J. Membrane Biol. 84, 183–190 (1985)

The Journal of
Membrane Biology

Interactions Involving the Cyanine Dye, diS-C_3-(5), Cytochrome *c* and Liposomes and their Implications for Estimations of $\Delta\psi$ in Cytochrome *c* Oxidase-Reconstituted Proteoliposomes

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Summary. The interference of cytochrome *c* with absorption and fluorescence changes of the cyanine dye, diS-C_3-(5), was investigated in the presence of liposomes and cytochrome *c*-oxidase reconstituted proteoliposomes. The apparent cytochrome *c*-dependent quenching of diS-C_3-(5) fluorescence, and the associated absorbance losses in the presence of liposomes and proteoliposomes in low ionic strength media, are due to destruction of the dye caused by cytochrome *c*-mediated lipid peroxidation. The rate of this reaction was further enhanced in the presence of hydrogen peroxide. Even in the absence of liposomes or proteoliposomes, a cytochrome *c*-induced breakdown of dye was observed in the presence of hydrogen peroxide.

The cytochrome *c* mediated absorbance and fluorescence losses of diS-C_3-(5) in liposomal or proteoliposomal systems are prevented by Ca^{2+} and La^{3+} ions, by ascorbate, by high ionic strength and by the antioxidant BHT. Under these conditions, the process of lipid peroxidation mediated by cytochrome *c* is inhibited either directly (e.g. by BHT) or indirectly, by preventing the binding of cytochrome *c* to lipid vesicles. The impact of these findings upon the experimental estimation of membrane potential in aa_3-reconstituted proteoliposomes is discussed.

Key Words cyanine dye · liposome · cytochrome *c* · cytochrome *c* oxidase · proteoliposomes · membrane potentials · absorbance and fluorescence of dye

Introduction

Among cyanine dyes, 3',3'-dipropylthiadicarbocyanine iodide, diS-C_3-(5), has been extensively used in measurements of membrane potentials ($\Delta\psi$) in cells, organelles and vesicles including liposomes (*see* Bashford & Smith, 1979; and Waggoner, 1976, 1979*a,b*, for reviews). While the interactions of cyanine dyes with red blood cells and cell components have been extensively characterized (Hoffman & Laris, 1974; Sims et al., 1974; Hladky & Rink, 1976*a–c*; Guillet & Kimmich, 1978; Tsien & Hladky, 1978), very little information is available on their interactions with liposomes and proteoliposomes (Waggoner et al., 1977; Krasne, 1980). Binding of diS-C_3-(5) to hemoglobin and other proteins including cytochrome *c* has been studied by Tsien and Hladky (1978). These authors have shown that such binding of dye results either in a red-shift of the absorption spectrum for bound monomers or in a blue-shift of the spectrum and the suppression of fluorescence for bound dimers. No such changes were observed in the case of cytochrome *c*, which did not bind diS-C_3-(5), nor affect its fluorescent behavior. On the other hand, when cytochrome *c* is bound but physically separated by several layers of eicosanoate (Cd-arachidate) from a monolayer containing cyanine dye I (a member of the diO family), the fluorescence of the dye was quenched by the distant cytochrome *c* as a result of Perrin-Forster energy transfer (Fromherz, 1970). In this case the fluorescence spectrum of cyanine dye I overlaps with the Soret band of cytochrome *c*. In other studies, Vanderkooi et al. (1973) have also shown that the fluorescence loss of 12-(9-anthroyl) stearic acid in a dispersion of cardiolipin induced by cytochrome *c* was due to such a transfer of electronic excitation energy (Forster, 1959).

For estimation of $\Delta\psi$ in cytochrome *c* oxidase-reconstituted proteoliposomes using the cyanine dye diS-C_3-(5), cytochrome *c* is required as an electron donor in addition to ascorbate. In view of its interactions with cyanine dye absorbance and fluorescence changes as outlined above, it is therefore necessary to investigate the factors and mechanism(s) involved in such interactions before diS-C_3-(5) can be used as a suitable $\Delta\psi$ probe in proteoliposomes containing cytochrome *c* oxidase.

It will be shown that an apparent cytochrome *c*-dependent quenching of diS-C_3-(5) fluorescence and associated absorbance loss in the presence of liposomes and proteoliposomes are due to destruction

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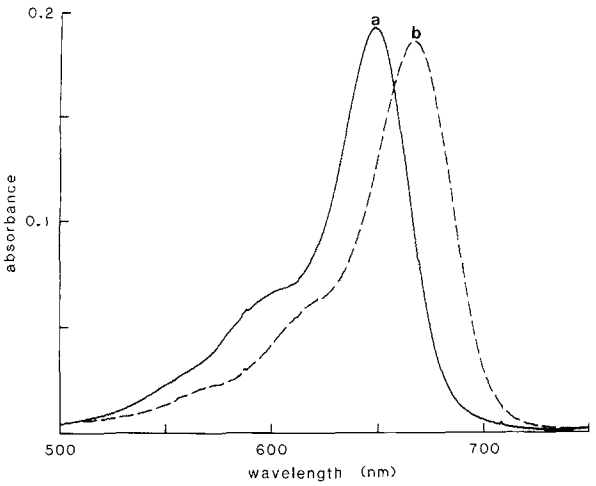

Fig. 1. Absorption spectra of diS-C_3-(5) in aqueous buffer and in the presence of liposomes. The reaction mixture included 1 μM diS-C_3-(5), 6.6 μM cytochrome *c* and liposomes containing 3.3 mg/ml lipid (*b* only). Medium was 50 mM Na-HEPES, pH 7.4, 28°C. (*a*) __, aqueous buffer. (*b*) _ _ _ _, buffer plus liposomes

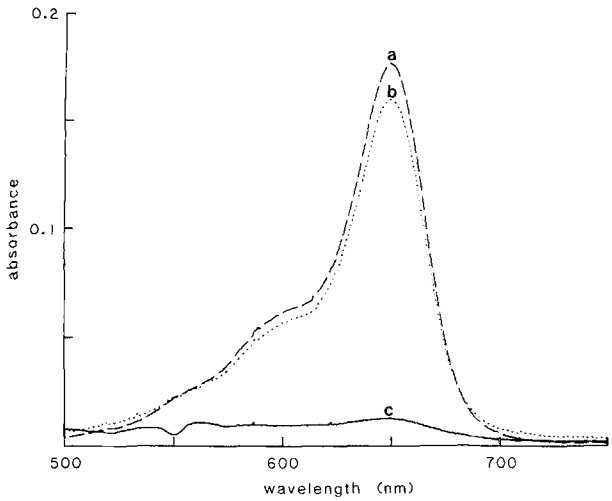

Fig. 2. Absorption spectra of diS-C_3-(5) in the presence of cytochrome *c*, H_2O_2, or both. All spectra were taken in the presence of 1 μM diS-C_3-(5) in 50 mM Na-HEPES (pH 7.4) at 28°C, after a 10-min preincubation period in the presence of the above compounds. *Symbols:* (*a*) _ _ _ _, plus 6.6 μM cyt. *c*; (*b*) ....., plus 8.8 μM H_2O_2; (*c*) __, plus 6.6 μM cyt. *c* and 8.8 μM H_2O_2

of the dye caused by cytochrome *c*-mediated lipid peroxidation. This process is also affected by the following factors: i) the redox state of cytochrome *c*, ii) the binding of cytochrome *c* to the membrane, iii) the presence of antioxidant, and iv) by Ca^{2+} and La^{3+} ions. The implications of our findings for measurements of $\Delta\psi$ in cytochrome *c* oxidase-reconstituted proteoliposomes are discussed.

Abbreviations: diS-C_3-(5), 3',3'-dipropylthiadicarbocyanine; BHT, butylated hydroxytoluene; diO-C_5-(3), 3',3'-dipentyloxacarbocyanine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; FCCP, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone; COV, cytochrome *c* oxidase vesicles

Materials and Methods

Cytochrome *c* (type VI, horse heart), sodium ascorbate, HEPES, valinomycin, asolectin (from soybean, type IV-S) and butylated hydroxytoluene (BHT), type III, were Sigma products. The cyanine dyes diO-C_5-(3) and diS-C_3-(5) were the generous gift of Dr. Alan Waggoner of Carnegie-Mellon University, Pittsburgh, Pa.

Liposome and Proteoliposome Formation

Essentially unilamellar-mixed soybean phospholipid liposomes and cytochrome *c* oxidase-reconstituted proteoliposomes were prepared in 50 mM HEPES-NaOH buffer (pH 7.4) either in the presence of 2% (wt/wt) butylated hydroxytoluene (BHT) or in its absence using the sonication method described by Proteau et al. (1983). In some experiments liposomes were also made in either 5 mM potassium phosphate buffer, pH 7.4 (low ionic strength medium) or in 50 mM of the same buffer (high ionic strength medium).

Spectroscopic Measurements

Absorbance measurements were made using a Beckman DU-7 HS UV/visible spectrophotometer integrated with an Apple-II plus microcomputer and a Bausch & Lomb DMP-29 X-Y plotter. The RS232 interface on the DU-7 permits data acquisition in real time and subsequent storage on floppy discs; data are later retrieved and transmitted to the X-Y plotter (Chanady et al., 1984). All spectra are corrected to a zero base line obtained in the presence of buffer, vesicles, and cytochrome *c*.

The fluorescence of diS-C_3-(5) was excited at 622 nm and the emission measured by a photomultiplier protected by an Oriel long-pass filter (No. 5133, 50%, 670 nm) and placed at 90° to the cuvette. The latter was a multipurpose cuvette assembly system as described by Kraayenhof et al. (1982) and fitted into a modified Gilford 2400 spectrophotometer. The output was plotted with a three-channel Cole-Parmer (Chicago, Illinois) recorder.

Results and Discussion

Absorption Spectra of diS-C_3-(5) in Aqueous Buffer and in the Presence of Liposomes

Consistent with the findings of Krasne (1980), a red-shift of 15 to 20 nm in the absorption spectrum of diS-C_3-(5) was observed in the presence of liposomes prepared from soybean phospholipids (Fig. 1, spectrum *b*). Except for this red-shifted (monomer) peak, no other differences were noticed in the absorption spectrum of the dye in aqueous buffer and in the presence of liposomes (*compare* spectrum *a* with spectrum *b* in Fig. 1). No such red-shift in the

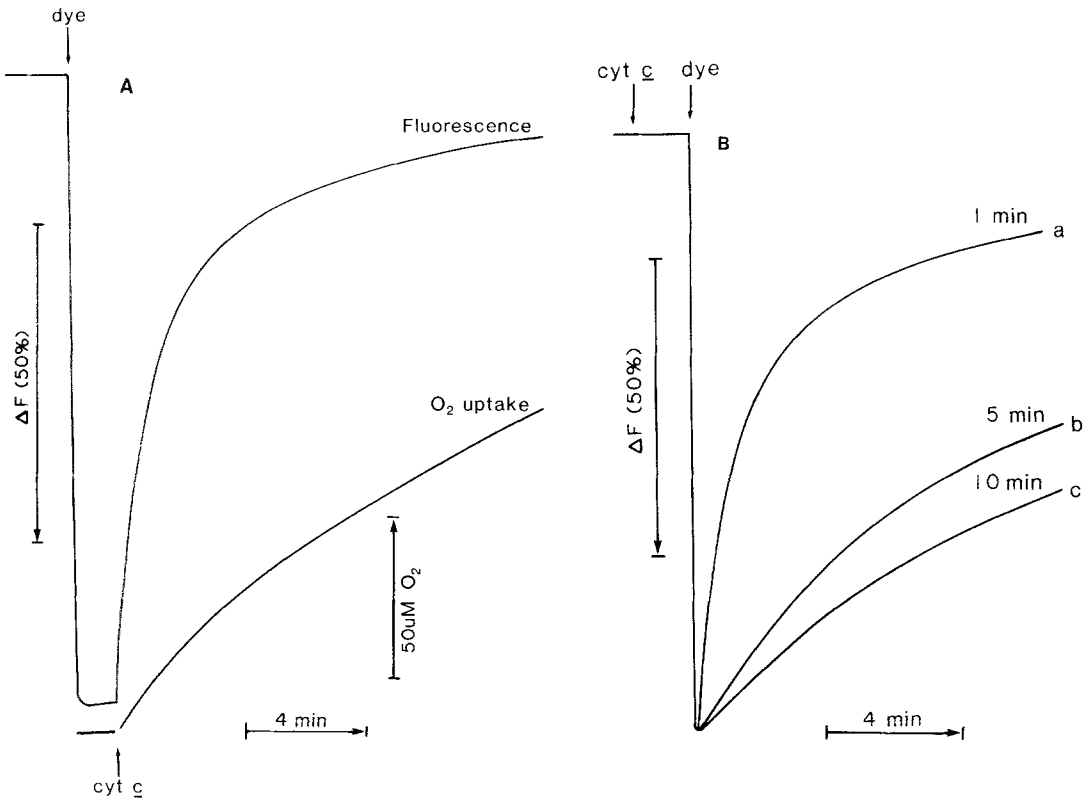

Fig. 3. *A*. Effect of cytochrome *c* on the kinetics of oxygen uptake and quenching of diS-C_3-(5) fluorescence in a liposomal system. In a total volume of 2.7 ml, the reaction mixture contained: 50 mM Na-HEPES, pH 7.4, 3.7 μM cytochrome *c*, 0.55 μM diS-C_3-(5) and liposomes containing 5 mg of phospholipid. *B*. Effect of preincubation times with cytochrome *c* on the rate of quenching of diS-C_3-(5) fluorescence mediated by cytochrome *c* in a low ionic strength buffer in the presence of liposomes. The components of the reaction mixture were the same as in Fig. 3*A*

spectrum of this dye was found in the presence of phosphatidylcholine: cholesterol (3 : 1) liposomes (Sims et al., 1974). The spectral shift of the dye in the presence of liposomes is dependent on the composition of liposomal phospholipids.

Effect of Cytochrome *c* and H_2O_2 on the Absorption Spectrum of the Dye in Aqueous Buffer

In the presence of a small amount of cytochrome *c*, no changes were noticed in the absorption spectrum of 1 μM diS-C_3-(5), in 50 mM Na-HEPES buffer (pH 7.4), after up to 10-min incubation (Fig. 2, spectrum *a*). A slight decrease in the absorbance of the dye occurred in the presence of H_2O_2 within the same time period (Fig. 2, spectrum *b*). A similar effect was seen by Whitin et al. (1980). However, in the presence of both cytochrome *c* and H_2O_2, the absorbance was completely abolished under the same conditions (Fig. 2, spectrum *c*). These results suggest that the dye is destroyed by H_2O_2 only when cytochrome *c* is also present. A product formed by interaction of H_2O_2 with cytochrome *c* was presumably responsible for this destruction (Mochan & Degn, 1969; Cadenas et al., 1980; Bernofsky & Wanda, 1983). No oxidation of dye occurred in buffer in the presence of oxidized cytochrome *c* alone.

Effect of Cytochrome *c* on the Absorbance and Fluorescence of diS-C_3-(5) in the Presence of Liposomes

Upon addition of diS-C_3-(5) to liposomes suspended in 50 mM HEPES-NaOH buffer (pH 7.4), its fluorescence was enhanced and stabilized within a few seconds (Fig. 3*A*). This fluorescence was quenched in a time-dependent manner upon addition of cytochrome *c*. In the absence of cytochrome *c*, no uptake of oxygen was observed; in its presence a marked O_2 uptake was noticed (Fig. 3*A*). This O_2 uptake is apparently due to lipid peroxidation (Klein, 1970) catalyzed by cytochrome *c* (*see* Cadenas et al., 1980, for review). As can be seen from Fig. 3*B*, the rate of fluorescence loss was also de-

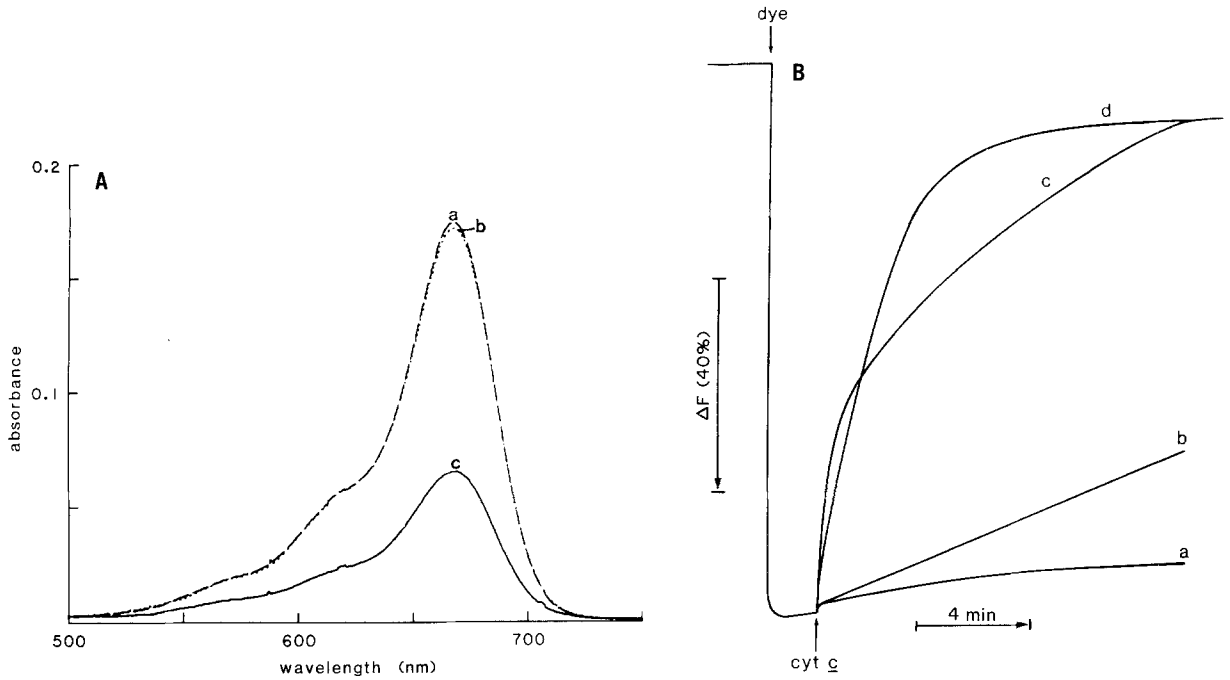

Fig. 4. *A*. Effect of ionic strength on cytochrome *c*-mediated absorbance decay of diS-C_3-(5) in a liposomal system. The reaction mixture contained *either* 5 mM K^+-phosphate, pH 7.4 (low ionic strength, spectrum *c*) *or* 50 mM K^+-phosphate (high ionic strength, spectra *a* and *b*), 1 μM diS-C_3-(5), 6.6 μM cytochrome *c* and liposomes containing 3.3 mg/ml phospholipid. After the addition of cytochrome *c*, the spectrum was taken either immediately (spectrum *a*) or after 10 min (spectra *b* and *c*). *B*. Effect of ionic strength upon cytochrome *c*-mediated quenching of diS-C_3-(5) fluorescence in a liposomal system. Liposomes were made in 50 mM potassium phosphate, pH 7.4 (high ionic strength buffer) and assayed either in the same buffer (trace *a*) or in 5 mM potassium phosphate (trace *d*). Liposomes prepared in 5 mM potassium phosphate, pH 7.4 (low ionic strength buffer) were assayed in the same medium (trace *c*) or in 50 mM K^+-phosphate (trace *b*). The components of other reaction mixtures were as given in the legend to Fig. 3*A*

pendent on the preincubation time with cytochrome *c*. When liposomes were preincubated with cytochrome *c* before the addition of dye, for increasing periods of time (Fig. 3*B*, traces, *a*, *b* and *c*) the rate of fluorescence loss was progressively diminished. This suggests that under the above conditions, the amount of available oxidant (lipid peroxide) had also decreased with time.

Factors Affecting the Cytochrome *c*-Dependent Absorbance and Fluorescence Changes of diS-C_3-(5)

(*a*) *Ionic Strength*

When liposomes prepared in 50 mM potassium phosphate buffer (pH 7.4) were assayed in the same medium, no change in the absorbance of dye was found after addition of cytochrome *c* when the spectrum was taken either immediately or after 10-min preincubation (Fig. 4*A*, spectra *a* and *b*). In a lower ionic strength medium (5 mM potassium phosphate, pH 7.4), the absorbance decreased considerably in the presence of cytochrome *c* under the above conditions (Fig. 4*A*, spectrum *c*).

The cytochrome *c*-dependent fluorescence loss was similarly affected by the ionic strength of the medium (Fig. 4*B*). In a high ionic strength medium, very little or no fluorescence loss was observed (Fig. 4*B*, trace *a*). In contrast, at low ionic strength both the rate and the extent of fluorescence loss were enhanced by cytochrome *c* (Fig. 4*B*, *cf.* traces *c* and *d*). The quenching of 12-(9-anthroyl) stearic acid fluorescence by cytochrome *c* was also affected by the ionic strength of the media (Vanderkooi et al., 1973). The initial rate of fluorescence loss was faster with liposomes prepared in 5 mM buffer than with those made in 50 mM K-phosphate. It is evident from traces *b* and *c* that the effect of cytochrome *c* on diS-C_3-(5) fluorescence was influenced by the ionic strength of the medium present both inside and outside the liposomes. Similar results were obtained when the ionic strength of the medium was varied by substitution of K_2SO_4 for potassium phosphate. It is interesting to note that the fluorescence and absorbance of the other cyanine dye, diO-C_5-(3) was unaffected by cytochrome

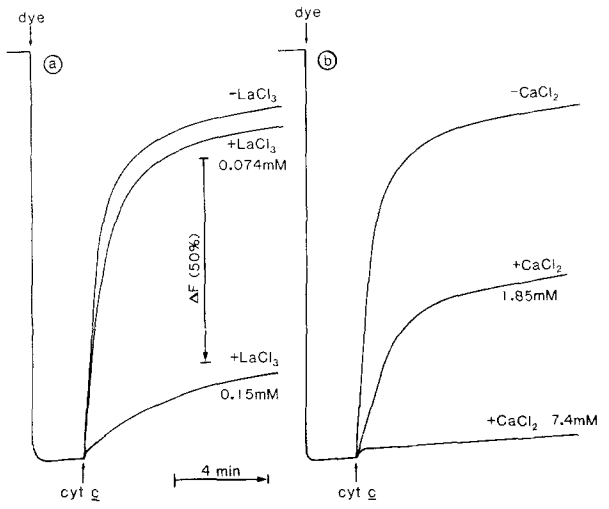

Fig. 5. Effects of La^{3+} and Ca^{2+} on cytochrome *c*-induced loss of diS-C_3-(5) fluorescence. The 2.7 ml reaction mixture contained: 50 mM Na^+-HEPES, pH 7.4, 3.7 μM cytochrome *c*, 0.55 μM diS-C_3-(5) and liposomes containing 5 mg phospholipid. The concentration of the other components of reaction mixture is indicated directly on the traces

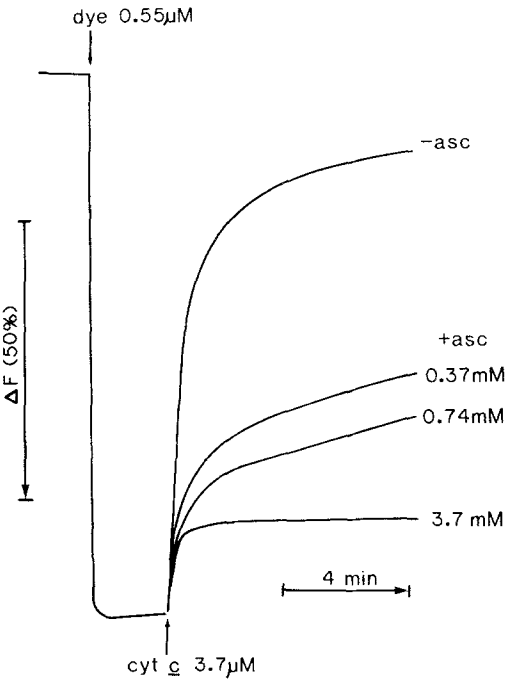

Fig. 6. Effect of varying the concentration of ascorbate upon cytochrome *c*-mediated quenching of cyanine dye fluorescence in low ionic strength buffer. Liposomes were made in 50 mM Na^+-HEPES, pH 7.4 and assayed in the same buffer. The 2.7 ml assay mixture contained: 50 mM Na-HEPES, pH 7.4, 3.7 μM cytochrome *c*, 0.55 μM diS-C_3-(5) and liposomes containing 5 mg phospholipid

c in the presence of liposomes under identical conditions.

Cytochrome *c* has been reported (Nicholls & Malviya, 1973; Nicholls, 1974; Cannon & Erman, 1980) to bind very tightly to asolectin liposomes in low ionic strength media but only weakly in high ionic strength media. All above results indicate that the binding of cytochrome *c* to liposomes is implicated in lipid peroxidation reactions. Therefore the ionic strength of the medium indirectly affects the rate of lipid peroxidation which in turn affects the loss of diS-C_3-(5) absorbance and fluorescence.

(*b*) *Divalent Cations*

The binding of cytochrome *c* to phospholipid vesicles (PLV's) can also be prevented by use of the divalent cations Ca^{2+} and La^{3+} (Fig. 5, *A* and *B*). This figure shows that $LaCl_3$ is at least *ten* times as effective as $CaCl_2$ in preventing cytochrome *c* binding. Cytochrome *c*-dependent abolition of diS-C_3-(5) fluorescence in the presence of liposomes can thus be effectively prevented by adding either $CaCl_2$ or $LaCl_3$ to the assay medium.

(*c*) *Ascorbate*

When PLV's were preincubated with varying concentrations of ascorbate prior to the addition of diS-C_3-(5), this prevented the loss of fluorescence after addition of cytochrome *c* (Fig. 6). In contrast to oxidized cytochrome *c*, reduced cytochrome *c* may bind liposomes less strongly (Nicholls & Malviya, 1973), and may also promote lipid peroxidation less effectively (*cf.* Mochan & Degn, 1969). However, it is also possible that ascorbate may directly or indirectly inhibit lipid peroxidation (Porter & Bright, 1983; Scarpa et al., 1984).

(*d*) *Antioxidant*

The effect of the antioxidant, butylated hydroxytoluene (BHT), on these processes was also examined. The results are shown in Figs. 7 and 8. When PLV's were prepared in the presence of the antioxidant BHT (*see* Materials and Methods) in a low ionic strength (50 mM Na-HEPES, pH 7.4) buffer, the effect of cytochrome *c* upon fluorescence was much smaller than with vesicles made without BHT (Fig. 7*B*), as were the effects upon the absorbance of the dye (*results not shown*).

On the basis of these results, it was concluded that cytochrome *c*-dependent absorbance and fluorescence changes in diS-C_3-(5) behavior in the presence of liposomes are modulated by the following

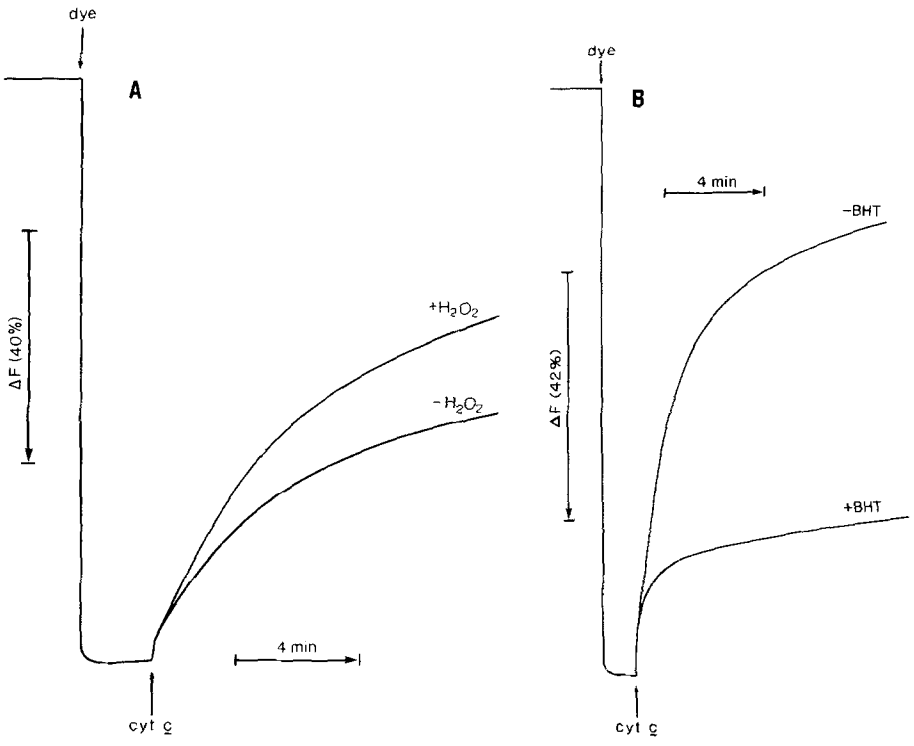

Fig. 7. *A*. Effect of hydrogen peroxide on cytochrome *c*-dependent quenching of diS-C_3-(5) fluorescence in the presence of liposomes. The concentration of H_2O_2 in the assay mixture was 44 μM, and the concentrations of other components of the reaction mixture are the same as in Fig. 6. *B*. Effect of the antioxidant, BHT, on the rate of cytochrome *c*-mediated quenching of diS-C_3-(5) fluorescence in a liposomal system. The concentration of BHT was 2% (wt/wt) and the other components of the reaction mixture were as given in Fig. 6

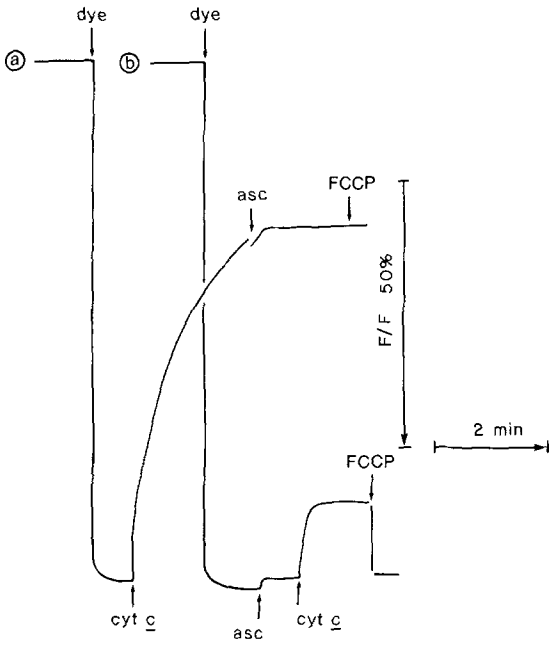

Fig. 8. Effect of the order of additions of cytochrome *c* and ascorbate on the reversible quenching of diS-C_3-(5) fluorescence ($\Delta\psi$ formation) in a low ionic strength medium by aa_3-reconstituted proteoliposomes. The reaction mixture in a total volume of 2.7 ml contained: 50 mM Na-HEPES, pH 7.4, 3.7 mM ascorbate, 3.7 μM cytochrome *c*, 0.6 μM diS-C_3-(5) and proteoliposomes containing 1 mg/ml soybean lipids and 0.85 nmol of cytochrome *c* oxidase. FCCP was used at a concentration of 0.74 μM

factors: (a) ionic strength of the medium, (b) Ca^{2+} and La^{3+} ions, (c) ascorbate and (d) lipid antioxidant, BHT.

Measurement of Membrane Potential in Cytochrome *c* Oxidase Vesicles by diS-C_3-(5)

DiS-C_3-(5) has been recently used in the estimation of membrane potentials ($\Delta\psi$) in proteoliposomes reconstituted with bacterial cytochrome oxidase (Kita et al., 1982; Matsushita et al., 1983; Koland et al., 1984). This prompted us to utilize this dye for the estimation of $\Delta\psi$ in mammalian cytochrome *c* oxidase-reconstituted vesicles, and some of the results are shown in Figs. 8 and 9. Oxidation of ascorbate in the presence of cytochrome *c* by COV's resulted in a quenching of diS-C_3-(5) fluorescence, which was reversed by FCCP (Fig. 8, trace *b*). The ionic strength of the medium and the order of addition of ascorbate and cytochrome *c* had marked effects upon this process. Even in proteoliposomes containing a very high level of cytochrome *c* oxidase, a cytochrome *c*-dependent 'quenching' of diS-C_3-(5) fluorescence was observed in the absence of ascorbate in low ionic strength media (Fig. 9, trace *b*). No such quenching of dye fluorescence was achieved by cytochrome *c* alone in high ionic strength media (Fig. 9, trace *a*). The small rapid initial quenching of

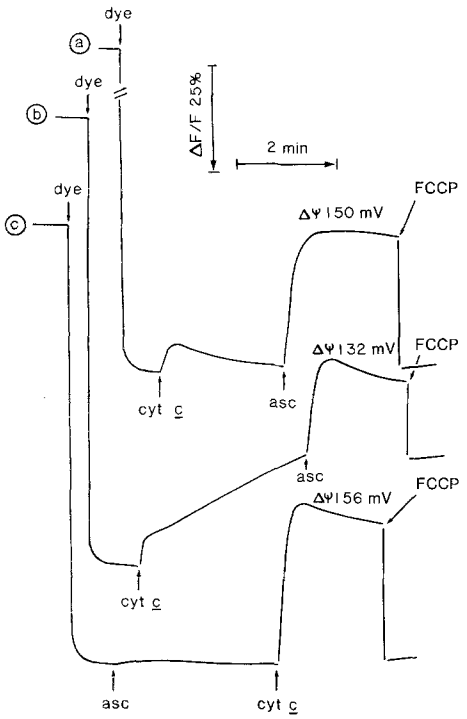

Fig. 9. Effect of ionic strength and the order of additions of ascorbate and cytochrome *c* on reversible fluorescence quenching of diS-C_3-(5) and associated $\Delta\psi$ formation by aa_3-reconsituted proteoliposomes. Proteoliposomes prepared in a low ionic strength buffer (50 mM Na-HEPES, pH 7.4) were assayed either in the same buffer (traces *b* and *c*) or in a high ionic strength buffer (50 mM Na-phosphate, pH 7.4, trace *a*). The concentrations of other components of the reaction mixture were as follows: 3.7 mM sodium ascorbate, 3.7 μM cytochrome *c*, 0.9 μM diS-C_3-(5), and proteoliposomes containing 1.85 mg/ml phospholipid and 85 nmol of cytochrome *c* oxidase. The $\Delta\psi$ values generated were estimated by comparison to standard curves (*not shown*) constructed from experiments in which fluorescence quenching was induced by imposition of K^- diffusion gradients of known magnitude in the presence of valinomycin

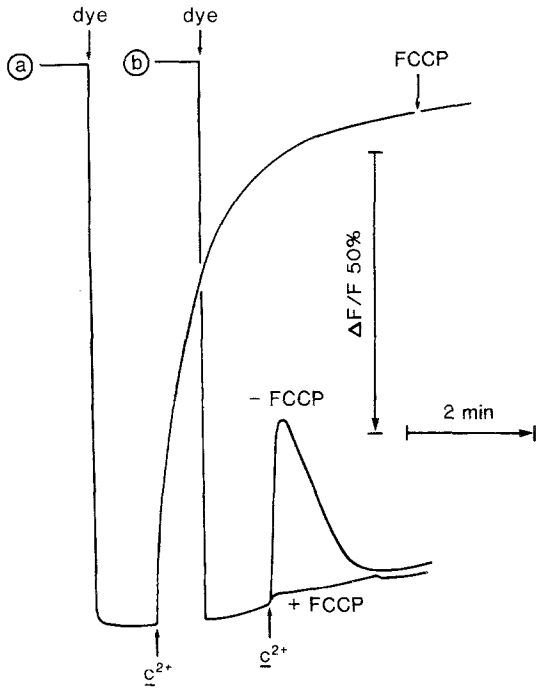

Fig. 10. Effect of ionic strength on ferro cytochrome *c*-dependent quenching of diS-C_3-(5) fluorecence and associated $\Delta\psi$ formation by aa_3-reconstituted proteoliposomes. Proteoliposomes made in 50 mM Na-HEPES, pH 7.4, were assayed either in the same buffer (trace *a*) or in 50 mM Na-HEPES plus 100 mM NaCl, pH 7.4 (trace *b*). The concentrations of the other components of the reaction mixture were: 10 μM reduced cytochrome *c*, 0.6 μM diS-C_3-(5) and proteoliposomes containing 1 mg/ml phospholipid and 1.6 nmol of cytochrome *c* oxidase. FCCP when present was used at a concentration of 0.74 μM

dye fluorescence caused by cytochrome *c* in both low and high ionic strength media may be due to a competitive binding of dye and cytochrome *c* to lipid vesicles.

In a low ionic strength medium, the addition of cytochrome *c* in the absence of ascorbate to proteoliposomes containing very low concentrations of cytochrome *c* oxidase, produced a marked loss of diS-C_3-(5) fluorescence. This loss was not reversed by FCCP (*compare* trace *a* of Fig. 8 with trace *a* of Fig. 9). No such effect was observed if ascorbate was added first, followed by cytochrome *c* (Fig. 8, trace *b* and Fig. 9, trace *c*). Addition of reduced cytochrome *c* to proteoliposomes containing very low levels of cytochrome *c* oxidase in a low ionic strength medium caused an 80 to 90% loss of cyanine dye fluorescence which was insensitive to FCCP (Fig. 10, *cf.* traces *a* and *b*). In a high ionic strength medium, an FCCP-sensitive quenching of dye fluorescence was observed upon addition of reduced cytochrome *c* (Fig. 10, trace *b*).

From these findings, it is evident that in order to measure membrane potentials ($\Delta\psi$) using diS-C_3-(5) in a proteoliposomal system with cytochrome *c* as electron donor, it is necessary to adhere to the following procedures:

(a) The estimation of $\Delta\psi$ must be carried out at high ionic strength and in well-buffered media.

(b) If it becomes necessary to make measurements at a low ionic strength (e.g. 50 mM Na-HEPES or 5 mM phosphate buffers), after the additions of vesicles and dye, ascorbate should be added first followed by cytochrome *c*.

(c) In a "pulsing" experiment which requires the use of reduced cytochrome *c* for measurement of

$\Delta\psi$, the reactions should be carried out in high ionic strength media and preferably in the presence of a lipid antioxidant.

(d) Alternatively, diO-C_5-(3) may be used rather than diS-C_3-(5) to estimate potentials in proteoliposome systems with cytochrome *c* as an electron donor.

This work was supported by Canadian NSERC Grant No. A-0412 to P. Nicholls. We are grateful to Prof. A.S. Waggoner of Carnegie-Mellon University, Pittsburgh, for a gift sample of cyanine dye.

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Received 1 August 1984; revised 6 November 1984