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Effect of Ionophores on Carrier-Mediated Electron Translocation in Ferricyanide-Containing Liposomes

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Ferricyanide-containing liposomes were used as a system to compare the electron- and proton-translocating properties of six redox reagents commonly used as electron donors for biochemical systems. The effects of different ionophore combinations on the ferricyanide-reduction rate were generally consistent with the expected proton- and electron-translocating properties of the mediators. The transmembrane pH gradient produced by hydrogen carriers was demonstrated. Nigericin or valinomycin plus carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone are capable of collapsing this gradient and of stimulating ferricyanide reduction mediated by this type of carrier. No pH gradient is produced with the electron carrier 1,1′-dibutylferrocene. In the presence of tetraphenylboron anion, which is needed for this carrier to act as an efficient mediator, addition of valinomycin alone is sufficient to obtain full stimulation of ferricyanide reduction. *NNN′N′*-Tetramethyl-*p*-phenylenediamine does not behave as a simple electron carrier. During *NNN′N′*-tetramethyl-*p*-phenylenediamine-mediated ferricyanide reduction protons are translocated across the membrane and accumulated in the vesicles. This is not due to the presence of demethylated impurities in the *NNN′N′*-tetramethyl-*p*-phenylenediamine sample, but may be the result of an accumulation of oxidation products other than the Wurster's Blue radical. These results suggest a reconsideration of studies on protonmotive forces across membranes where *NNN′N′*-tetramethyl-*p*-phenylenediamine is used as a mediator.

Interest in vectorial aspects of biological electron-transport reactions has been greatly stimulated by the chemiosmotic hypothesis (Mitchell, 1966). The study of such reactions in intact systems with natural substrates is, however, complicated by the number of electron-transfer steps involved, and artificial electron donors are often used to simplify the analysis. An understanding of possible proton- and electron-translocator properties of these mediators is therefore essential for the interpretation of experimental results.

Ferricyanide-containing liposomes provide a model for transmembrane electron transport (Hinkle, 1970) and have previously been used to study mediator properties (Hinkle, 1970, 1973; Deamer *et al.*, 1972; Hauska & Prince, 1974). In the present work we have used this system to carry out a systematic comparison of electron and proton translocation mediated by six redox reagents commonly used as electron donors for biochemical systems. We have specified those combinations of ionophores that give maximal stimulation of ferricyanide reduction, and we have followed the proton translocation associated with the electron flow in each case.

Abbreviations used: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid; cardiolipin, 1,3-bisphosphatidylglycerol.

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Experimental

Materials

Purified phospholipids, including grade-I egg-yolk phosphatidylcholine and grade-I bovine heart cardiolipin, were obtained from Lipid Products, Nutfield Nurseries, Redhill, Surrey, U.K.

The mediators phenazine methosulphate and 2,6-dichlorophenol-indophenol, together with tetraphenylboron (sodium salt), valinomycin and 9-aminoacridine, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; 2,3,5,6-tetramethyl-*p*-phenylenediamine was from Aldrich-Europe, Beerse, Belgium, benzoquinone was from Merck, Darmstadt, Germany, and 1,1′-dibutylferrocene was from K & K Laboratories, Cleveland, OH, U.S.A. Carbonyl cyanide *p*-trifluoromethoxyphenylhydra-

zone was obtained from Boehringer, Mannheim, Germany. Nigericin was generously provided by Dr. J. W. Chamberlain of the Lilly Research Co., Indianapolis, IN, U.S.A. *NNN'N'*-Tetramethyl-*p*-phenylenediamine was purchased from BDH Chemicals, Poole, Dorset, U.K. and was recrystallized from ethanol as described by Sanadi & Jacobs (1967). $Na_3Fe(CN)_6$ was from ICN Pharmaceuticals, Plainview, NY, U.S.A. Solutions of the mediators were prepared just before use.

Methods

Preparation of liposomes. Phosphatidylcholine (50mg) and cardiolipin (12.5mg) in chloroform/methanol (3:1, v/v) were dried under N_2, and 2.5ml of 0.2M-$Na_3Fe(CN)_6$/10mM-potassium phosphate, pH7.0, was added. The solution was sonicated under N_2 for 15min at 60W in a Branson sonifier equipped with a microtip and cooling device (Hansen *et al.*, 1978). The sonicated suspension was placed on a Sephadex G-25 column (2cm×20cm) (Pharmacia, Uppsala, Sweden) equilibrated with the buffer used in the assay. The slightly turbid liposome suspension was collected from the column in 5ml of eluate.

Spectrophotometry and pH measurements. Quenching of 9-aminoacridine was measured with a Perkin–Elmer MPF-2A fluorescence spectrophotometer, with an excitation wavelength of 380nm and emission wavelength of 460nm. Spectrophotometry was carried out with a Cary 118C spectrophotometer. Ferricyanide reduction was followed at 420nm [ε 1000 litre·mol^{-1}·cm^{-1} (Kolthoff & Tomsicek, 1935)], and the concentration of Wurster's Blue was measured at 563nm [ε 15000 litre·mol^{-1}·cm^{-1}. (Michaelis *et al.*, 1939)]. The pH in the cuvette was measured with a Radiometer pH-meter 26 (electrode GK 2401C).

Results and Discussion

Ferricyanide-containing liposomes with externally added ascorbate were used as a model system to study mediator properties. In the absence of any mediator, trapped ferricyanide is reduced by ascorbate at a very low rate, which is not appreciably increased on addition of ionophores. But as previously reported (Hinkle, 1970, 1973; Deamer *et al.*, 1972; Hauska & Prince, 1974), an increased rate of reduction is obtained in the presence of lipophilic mediators. The addition of certain ionophores induces a dramatic increase in the mediated ferricyanide reduction.

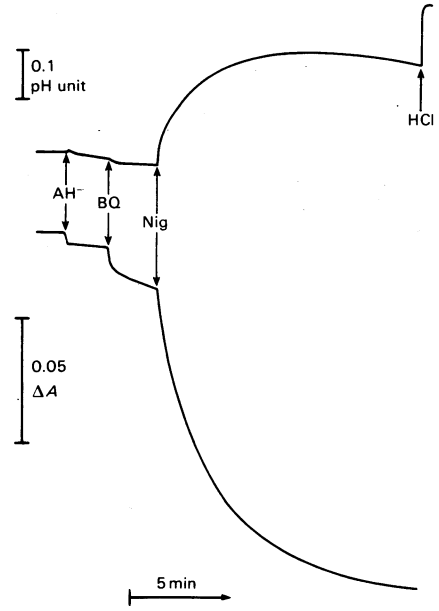
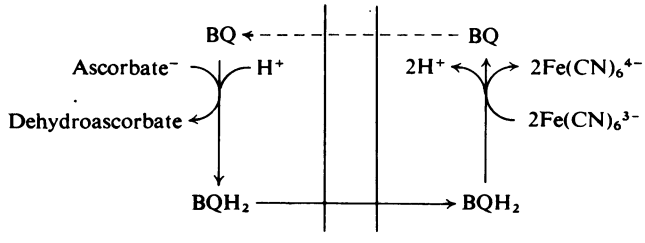


Fig. 1. *Effect of nigericin on benzoquinone-mediated reduction of liposomal ferricyanide and associated pH changes in the medium*

Ferricyanide reduction is measured at 420nm and external pH changes by a combined electrode. The cuvette contained 2.2ml of 67.5mM-Na_2SO_4/7.5mM-K_2SO_4/0.2mM-Tes/0.2mM-EDTA, pH7.0, and 300μl of liposomes. The concentration of liposomal ferricyanide was 170μM. At the arrows the reagents indicated were added. The final concentrations of ascorbate (AH^-), benzoquinone (BQ) and nigericin (Nig) were 4mM, 0.32mM and 0.24μg/ml respectively; 50nequiv. of HCl was added at the end of the experiment.



Scheme 1

Three types of mediators were used in the present study: (*a*) hydrogen carriers, (*b*) simple electron carriers and (*c*) a hydride carrier.

(*a*) *Hydrogen carriers*

Benzoquinone (BQ) was chosen as an example of a hydrogen carrier. It is expected to cross the membrane in an electroneutral cycle transferring two electrons and two protons into the liposome (Scheme 1).

The protons will accumulate inside and ultimately establish an equilibrium with the driving force of the electron-transfer reaction. Therefore any agents that can collapse this pH gradient and compensate for the charge of the protons should increase the rate of ferricyanide reduction.

Fig. 1 shows an experiment with benzoquinone as mediator. Simultaneous measurements of the rate of reduction followed at 420nm and the change in medium pH are shown. The benzoquinone-stimulated reduction is associated with only very small changes of medium pH. Addition of nigericin, which promotes an electroneutral exchange between H^+ and K^+, greatly stimulates reduction. On its addition a jump towards a more acidic pH followed by a slower acidification reaction is observed. The jump indicates a release of protons from the interior of the vesicles, and the slow phase can be accounted for by the proton released in the overall reaction:

$$\text{Ascorbate}^- + 2\text{Fe(CN)}_6^{3-} \rightarrow \text{Dehydroascorbate} + 2\text{Fe(CN)}_6^{4-} + \text{H}^+ \quad (1)$$

To obtain more direct evidence for proton accumulation we have measured the change in internal pH by means of 9-aminoacridine fluorescence quenching (Casadio & Melandri, 1977). Such fluorimetric measurements show that, as expected, benzoquinone-mediated ferricyanide reduction results in an accumulation of protons in the absence of ionophores (Fig. 2, trace B). Addition of nigericin releases the accumulated protons from the liposomes into the medium (Fig. 1).

The stimulation pattern of benzoquinone-mediated reduction obtained with different combinations of ionophores is summarized in Table 1. Addition of valinomycin, which increases the permeability to K^+, or the proton carrier carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone alone did not affect the benzoquinone-mediated ferricyanide reduction (results not shown), but a strong stimulation of the ferricyanide reduction was observed when both ionophores were present (Table 1).

The effects of the same ionophores on the reduction rate with two other commonly used hydrogen carriers 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) and 2,6-dichlorophenol-indophenol (DCPIP) are shown in Table 1. The stimulation patterns are identical with the benzoquinone-mediated reduction. Direct pH measurements and fluorescence quenching of 9-aminoacridine confirm that the proton movements with 2,3,5,6-tetramethyl-*p*-phenylenediamine or 2,6-dichlorophenol-indophenol are the same as with the benzoquinone-mediated ferricyanide reduction (results not shown). It should be noted that 2,6-dichlorophenol-indophenol does not act as a protonophore itself in our system, since both carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin are needed to stimulate reduction. 2,6-Dichlorophenol-indophenol is, however, a potent uncoupler of photophosphorylation in chloroplasts (Hill *et al.*, 1976).

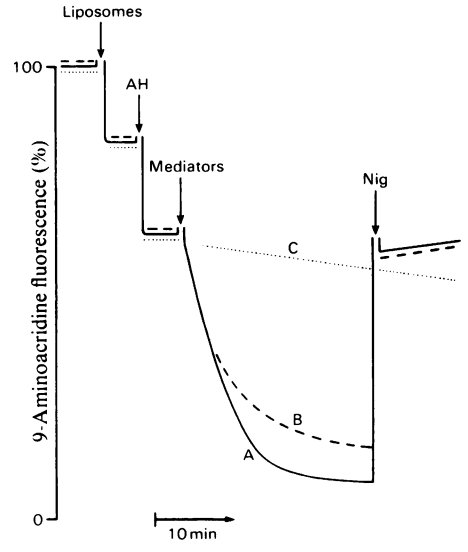


Fig. 2. *Quenching of 9-aminoacridine fluorescence in ferricyanide-containing liposomes*

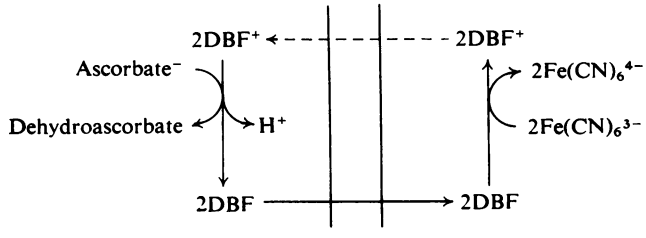
The cuvette contained 2.2ml of 8μM-9-aminoacridine/67.5mM-Na_2SO_4/7.5mM-K_2SO_4/0.2mM-Tes/0.2mM-EDTA, pH7.0. Liposomes (300μl) and 4mM-ascorbate (AH^-) were added as indicated. The reduction of liposomal ferricyanide (170μM) was initiated by adding 1μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine (trace A), 0.32mM-benzoquinone (trace B) or 4μM1,1'-dibutylferrocene (trace C). Nigericin (Nig) was added to a final concentration of 0.24μg/ml.

(*b*) *Electron carriers*

Dibutylferrocene (DBF), a lipophilic one-electron carrier, was introduced as a mediator in studies on ferricyanide-containing liposomes by Hinkle (1973). With externally added ascorbate this mediator in its reaction with ascorbate should produce one proton outside the liposomes each time two electrons have been transferred to ferricyanide. But no change in internal pH is expected to occur (Scheme 2). With a catalytic amount of the mediator, an efflux of the dibutylferrocinium cation must take place in order to

Table 1. *Effects of different ionophores on mediated electron transfer from ascorbate to ferricyanide enclosed in liposomes*
Ferricyanide reduction was measured at 420nm. A portion (0.3ml) of the liposome preparation (see under 'Methods') was added to a cuvette containing 2.2ml of 90mM-sodium phosphate/10mM-potassium phosphate buffer, pH7.0. The concentration of liposomal ferricyanide in the cuvette was 170μM, and the ascorbate concentration was 8mM. The final concentrations of the mediators benzoquinone, 2,3,5,6-tetramethyl-*p*-phenylenediamine, 2,6-dichlorophenol-indophenol, phenazine methosulphate, 1,1'-dibutylferrocene and *NNN'N'*-tetramethyl-*p*-phenylenediamine were 0.32mM, 0.4μM, 0.4μM, 10μM, 4μM and 1μM respectively. The ionophores valinomycin (Val), tetraphenylboron anion (TPB^-), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and nigericin (Nig) were added to a final concentration of 0.32μg/ml, 10μM, 1μM and 0.24μg/ml respectively. A very strong (more than 10-fold) stimulation of the ferricyanide reduction by the ionophore(s) is indicated by +++, a strong (5–10-fold) stimulation by ++, a weak (1–5-fold) stimulation by +, and a very weak stimulation by ±. The symbol − indicates that the presence of the ionophore(s) did not affect the rate of the electron-transfer reaction. Valinomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or tetraphenylboron anion added alone could not stimulate the reduction rate appreciably with any of the mediators used (not shown). The degree of stimulation depended strongly on the concentration of both the mediator and ascorbate. The conditions used were optimized to give a ionophore-stimulated rate of about 2μM-ferricyanide reduced/s.

Mediator type	Examples	Effect of: Nig	FCCP+Val	Val+TPB^-	FCCP+TPB^-	Nig+TPB^-
(*a*) Hydrogen carriers	Benzoquinone	+++	+++	−	−	+++
	2,6-Dichlorophenol-indophenol	+++	+++	−	−	+++
	2,3,5,6-Tetramethyl-*p*-phenylenediamine	+++	+++	−	−	+++
(*b*) Hydride carrier	Phenazine methosulphate	−	++	+++	+	±
(*c*) Electron carriers	1,1'-Dibutylferrocene	−	±	+++	±	±
	NNN'N'-Tetramethyl-*p*-phenylenediamine	++	++	+++	±	++



Scheme 2

close the mediator cycle; a negative potential inside the liposomes is thus created and this will slow down the ferricyanide reduction. Any agent that can collapse this membrane potential should stimulate the 1,1'-dibutylferrocene-mediated ferricyanide reduction.

Fig. 3 shows that the 1,1'-dibutylferrocene-mediated electron transfer is, in fact, stimulated by valinomycin. Addition of tetraphenylboron anion, which increases the permeability to the dibutylferricinium cation (Hinkle, 1973), greatly increases the valinomycin-stimulated reduction rate. This strong stimulation by tetraphenylboron anion seems to indicate that 1,1'-dibutylferricinium cation cannot by itself easily penetrate the membrane. The change in external pH is that expected solely from the ascorbate oxidation. Measurements of 9-aminoacridine fluorescence quenching confirms that no protons are accumulated during the 1,1'-dibutylferrocene-mediated ferricyanide reduction (Fig. 2, trace C). Table 1 shows that valinomycin plus tetraphenylboron anion was the only ionophore combination able to cause maximal stimulation of 1,1'-dibutylferrocene-mediated ferricyanide reduction. This is in contrast with the results of Hinkle (1973), who found a stimulation by carbonyl cyanide *m*-chlorophenylhydrazone in the presence of tetraphenylboron anion. The presence of a very high internal K^+ concentration in Hinkle's system may explain this difference.

(*c*) *Hydride carrier*

Phenazine methosulphate (PMS) was used as an example of a hydride carrier; on oxidation it loses two electrons and one proton. The expected redox

reactions taking place in ferricyanide-containing liposomes are shown in Scheme 3. If a catalytic amount of the mediator is used, the electron transfer results in generation of both a pH gradient and a membrane potential. Both these have to be collapsed in order to obtain full stimulation and it is expected that nigericin and valinomycin are both needed for that purpose. The observation that efficient stimulation with this combination is only obtained when the tetraphenylboron anion is also present [Table 1 and Hinkle (1973)] indicates that the efflux of accumulated phenazinium cation is stimulated by the presence of this lipophilic anion. The profound stimulation observed with valinomycin plus tetraphenylboron in the absence of nigericin (Table 1) is not easily accounted for by the model presented in Scheme 3, but the existence of a neutral lipophilic complex between protonated phenazinium cation and tetraphenylboron anion might explain this behaviour.

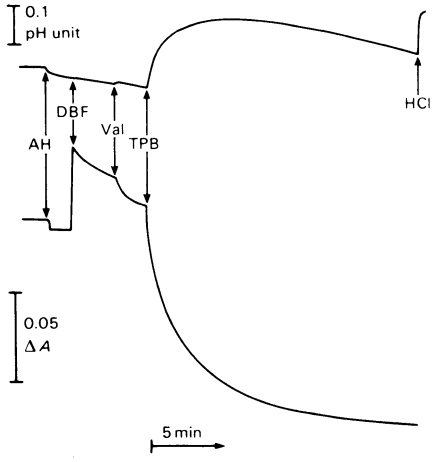


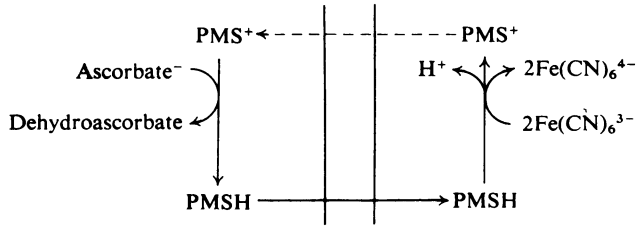
Fig. 3. *Effect of valinomycin and tetraphenylboron anion on dibutylferrocene-mediated reduction of liposomal ferricyanide and associated pH changes in the medium*
Experimental conditions were as in Fig. 1. The final concentrations of ascorbate (AH^-), dibutylferrocene (DBF), valinomycin (Val) and tetraphenylboron anion (TPB^-) were: 4mM, 4μM, 0.32μg/ml and 10μM respectively.

NNN'N'-Tetramethyl-p-phenylenediamine

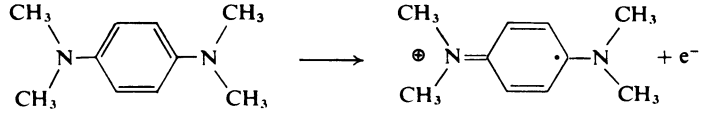
On oxidation of *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) the cation radical Wurster's Blue is formed (Scheme 4).

No protons are involved in this reaction and *NNN'N'*-tetramethyl-*p*-phenylenediamine is commonly supposed to be a simple electron carrier. As expected the *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated reduction is similar to the reduction with 1,1'-dibutylferrocene in the sense that it is strongly stimulated by valinomycin plus tetraphenylboron anion (Table 1). However, it differs from 1,1'-dibutylferrocene in that the reduction rate is also stimulated by nigericin or the valinomycin–carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone combination (Table 1).

Fig. 4 shows that the change in medium pH with *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated ferricyanide reduction is similar to that with benzoquinone-mediated ferricyanide reduction, indicating that protons are released on addition of nigericin. From Fig. 2 it is apparent that ferricyanide reduction mediated by *NNN'N'*-tetramethyl-*p*-phenylenediamine, but not that mediated by 1,1'-dibutylferrocene, is associated with proton accumulation. Thus



Scheme 3



Scheme 4

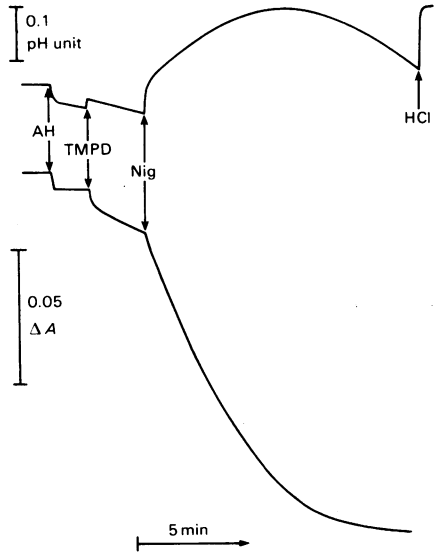


Fig. 4. *Effect of nigericin on the NNN'N'-tetramethyl-p-phenylenediamine-mediated reduction of liposomal ferricyanide*

Experimental conditions were as in Fig. 1. *NNN'N'*-Tetramethyl-*p*-phenylenediamine (TMPD) was added to a final concentration of 1 μM.

it is concluded that when *NNN'N'*-tetramethyl-*p*-phenylenediamine is used as a mediator it may act either as an electron carrier (as shown by the stimulation by valinomycin plus tetraphenylboron anion; Table 1) or by more complex pathways as a hydrogen/hydride carrier.

Using ferricyanide-containing liposomes and 9-aminoacridine, Hauska & Prince (1974) did not obtain fluorescence quenching with *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated ferricyanide reduction. The reason for this difference is not clear. However, their experimental conditions differ somewhat from ours and it is not stated whether the ferricyanide reduction in their system could be stimulated by ionophores.

The proton-translocator properties of *NNN'N'*-tetramethyl-*p*-phenylenediamine could occur as a result of the presence of demethylated impurities in the *NNN'N'*-tetramethyl-*p*-phenylenediamine solution (see, e.g., Wikström, 1978). The purity of the recrystallized sample used in the present study was tested by n.m.r. spectroscopy. The presence of demethylated impurities would result in more than one methyl line in the ^{13}C spectrum and would also result in splitting of the methyl lines of the H spectrum. No such splitting was detectable. The noise level of the spectrum permitted us to conclude that the sample contained less than 1% demethylated *NNN'N'*-tetramethyl-*p*-phenylenediamine. The proton-translocator properties of *NNN'N'*-tetramethyl-*p*-phenylenediamine is thus not likely to be the result of such impurities.

Dismutation and a further oxidative degradation of Wurster's Blue can perhaps give products with proton-translocator properties. Among free radicals this radical is a very stable one, but the rate at which it undergoes dismutation reactions is not always negligible (Clark, 1960). Therefore the stability of Wurster's Blue was investigated by measurements at 563 nm. If *NNN'N'*-tetramethyl-*p*-phenylenediamine is added to a 1 mM-ferricyanide solution the blue colour initially formed disappears in a few minutes. Moreover 1 mol of *NNN'N'*-tetramethyl-*p*-phenylenediamine can reduce more than 1 mol of liposomal ferricyanide (results not shown).

Fig. 5 shows that the *NNN'N'*-tetramethyl-*p*-phenylenediamine is converted into Wurster's Blue immediately after addition to a suspension of ferricyanide-containing vesicles, but the blue colour is subsequently bleached at a low rate. Fig. 5 also shows the effect of adding ascorbate after *NNN'N'*-tetramethyl-*p*-phenylenediamine has been converted into Wurster's Blue by intravesicular ferricyanide. The ascorbate addition causes a reduction of Wurster's Blue and simultaneously a reduction of ferricyanide takes place (results not shown). Fig. 5 shows that the rate of electron transfer depends strongly on the time *NNN'N'*-tetramethyl-*p*-phenylenediamine has been exposed to oxidation. Ap-

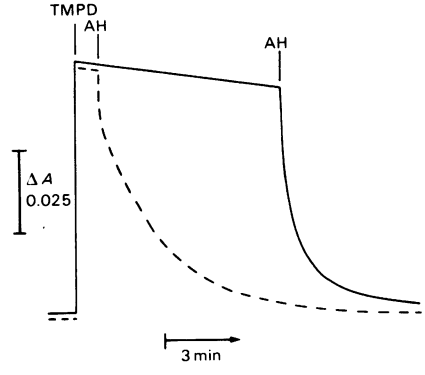


Fig. 5. *Stability of Wurster's Blue formed in ferricyanide-containing liposomes*

The formation of Wurster's Blue was measured at 563 nm. The cuvette contained 2.2 ml of 67.5 mM-Na_2SO_4/7.5 mM-K_2SO_4/0.2 mM-Tes/0.2 mM-EDTA, pH 7.0, and 300 μl of liposomes. The reagents indicated were added at the points marked. In each experiment the final concentrations of ascorbate (AH^-) and *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) were 5 mM and 5 μM respectively.

parently some degradation products, which stimulate the ferricyanide reduction, are accumulated.

Our suggestion that secondary oxidation of *NNN'N'*-tetramethyl-*p*-phenylenediamine occurs is supported by the observations of Nicholls & Hildebrandt (1978), who found that *NNN'N'*-tetramethyl-*p*-phenylenediamine is oxidized further than the Wurster's Blue stage by cytochrome aa_3 in the presence of cyanide.

The results presented indicate that lipophilic cations, such as the phenazinium cation, the ferricinium cation and the Wurster's Blue cation ($TMPD^+$), do not easily penetrate the membrane in the absence of a lipophilic anion such as tetraphenylboron. Permeation of protonated *NNN'N'*-tetramethyl-*p*-phenylenediamine ($TMPDH^+$) is therefore also likely to occur at a relatively low rate. However, the possibility that an influx of this cation might account for proton translocation observed with *NNN'N'*-tetramethyl-*p*-phenylenediamine cannot be excluded.

NNN'N'-Tetramethyl-*p*-phenylenediamine has been used as a mediator in many experiments on the protonmotive force generated across the membranes of mitochondria and chloroplasts (see, e.g., Hauska *et al.*, 1977; Wikström & Saari, 1977; Wikström, 1978; Sigel & Carafoli, 1978; Sorgato & Ferguson, 1978). Generally the conclusions reached are based on the concept of *NNN'N'*-tetramethyl-*p*-phenylenediamine as a pure electron carrier, but our results show that *NNN'N'*-tetramethyl-*p*-phenylenediamine can act as a proton translocator and suggest that this aspect of *NNN'N'*-tetramethyl-*p*-phenylenediamine-mediated electron transport should be considered in the interpretation of the results.

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