl ester incorporated into the phosphatidylcholine bilayer was similar to that observed with imidazole present. The absorbance spectra of the hemin dimethyl ester in vesicles prepared in phosphate were characteristically high spin as would be predicted (see Fig. 3). At the concentration of reduced indigotetrasulfonic acid and $K_3Fe(CN)_6$ as indicated for the experiment of Fig. 2, hemin dimethyl ester catalyzed electron transfer in phosphate buffer for vesicles prepared in phosphate buffer at the same rate as that observed with imidazole. The reaction in phosphate buffer was found to be independent of pH between pH 5 and 10.

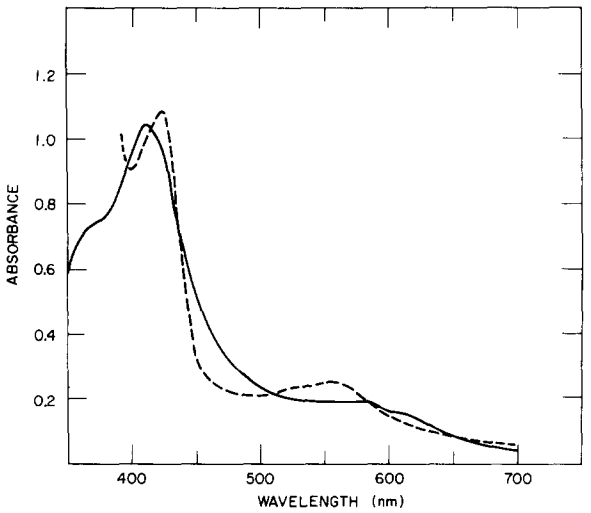


Fig. 3. Absorbance spectrum of hemin dimethyl ester in egg yolk phosphatidylcholine vesicles. The sample contained 20 nmol of hemin dimethyl ester and approx. 10 mg of egg phosphatidylcholine in 2.85 ml of buffer which had 0.175 M NaCl, 0.025 M KCl and 0.2 M phosphate buffer, pH 7.0. (———) Ferric form. (- - - - -) Ferrous form obtained by addition of $Na_2S_2O_4$. The reference cuvette contained buffer only. Although sample scattering makes a contribution to the absorbance (increasing at lower wavelengths), the hemin dimethyl ester has sufficiently narrow bands that its spin state and concentration are readily evaluated.

Effect of valinomycin and FCCP

Because the hemin dimethyl ester catalysis proceeds rapidly until all the external reductant is oxidized, and because the [^{14}C]sucrose-loaded vesicles showed that there was no breakage of the liposomes during electron transport, it seemed clear that an anion must accompany the ferric form of hemin dimethyl ester across the bilayer in order to preserve electrical neutrality. However, because valinomycin plus potassium and uncouplers like FCCP are often used in in vivo studies to test for possible $\Delta\psi$ formation and/or proton gradients, their effects were tested here.

Valinomycin was added to a standard hemin dimethyl ester containing vesicle preparation before mixing with reduced indigotetrasulfonic acid. The potassium concentration was 0.6 M both inside and outside the vesicles. Several valinomycin concentrations were tested (0.3–1 μM, see Fig. 4). No effect by valinomycin was observed either on the rate or extent of the reaction. In a similar set of experiments, in which FCCP was added to final concentrations of 0.8 and 1.7 μM, no effect on the hemin dimethyl ester-catalyzed rate was observed. These results are also shown in Fig. 4.

Tests for anion transport

Although OH^- is the obvious candidate for the anion transported because it is known to be coordinated to ferric porphyrin complexes at pH 7 in the absence of other strong ligands [30], we wanted to test also whether any other anions were being transported.

The only anions present at high concentration in the phosphate buffer system at pH 7 are $H_2PO_4^-$ and HPO_4^{2-}. To test whether one of these (or both) was being transported from inside the vesicles to the outside to allow electrically neutral electron transport, the composition inside the vesicles was adjusted such that 0.04 M ^{33}P was present along with 0.1 M citrate to act as a nonpermeant buffer at pH 7.0. Citrate was

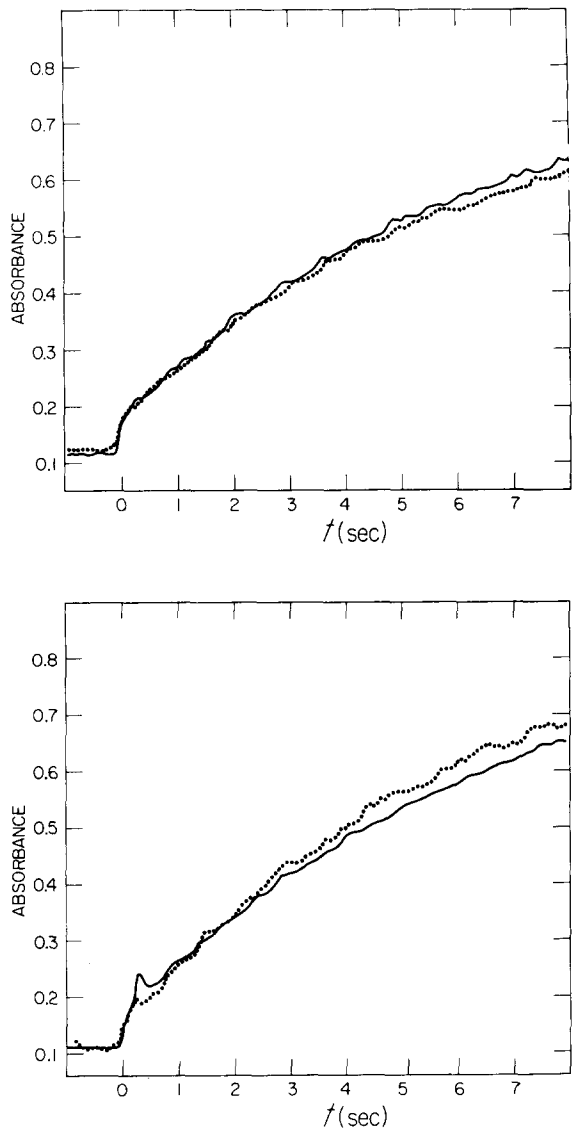


Fig. 4. (Top) Effect of valinomycin (in the presence of 0.6 M potassium) on hemin dimethyl ester-catalyzed electron transport across egg phosphatidylcholine vesicles. (———) Complete system without valinomycin. (· · · · · ·) Complete system with valinomycin added to a concentration of 1 μM. Vesicles contained 7 nmol hemin dimethyl ester per 10 mg phospholipid. Vesicles were added to the dye solution at time zero. (Bottom) Effect of FCCP on hemin dimethyl ester-catalyzed electron transport across egg phosphatidylcholine vesicles. (———) Complete system without FCCP. (· · · · · ·) Complete system with FCCP added to a concentration of 1.7 μM. Vesicles contained 2.8 nmol hemin dimethyl ester per 10 mg phospholipid.

chosen because its possible transport as an anion seemed very unlikely (three negative charges at pH 7). The results clearly showed that when this system was added to the reduced indigotetrasulfonic acid solution, the electron-transport reaction proceeded normally but no significant phosphate was transported out of the vesicles (less than 5%). In agreement with this result, other radioactive-labelling experiments with vesicles containing ferric hemin dimethyl ester showed that phosphate was not exchanged across the bilayer at a measurable rate.

Another anion which is present at very low concentrations is CN^-. Even though this anion is in a tight complex with the Fe^{3+} and Fe^{2+} within the vesicles, there is a very small concentration of free CN^-. Although the absorbance spectrum of hemin dimethyl ester in the phosphate buffer system (see Fig. 3) clearly indicated the presence of high-spin iron (the cyanide complexes are low spin), we thought a more sensitive test would be to assay for CN^- transport by chemical measurement of any CN^- carried to the outside of the vesicles. The method of measurement is described in Methods and Materials. Less than 10% equivalent of CN^- was found external to the vesicles after completion of electron transport in these experiments. Thus, CN^- is not transported significantly.

To determine if OH^- was the anion transported as expected, we felt the simplest evidence would be obtained by measuring the pH of the reaction media as electron transfer ensued. Fig. 5 (top) shows that in control experiments where the reduced indigotetrasulfonic acid is simply titrated with $K_3Fe(CN)_6$ in the presence of liposomes, the pH of the media decreases by 0.16 pH units due to the reaction:

$$2K^+ + ITSAH_2 + 2K_3Fe(CN)_6 \rightleftharpoons ITSA + 2H^+ + 2K_4Fe(CN)_6$$

where $ITSAH_2$ represents reduced indigotetrasulfonic acid and ITSA its oxidized form. However, in the hemin dimethyl ester-catalyzed electron-transport system, no significant pH decrease is observed (see Fig. 5, middle), even though electron transport is rapid (Fig. 5, bottom). The very slow decrease in pH that occurs at longer times is probably due to proton leakage. The natural leakage of protons from such liposomes has been measured [31] and is about 100-fold slower than our measured electron-transport

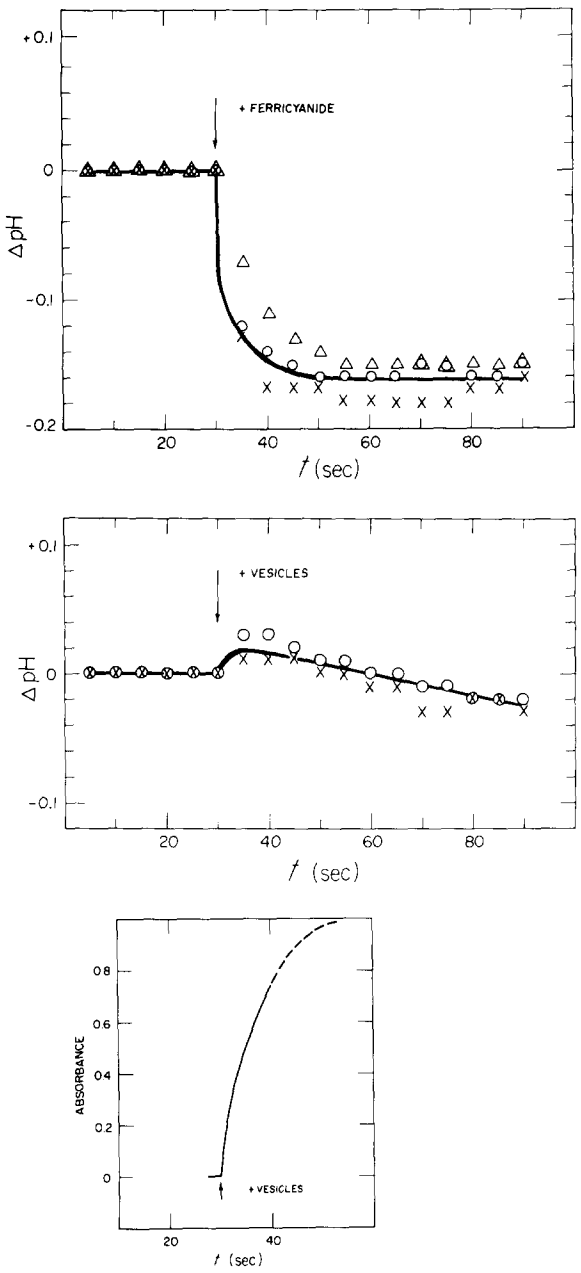


Fig. 5. (Top) pH Change due to the reaction (0.25 ml) of $K_3Fe(CN)_6$ (600 nmol) added directly to 300 nmol reduced indigotetrasulfonic acid in 6 ml of $2 \cdot 10^{-4}$ M potassium phosphate buffer. The pH of the system was recorded at 5-s intervals. The results of three separate experiments are plotted, each with an initial pH near 7.00 (Middle) pH Change upon mixing 0.5 ml of ferricyanide (600 nmol) and hemin dimethyl ester (17 nmol) containing egg phosphatidylcholine vesicles (20 mg) with 300 nmol reduced indigotetrasulfonic acid in 6 ml of $2 \cdot 10^{-4}$ M potassium phosphate buffer at an initial pH near 7.00. The results of two separate experiments are recorded. (Bottom) Absorbance change due to hemin dimethyl ester-catalyzed electron transfer under the same conditions for which the pH measurement of the middle pH trace was conducted. Although we could not follow both parameters in the same experiment, all conditions were the same and the samples used in the pH measurements became visibly densely blue at the same rate as that herein recorded.

TABLE I

MEASURE OF pH CHANGE DURING ELECTRON TRANSPORT

An average of three experiments were conducted for each system. The extremity of the values obtained are indicated by the ±value. All systems stabilized within 30 s. The buffer outside the vesicles contained 0.4 M KCl and $2 \cdot 10^{-4}$ M K_2HPO_4 at an initial pH of 7.00. ITSA, indigotetrasulfonic acid; $ITSAH_2$, reduced indigotetrasulfonic acid.

System	ΔpH
Basic redox reaction	
$ITSAH_2$ + vesicles + ferricyanide (external to vesicles)	−0.16 ± 0.01
Controls	
ITSA + buffer	0.01 ± 0.01
ITSA + ferricyanide	0.01 ± 0.01
ITSA + vesicles (loaded with ferricyanide but no hemin dimethyl ester)	0.01 ± 0.02
ITSA + hemin dimethyl ester vesicles	0.01 ± 0.01
$ITSAH_2$ + vesicles (loaded with ferricyanide but no hemin dimethyl ester)	0.01 ± 0.01
Complete	
$ITSAH_2$ + hemin dimethyl ester vesicles (loaded with ferricyanide)	0.01 ± 0.01

rates. Other controls conducted relevant to these measurements are shown in Table I. Thus, OH^- coordinated to ferric hemin dimethyl ester would seem to be the anion transported. It should be noted that the internal liposome space has a high buffer concentration (0.2 M phosphate), so that even though many OH^- equivalents may be transported out during electron transfer, the change in internal pH is not extensive.

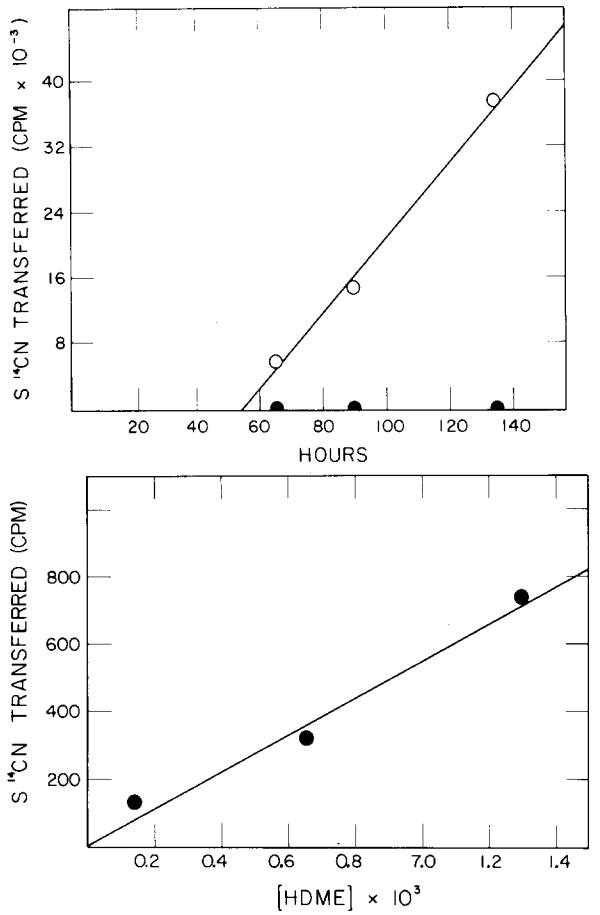


Fig. 6. (Top) SCN^- transfer across a chloroform phase catalyzed by hemin dimethyl ester. $2 \cdot 10^6$ cpm of $KS^{14}CN$ (60 mCi/mmol) were added to one of the aqueous compartments of a U-tube containing 0.6 ml of aqueous 1 mM KSCN, 1 mM phosphate, pH 7, on each side of 0.6 ml of chloroform. The solutions were agitated many hours with a stir bar and 25 μl aliquots were removed periodically and counted. When the chloroform layer contained $1 \cdot 10^{-3}$ M hemin dimethyl ester, 55 h were required for the first $S^{14}CN^-$ to migrate through the chloroform layer to the nonradioactive aqueous phase. (○) Hemin dimethyl ester, (●) control. (Bottom). Dependence upon hemin dimethyl ester (HDME) concentration of SCN^- transfer across a chloroform layer. $6 \cdot 10^5$ cpm of $KS^{14}CN$ were added to 1 ml of an aquous solution of $1.7 \cdot 10^{-4}$ M KSCN and 1 mM phosphate, pH 7. This was shaken vigorously with 1 ml of chloroform. After the layers settled, the U-tube was filled as described previously, except that the chloroform used was that saturated with KSCN as prepared above. The system was stirred for 39 h and then 100-μl aliquots were counted. The amounts of $S^{14}CN^-$ cpm transferred across the chloroform layer at three different hemin dimethyl ester concentrations are shown. Without hemin dimethyl ester in the chloroform layer the $S^{14}CN^-$ cpm transferred were insignificant.

As an independent measure of the ability of ferric hemin dimethyl ester to carry anions across an apolar phase, we tested for the exchange of $S^{14}CN^-$ between two aqueous phases in a U-tube separated by a chloroform layer containing hemin dimethyl ester. SCN^- was chosen because it is easily assayed when radioactively labelled, it binds to ferric hemin dimethyl ester much like OH^-, and it did not cross the chloroform layer on its own. Fig. 6 (top) shows that hemin dimethyl ester does catalyze the exchange of $S^{14}CN^-$ between the two aqueous phases and Fig. 6 (bottom) shows the dependency of the SCN^- exchange on hemin dimethyl ester concentration. Thus, the ability of ferric hemin dimethyl ester to catalyze appropriate anion transport across hydrophobic phases is clear.

Dependence of the rate of electron transfer on porphyrin structure

Because one would assume that hemin dimethyl ester would much more readily diffuse across the lipid bilayer than unesterified heme which has two free carboxyl groups, we tested the latter compound for catalysis. The concentration of hemin in the phosphatidylcholine vesicles was varied from 1.1 to 50 nmol hemin per 10 mg phospholipid. The results showed that in imidazole and phosphate buffer systems even at high hemin concentrations, either no electron transfer occurred, or a very low rate of electron transfer was catalyzed which was nearly indistinguishable from the background rate. To evaluate whether protonation of the carboxyl groups would have any effect, hemin was also tested in the phosphate buffer system at pH 5; no catalysis was observed.

Several other porphyrin complexes were tested which we felt should not be active. The iron-free porphyrin, i.e., protoporphyrin IX dimethyl ester, at a fairly high concentration (11 nmol per 10 mg of phospholipid) gave an electron-transfer rate nearly indistinguishable from the background rate. Also, nickel tetraphenylporphyrin, another porphyrin not likely to undergo a redox change, was incorporated into the egg phosphatidylcholine vesicles (about 50% incorporation) to give final concentrations of 2.86 and 5.7 nmol per 10 mg of phospholipid. This metalloporphyrin showed no catalysis of electron transfer.

Finally, we tested another iron-porphyrin complex we felt should be catalytically active. Vesicles con-

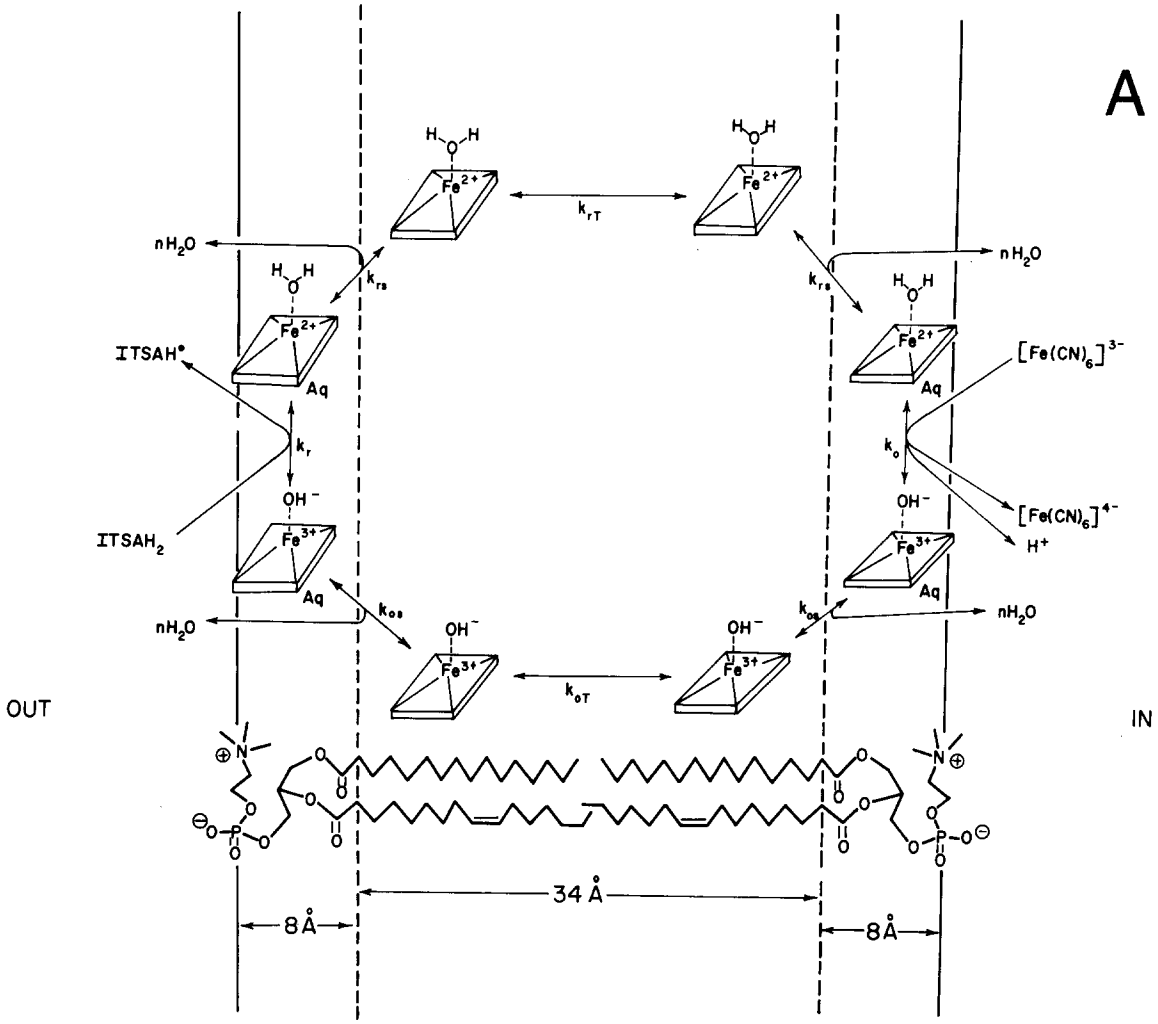
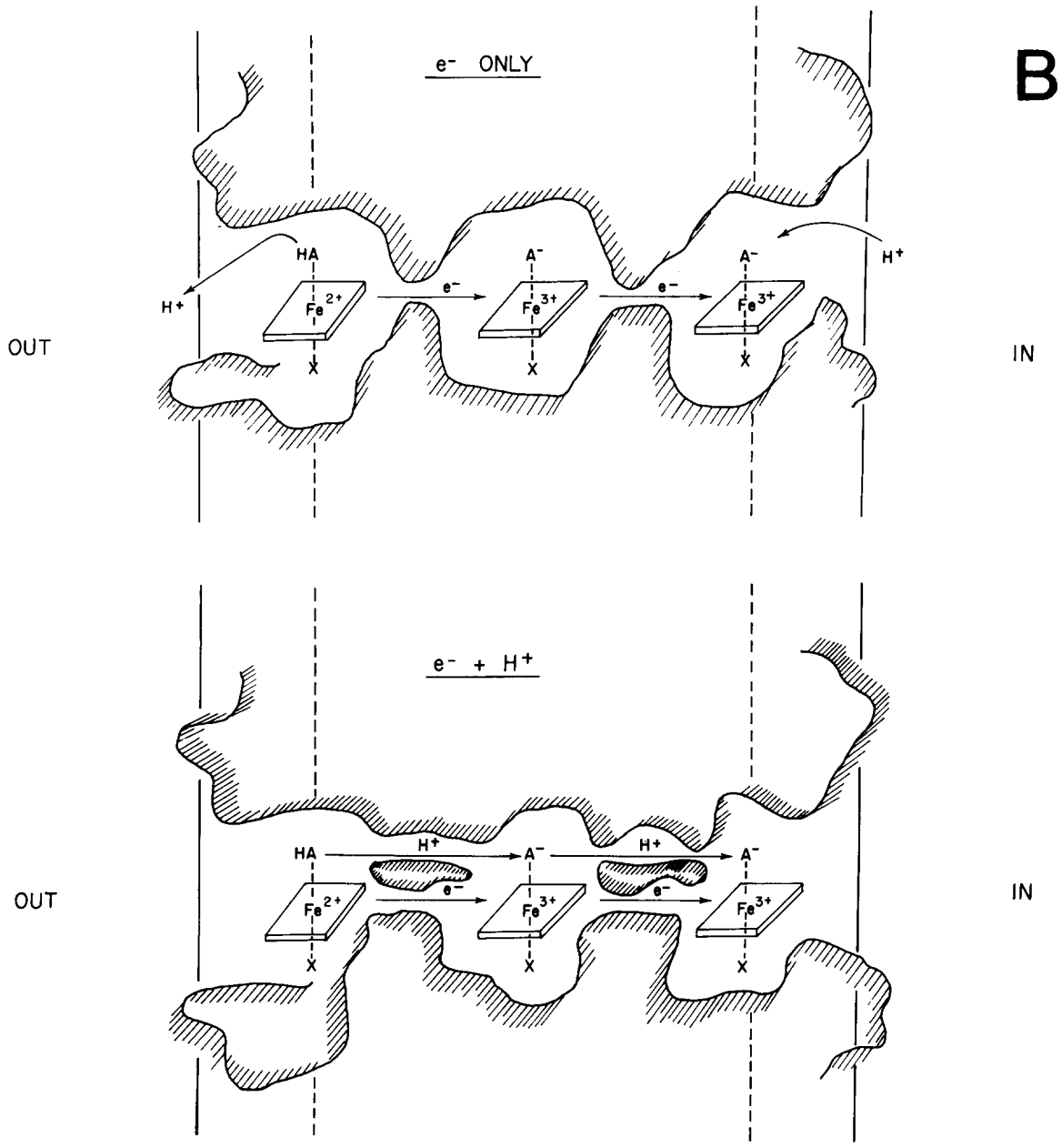


Fig. 7. (A) Schematic representation of hemin dimethyl ester-catalyzed electron transport across egg phosphatidylcholine vesicles in the phosphate buffer system. The rhombi are drawn to represent the expected size of the conjugated π-system of the porphyrin ring relative to the phospholipid bilayer. Aq is used to represent the fully hydrated iron-porphyrin complex. Although the liposomes are known to have substantial packing differences between the outer and inner halves of the bilayer, these are ignored here. (B) Schematic representation of hypothetical in vivo iron porphyrin electron-transport systems. The shaded areas represent part of the protein(s) that binds the iron porphyrin. The porphyrin complex is drawn as an octahedral low-spin structure (six coordinated ligands). The symbol A could be an imidazole side chain of histidine.

taining iron tetraphenylporphyrin (75% incorporation) were prepared, giving final concentrations of 2.85 and 5.7 nmol per 10 mg of phospholipid. A faster rate of dye oxidation was consistently observed with this system which was about twice that of the hemin dimethyl ester rate at the same concentration.

Dependence of rate of electron transport on the phospholipid bilayer composition

Because natural membranes also have negatively charged lipids, we measured electron transport by hemin dimethyl ester in egg phosphatidylcholine vesicles containing 20% cardiolipin. In these hybrid



vesicles the rate of hemin dimethyl ester-catalyzed electron transport was between 2- and 3-fold faster than that seen in egg phosphatidylcholine vesicles. Interestingly, when egg phosphatidylcholine vesicles were prepared with phosphatidylethanolamine added (50%), the hemin dimethyl ester-catalyzed reaction in imidazole buffer was slowed 5-fold.

Discussion

The results show that hemin dimethyl ester and iron tetraphenylporphyrin catalyze high rates of electron transfer across egg phosphatidylcholine bilayers. From the experimental data presented, this is a well behaved model system in which the rate of electron

transfer exhibits a first-order dependence on the iron porphyrin concentration. Thus, there is no indication that any aggregation effects play a role. Indeed, the same high rates of electron transfer per hemin dimethyl ester are observed at such low concentrations of catalyst that there are fewer than one such molecule per vesicle. Furthermore, generalized defects in the bilayer structure cannot be involved because porphyrins which did not have the ability to undergo a redox change in the potential range between the midpoint potentials of the aqueous redox materials (approx. −0.1 to +0.4 V vs. standard hydrogen electrode), e.g., protoporphyrin IX dimethyl ester and nickel tetraphenylporphyrin, did not show catalysis.

Of special interest is the result that hemin, which has two free carboxyl groups, is not catalytic even at high concentration. Thus, the hydrophilic carboxyl groups are apparently sufficient to anchor the porphyrin at the aqueous interface even when the carboxyl groups are substantially protonated (pH 5 results).

Many aspects of the experimental data clearly point to the fact that hemin dimethyl ester functions in catalysis by an electroneutral diffusion mechanism: (a) The catalyzed reaction rapidly goes to completion without breaking the vesicles. If the electron transfer were electrogenic, less than 5% would occur before an equilibrium with $\Delta\psi$ would be reached. (b) Potassium plus valinomycin does not affect the rate or extent of the reaction. (c) FCCP does not affect the initial rate or extent of the reaction. (d) Although phosphate and CN^- are not transported, the lack of pH change external to the vesicles during electron transport is consistent with the expectation that ferric hemin dimethyl ester not only coordinatively binds one OH^- [30], but can also readily diffuse across the bilayer in this state. (e) Ferric hemin dimethyl ester will transport SCN^- (similar to OH^- in coordination properties) through an apolar phase (chloroform) in a bulk transport system. (f) Hemin, which has two free carboxyl groups, is not catalytic, indicating that the distance between these molecules at the two opposing bilayer interfaces is too large for an electron to move without being carried.

The system studied here is very different than the ferrocene system studied by Hinkle [18,19]. The reason is clear. The iron in the ferrocene that Hinkle studied is coordinatively saturated with the two cyclopentadienyl anions and very shielded from reacting with any other ligands (e.g., OH^-) even when oxidized. On the other hand, ligand interaction is always required in heme groups which are otherwise coordinatively unsaturated. This mechanistic difference is again reflected in the relative rates of electron transfer. Our rates are 10-fold faster than those in the ferrocene system. Thus, the results of our studies on heme models are exceedingly relevant to questions involving heme proteins in in vivo electron-transport systems of which there are many.

A summary scheme which is consistent with the results reported here is shown in Fig. 7A. Note that the scheme of Fig. 7A can be viewed as a proton- and electron-carrying cycle simply by coordinating water to the ferrous form of the iron porphyrin. This is, in fact, likely to occur, since the high-spin Fe^{2+} is pentacoordinated in a tetragonal bipyramidal structure [32,33]. Thus, a fifth ligand must be present and water is the best candidate of those potential ligands available. The change in acidity that would occur during catalyzed electron transfer would not limit electron transfer until the redox reaction were nearly complete because of the high buffering capacity within the vesicles and because the $E^{0\prime}$ values of indigotetrasulfonic acid and $K_3Fe(CN)_6$ differ by 0.5 V, which is a large driving force.

It should also be noted that whereas ferric hemin dimethyl ester would be expected to catalyze OH^- exchange (or ferrous hemin dimethyl ester would catalyze proton exchange across the bilayer), net OH^- movement out of (or net proton movement into) the liposome cannot occur without net electron transfer into the vesicle. In this sense, the system behaves like the quinone-catalyzed electron- and proton-transporting systems studied by Hinkle [18,19] and Hauska and co-workers [22–24]. Valinomycin plus potassium and FCCP were also without significant effect in those systems [18,23].

In the case of hemin dimethyl ester catalysis in imidazole buffer, it is possible that the anion transported is imidazolate, which has a pK'_a for protonation of about 11 when bound to ferric porphyrins in water [34–36]. Because such a species would be protonated readily when the iron is reduced on the outside of the vesicle and then return to the inside while still coordinated in its neutral form, the com-

plex could also be viewed effectively as a proton and electron carrier. The imidazole system is of interest as a model for several reasons, one of which is due to the fact that the imidazolate anion is thought to play a role in several metalloprotein systems [37–41].

A major reason for interest in the model system reported here is because of its relevance to in vivo electron transport. For example, multiple cytochrome *b* components are often thought to span the inner membrane of mitochondria or other energy-transducing membranes and provide the 'electron wire' part (electrons only) of a Mitchell loop [42,43]. Cytochrome oxidase is another such system where metal centers (copper included in this case) are viewed as playing such a discriminating role of allowing only electrons (no protons) to be transported. If one imagines that these multi-metal-centered systems are strung out as shown in Fig. 7B, then the only difference between this in vivo model and that of Fig. 7A is the role of diffusion in the latter. In place of a free diffusion in the in vivo system is an imagined hopping of the electron from center to center. This hopping is presumed to occur at distances of 5–7 Å between the porphyrin edges. But what prevents the very small proton from migrating as well? To be sure, all the ligands coordinated to heme and copper in the electron transport pathways of bioenergetic membranes are not known with certainty, but most are acid-base groups and the imidazole group of histidine is almost always one of the ligands involved. Moreover, a ligand like imidazole will have other groups hydrogen-bonded to it, thus facilitating proton movement [44]. In order for such a hydrogen-bond network to function in vivo, a gate, perhaps involving conformational change in the protein, would be required to insure coupling between electron and proton flow through heme centers and to prevent rapid proton leakage [45]. But perhaps small hydrophobic barriers exist in the in vivo systems permitting electrons to hop (outer sphere electron transport) but not protons. The model system studied here makes it clear that if transmembrane potentials develop in vivo due to electron transport through membrane-bound heme proteins, then some such unique environment to prevent proton movement must exist for otherwise electrons and protons would be expected to move in parallel.

Thus, in conclusion this model system should be particularly useful for studying coupled electron and anion or cation movement, electron-transfer mechanisms at aqueous interfaces of lipid bilayers, effect of bilayer composition on electron and anion transport, the dynamics of diffusion of metalloporphyrins across a bilayer, and reactions between multiheme centers within a bilayer.

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