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Mechanism of uptake of the fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) by phospholipid vesicles

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The fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) is taken up by liposomes of egg phosphatidylcholine in response to the imposition of a transmembrane potential. Entry of DMP^+ into the bilayer driven by the transmembrane potential is accompanied by a change in the fluorescence emission maximum of the dye. This change reflects the movement of the dye molecules from the headgroup region of the bilayer into the region of the fatty acyl chains. It is released into the external aqueous phase on discharge of the transmembrane potential. Partition of the dye into the phospholipid bilayer is favoured by the presence of negatively charged lipids, such as dioleoylphosphatidic acid and dicetyl phosphate, in the bilayer. Stearylamine opposes entry of the dye into the bilayer. Tetraphenylboron (TPB^-) increases the partitioning of DMP^+ into the phospholipid bilayer even in the absence of a transmembrane potential. The fluorescence emission maximum of DMP^+ under these conditions is similar to that observed in the absence of TPB^- following imposition of the transmembrane potential. It is suggested that TPB^- facilitates the entry of DMP^+ into the fatty acyl chain regions of the phospholipid bilayer.

Introduction

The fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) was introduced by Bereiter-Hahn to measure the metabolic state of mitochondria in situ [1]. He concluded that the fluorescence changes observed as the metabolic state of mitochondria was altered were not primarily due to ion movements or pH changes. Subsequently, Rafael [2] and Mewes and Rafael [3] concluded that the dye responded to the transmembrane potential in mitochondria. Examination of the response of the dye in bacterial cells by Midgley and co-workers [4–7] revealed that DMP^+ taken into the cells was actively extruded from the cells, possibly by a cation/proton antiport mechanism. Recent work in our laboratory suggested that uptake of DMP^+ into an *acrA* strain of *Escherichia coli* was likely driven both by the establishment of a membrane potential and by a H^+/DMP^+ antiport system [8,9].

In the present paper, we have examined the nature of the uptake mechanism for DMP^+ using phospholipid vesicles (liposomes). We have confirmed that the driving force for uptake is the transmembrane potential. Furthermore, we have found that the observed changes in the fluorescence intensity of DMP^+ reflect both absorption and desorption of DMP^+ by the phospholipid bilayer and movements of the dye within the bilayer itself.

Materials and Methods

Preparation of phospholipid vesicles (liposomes)

Egg yolk phosphatidylcholine (Sigma, Type V-E) was lyophilized and resuspended at a concentration of 35 mg/ml in 2 mM Hepes buffer (pH 7.0) containing 0.3 M KCl (unless otherwise indicated). Dicetyl phosphate (P-L Biochemicals), stearylamine (P-L Biochemicals) or DOPA (Avanti Polar-Lipids) were incorporated into liposomes at a concentration of 10% (mol/mol) by addition to egg yolk PC in chloroform/

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Abbreviations: DMP^+, 2-(4-dimethylaminostyryl)-1-ethylpyridinium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPB^-, tetraphenylboron; PC, phosphatidylcholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; ANS^-, 1-anilinonaphthalene-8-sulfonate.

methanol (2:1, v/v) prior to lyophilizing. The mixture was sonicated with a bath-type sonicator (Branson 1200) until it had cleared, and then heated at 40°C in a water bath for 20 min, followed by cooling on ice. Excess K^+ was then removed by passing the sample through a 10×1 cm Sephadex G-50 (Fine) gel-filtration column equilibrated with 2 mM Hepes buffer at pH 7.0 containing 0.6 M sucrose. Aliquots (0.05 ml) of the K^+-loaded, column-processed liposomes were added directly to fluorescence assay systems. Where efflux of potassium was measured with a K^+-electrode (Orion) the reaction mixtures contained, in a final volume of 5.0 ml, 0.5 ml of the liposome preparation, 2 mM Hepes (pH 7.0) and 0.1 M NaCl unless otherwise indicated. In experiments using bovine serum albumin-loaded liposomes, 1% (w/v) bovine serum albumin was added to the phospholipid suspensions prior to sonicating.

Liposome volume and phospholipid content were determined as previously described [10].

Fluorescence measurements

The fluorescence of DMP^+ and SPQ was measured in an SLM Aminco SPF 500C spectrofluorometer (Urbana, IL) interfaced to a Hewlett Packard Model 7470A plotter. DMP^+ fluorescence was excited at 467 nm (5 nm band pass) and emission was measured at 557 nm (20 nm band pass). Fluorescence changes were monitored following addition of DMP^+ to the assay systems as indicated in the figure legends. The reaction mixtures, in a final volume of 2 ml, contained 0.05 ml liposome preparation and 2 mM Hepes (pH 7.0). The final concentrations of other reagents are indicated in the figure legends.

SPQ fluorescence was monitored in liposomes preloaded with 1 or 5 mM SPQ in the presence or absence of KCl. Excess dye was removed by the column filtration method. The quenching of SPQ fluorescence by Cl^- was monitored in response to external additions of Cl^-. Enhancement of SPQ fluorescence was measured in response to Cl^- efflux. The excitation and emission wavelengths for SPQ were 350 nm and 445 nm, respectively.

Pyranine fluorescence was measured as previously described [10].

Measurement of binding and release of DMP^+ from liposomes

K^+-loaded liposomes (0.5 ml) were added to DMP^+ fluorescence assay systems containing 2 mM Hepes (pH 7.0), in a final volume of 2.0 ml. The assay was initiated by the addition of 12.5 μM DMP^+. Following incubation for 2 min, duplicate samples (0.2 ml) were removed from the reaction mixture, applied to 1-cm columns of Sephadex G-50 (Fine) contained in 1 ml tuberculin syringes and centrifuged at 1200 rpm for 1 min in a bench top centrifuge. Each column was subsequently washed with 2×0.2 ml of buffer to elute the liposome fraction. Free dye was then eluted by washing the column with 5×0.2-ml aliquots of buffer. Bovine serum albumin (0.5% (w/v) final concentration) was added to each 'free dye' fraction to give a final volume of 2.0 ml, and the fluorescence of each fraction was measured. This procedure was repeated for samples taken after the addition of 20 ng valinomycin and, finally, 20 ng nigericin. The fluorescence yield was calibrated by addition of aliquots of a standard DMP^+ solution to 0.5% bovine serum albumin in the same buffer system.

Partition coefficients for DMP^+ and ANS^- with liposomes were determined by the method of Huang and Haugland [11].

Results

Characteristics of egg phosphatidylcholine liposomes

Liposomes were prepared by bath sonication of egg phosphatidylcholine in the appropriate buffer. The ^{31}P nuclear magnetic resonance spectrum showed the presence only of small unilamellar vesicles. The trapped volume of the liposomes was measured using [^{14}C] sucrose. A value of 0.34 μl/μmol egg phosphatidylcholine was obtained. A value of 0.27 μl/μmol egg phosphatidylcholine was obtained by measuring with a K^+-electrode the amount of K^+ released on dilution of liposomes loaded with 0.3 M KCl into buffer in the presence of valinomycin.

Uptake of DMP^+ by egg phosphatidylcholine liposomes

Addition of DMP^+ to a suspension of egg phosphatidylcholine liposomes loaded with 0.3 M KCl and suspended in K^+-free buffer gave a small increase in fluorescence intensity which was greatly increased by the addition of valinomycin to induce efflux of K^+ and to establish a transmembrane potential (Fig. 1, curves 1–3). The valinomycin-induced increment in fluorescence intensity was abolished by subsequent addition of nigericin to re-equilibrate K^+ across the liposome membrane. Nigericin added before valinomycin did not induce an increase in fluorescence intensity and abolished the ability of valinomycin to do this (Fig. 1, curve 4). The fluorescence intensities of both the initial and the valinomycin-induced phases were dependent on the concentration of dye added to the medium. This is consistent with a greater amount of DMP^+ partitioning into the phospholipid bilayer as dye concentration was increased.

The fluorescence emission spectrum of the dye was measured at different time intervals in the course of an experiment similar to that shown in Fig. 1 (Fig. 2). The liposome suspension showed no significant fluorescence emission between 475 and 750 nm (curve 1).

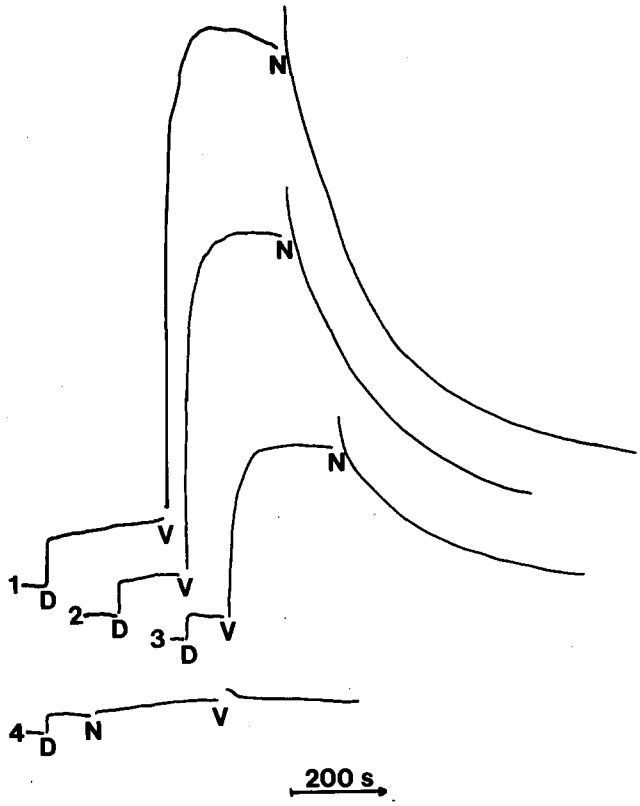

Fig. 1. Change in fluorescence of DMP^+ in egg PC liposomes loaded with 0.3 M KCl. Curves 1 and 2, 1.88 and 1.25 μM DMP^+, respectively. Curves 3 and 4, 0.63 μM DMP^+. V, addition of 5 ng valinomycin. N, addition of 5 ng nigericin. D, addition of DMP^+. Fluorescence in this and the other figures is expressed in arbitrary units.

Addition of DMP^+ to the liposomes gave a small peak of fluorescence with maximum emission at about 577 nm (curve 2). Fluorescence intensity was increased by addition of valinomycin with maximum emission occurring initially at this wavelength (curve 3) but shifting to 568 nm with time (curves 4 and 5). Addition of nigericin then decreased fluorescence emission with the emission peak returning to a higher wavelength (curve 6). These results suggested that the establishment of a membrane potential increased the binding or partitioning of dye in the phospholipid bilayer but that the bound dye then moved into another environment with time.

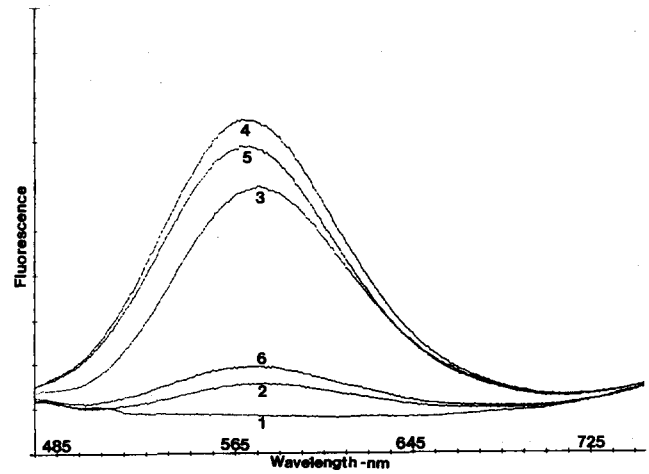

Fig. 2. Fluorescence emission spectrum of DMP^+ in egg PC liposomes. Curve 1, liposomes in 2.0 mM Hepes (pH 7.0). Curve 2, 0.63 μM DMP^+ added to the cuvette. Curves 3–5, 5 ng valinomycin added to the cuvette and spectra scanned at 1-min intervals. Curve 6, 5 ng nigericin added to the cuvette. Fluorescence was excited at 467 nm.

The alternative explanation that the valinomycin- and nigericin-induced changes were a consequence only of environmental changes in the dye initially bound was eliminated by the results of the following experiment.

The concentration of free dye in the medium during the course of an experiment like that of Fig. 1 was measured. Samples were removed at intervals and the liposomes separated from the external medium by chromatography on a column of Sephadex G-50. The amount of free dye was estimated following addition of bovine serum albumin. DMP^+ binds to this protein with greatly increased dye fluorescence.

Addition of DMP^+ to the KCl-loaded liposome suspension resulted in the binding of 10–12% of the added dye (see Materials and Methods for details of this experiment). Valinomycin now induced the removal of DMP^+ from the medium to leave only 10% unbound. Nigericin caused release of about 70% of the dye accumulated in the presence of valinomycin. Thus, most of the valinomycin-induced change in fluorescent intensity of DMP^+ is due to uptake of the dye.

The initial phase of increase in fluorescence intensity seen on addition of DMP^+ to the liposome suspension is likely to be due to partitioning of the dye into the phospholipid bilayer. This was shown by measuring the wavelength of maximum fluorescence emission by DMP^+ as a function of the polarity of its environment. As shown in Table I, this wavelength maximum decreased as the polarity of the environment of the dye decreased. The dye bound to liposomes in the absence

TABLE I

Effect of polarity of the medium on the wavelength of maximum fluorescence emission of DMP^+

The excitation wavelength was 467 nm.

Medium	Polarity [a]	Wavelength (nm)
Water	94.6	592–593
Ethanol	79.6	586–587
Phosphatidylcholine liposome [b]		577–578
Chloroform	63.2	565

[a] Polarity is expressed on the Z scale, an empirical measure of solvent polarity [12,13].

[b] DMP^+ was added to the liposome suspension in the absence of a transmembrane potential.

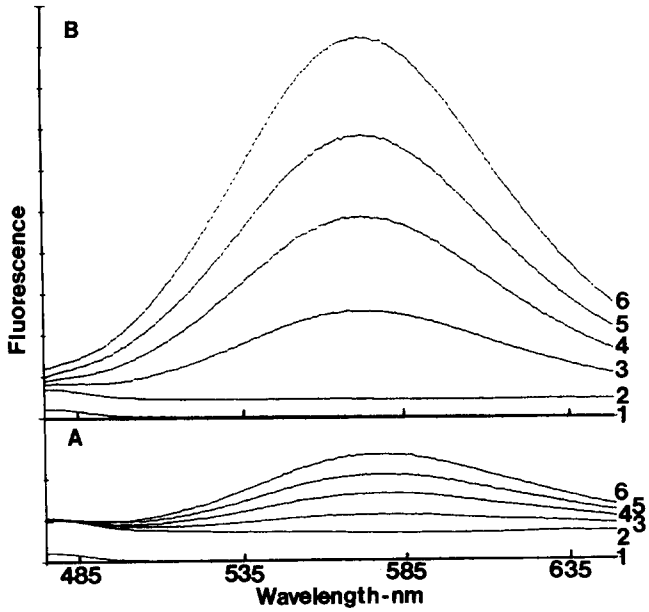

Fig. 3. Fluorescence emission spectrum of DMP^+ in egg PC liposomes (Panel A) and liposomes prepared from egg PC with 10% (mol/mol) DOPA (Panel B). Curve 1, no DMP^+ present. Curves 2–6, successive additions of 0.63 μM DMP^+.

of a transmembrane potential was located in an environment with a polarity intermediate between that of ethanol and chloroform.

The influence of the surface charge of the liposome on the partitioning of the dye into the phospholipid bilayer was examined. Liposomes were prepared with and without 10% (mol/mol) dioleoylphosphatidic acid (DOPA) to study the effect of a negative surface charge. The partitioning of DMP^+ into liposomes was greatly increased by the presence of DOPA as shown by the enhancement in the fluorescence emission spectra in Fig. 3. Negatively-charged dicetyl phosphate (10% mol/mol) gave similar results to DOPA. Partitioning of DMP^+ into liposomes was prevented by the incorporation of positively-charged stearylamine at 10% (mol/mol). Partition coefficients for DMP^+, and for ANS^-, into phosphatidylcholine liposomes containing 10% (mol/mol) DOPA, dicetyl phosphate or stearylamine are shown in Table II. As suggested by the data of Fig. 3, DOPA and dicetyl phosphate increased the partition coefficient for DMP^+ into egg phosphatidylcholine liposomes. Conversely, DOPA and dicetyl phosphate decreased, and stearylamine increased, the partition coefficient of negatively charged ANS^-.

Driving force for fluorescence-intensity changes with DMP^+

The results of Fig. 1 suggested that the entry of DMP^+ into the phospholipid bilayer, with a consequent increase in fluorescence intensity, was enhanced by the imposition of a transmembrane potential. This possibility was examined in more detail (Fig. 4). Lipo-

TABLE II

Partition coefficients of DMP^+ and ANS^- into liposomes of different composition

The partition coefficients were determined as described in Materials and Methods. PC, egg phosphatidylcholine; DOPA, dioleoylphosphatidic acid; DCP, dicetyl phosphate; SA, stearylamine. DOPA, DCP and SA, when present, were at 10% (mol/mol) of the concentration of PC.

Compound	Partition coefficient			
	PC	PC+DOPA	PC+DCP	PC+SA
DMP^+	$5.1 \cdot 10^4$	$1.9 \cdot 10^6$	$3.9 \cdot 10^5$	
DMP^+/TPB^- [a]	$2.9 \cdot 10^6$			
ANS^-	$3.6 \cdot 10^5$	$1.1 \cdot 10^5$	$1.4 \cdot 10^5$	$1.1 \cdot 10^6$

[a] Equimolar ratio.

somes loaded with 50, 100, 200 and 300 mM KCl were incubated with DMP^+ in KCl-free buffer. Addition of valinomycin resulted in fluorescence enhancement, the magnitude of which was proportional to the size of the KCl gradient. The kinetics of K^+ release following addition of valinomycin (Fig. 5, curve 3) were compared with the rate of the DMP^+ response (curve 1). The fluorescence response of DMP^+ was more rapid than the rate of K^+ release. This is consistent with a membrane potential-driven fluorescence response since relatively few K^+ ions need to exit to establish a significant membrane potential [14]. Nigericin induced a more rapid loss of K^+ from the liposomes than valinomycin (curves 3, 4). This is expected from the non-electrogenic action of nigericin which catalyzes electroneutral exchange of H^+ for K^+ [14]. Thus,

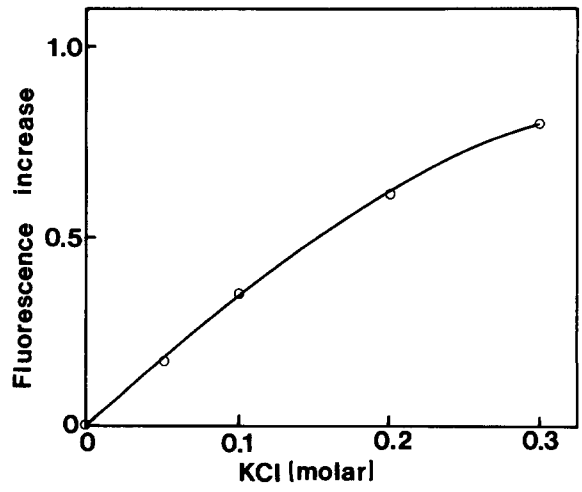

Fig. 4. Change in the fluorescence intensity of DMP^+ as a function of the K^+ gradient in KCl-loaded egg PC liposomes. Liposomes containing 50, 100, 200 or 300 mM KCl were diluted into KCl-free buffer. The increase in fluorescence intensity following addition of 5 ng valinomycin was measured. The KCl concentration within the liposome was confirmed by measuring the internal volume with [^{14}C]sucrose and K^+ released using an ion-specific electrode (see Materials and Methods).

nigericin addition before valinomycin caused a rapid loss of K^+ from the liposomes without altering the fluorescence behaviour of DMP^+ (curves 4, 5).

The use of KCl-loaded liposomes raised the possibility that the movement of other ions (e.g., Cl^-) could complicate the interpretation that membrane potential was the main driving force for uptake of DMP^+ into the liposome bilayer. We have previously used liposomes loaded with the fluorescent dye pyranine to study the uptake of H^+ into the internal aqueous phase of the liposome [10]. This procedure was used here. Dilution of liposomes loaded with pyranine and 0.3 M KCl into 0.1 M NaCl containing buffer resulted in a slow acidification of the vesicle interior concomitant with K^+ leakage from the vesicles. Addition of nigericin and valinomycin, but not DMP^+, gave rapid

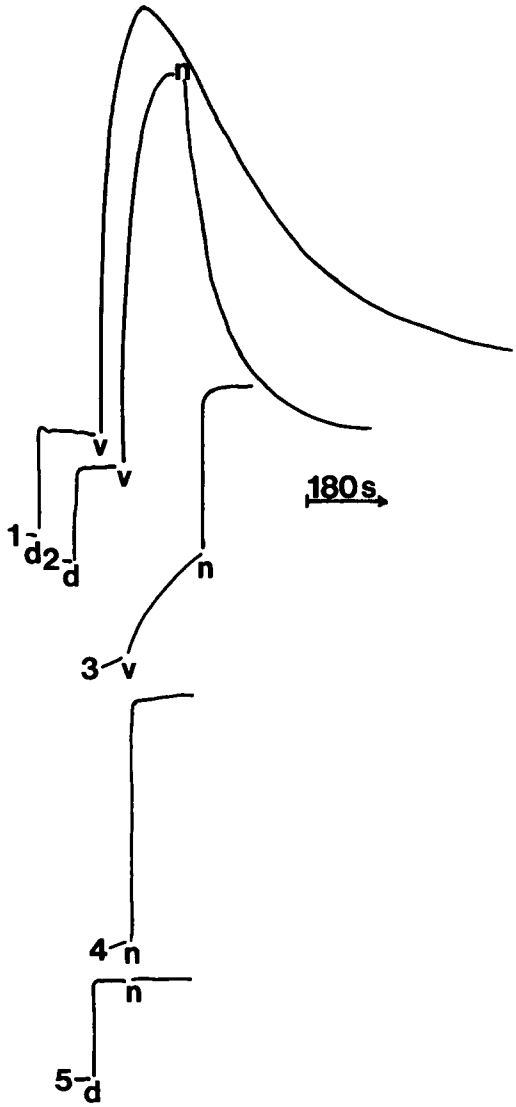

Fig. 5. Comparison of changes in fluorescence of DMP^+ and K^+ efflux from egg PC liposomes. Curves 1, 2 and 5, DMP^+ fluorescence. Curves 3 and 4, K^+ efflux from liposomes containing 0.3 M KCl. Symbols as in Fig. 1. d, addition of DMP^+.

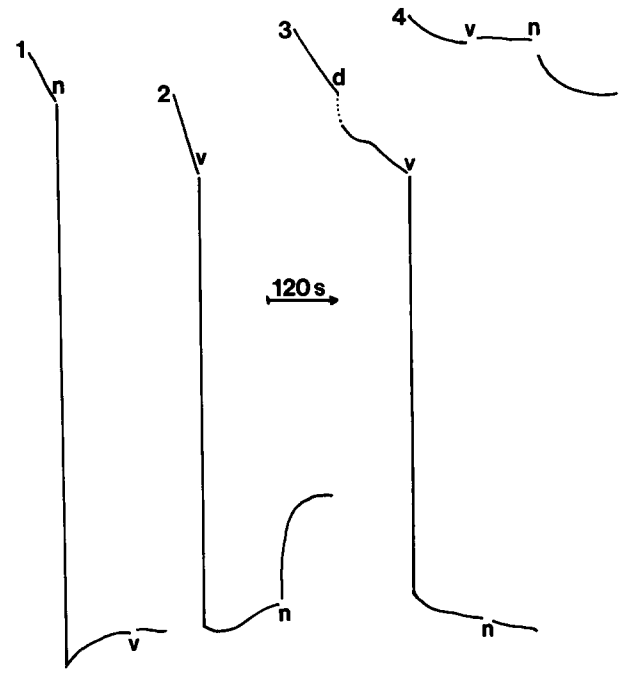

Fig. 6. Changes in fluorescence of pyranine in 0.3 M KCl-loaded, egg PC liposomes. In curves 1 and 2, the reaction mixtures contained 0.1 M NaCl. In curve 4, the reaction mixture contained 0.3 M KCl. Symbols as in Fig. 1.

movement of H^+ into the liposome interior (Fig. 6). This did not occur if the KCl-loaded liposomes were diluted into 0.3 M KCl (curve 4). Acidification in the presence of nigericin is expected since this ionophore catalyzes K^+/H^+ antiport [14]. Movement of H^+ following addition of valinomycin is consistent with H^+ entering the vesicle in response to the valinomycin-initiated membrane potential. There must be a path for rapid H^+ movement across the liposomal bilayer [10].

Movement of Cl^- was examined using the fluorescent dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) [15]. The validation of the use of this dye to measure Cl^- movements in liposomes is shown in Fig. 7A. Liposomes were preloaded with 5 mM SPQ in the absence of KCl. Several consecutive additions of 2.5 mM NaCl resulted in quenching of the fluorescence of SPQ as Cl^- entered the vesicles slowly (curve 1). Addition of tri-n-butyltin chloride, a Cl^-/OH^- antiporter [16] or monensin, which catalyzes Na^+/H^+ antiport [14], enhanced the rate of Cl^- entry (curves 2, 3). In a similar manner, the slow Cl^- movement on addition of KCl to the liposome suspension (curve 5) was enhanced by the addition of tri-n-butyltin chloride and nigericin (curves 5, 6).

In Fig. 7B, the kinetics of Cl^- movement from SPQ-and KCl-loaded liposomes (curves 8, 9) are compared with the kinetics of the DMP^+ response (curve 7). Addition of valinomycin or nigericin caused some loss of Cl^- from the loaded liposomes but the rate was slower than the DMP^+ response. Loss of Cl^- from 0.3

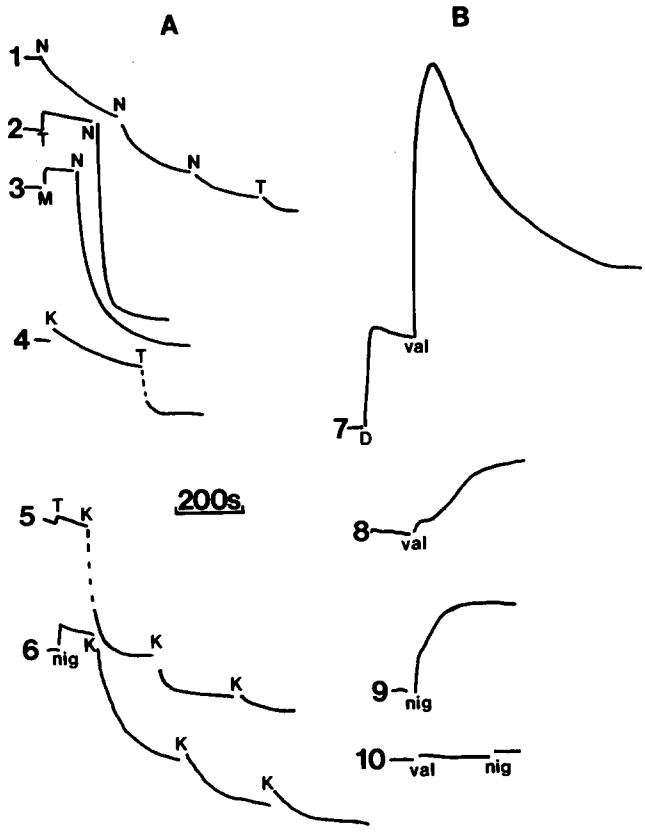

Fig. 7. (Panel A) Quenching of SPQ fluorescence in egg PC liposomes preloaded with 5 mM SPQ in the absence of KCl. N, addition of 2.5 mM NaCl. T, addition of 0.125 μM tri-n-butyltin chloride. K, addition of 7.5 mM KCl in curve 4, 15 mM KCl in curves 5 and 6. nig, addition of 5 ng nigericin. (Panel B) Changes in DMP^+ fluorescence (curve 7) and SPQ fluorescence (curves 8–10) in egg PC liposomes preloaded with 1 mM SPQ and 0.3 M KCl. Curve 10, the reaction mixture contained 0.3 M NaCl. val, addition of 5 ng valinomycin.

M KCl-loaded liposomes on addition of valinomycin or nigericin was not detected if they were diluted into 0.3 M NaCl (curve 10).

The experiments in this section indicated that membrane potential was the main driving force for the uptake of DMP^+. There was no obvious correlation between changes in the fluorescence intensity of DMP^+ and the movement of H^+ or Cl^-.

Effect of tetraphenylboron (TPB^-) on the interaction of DMP^+ with egg phosphatidylcholine liposomes

Fig. 8 illustrates the effect on DMP fluorescence of adding 0.125 μM TPB^- to 0.3 M KCl-loaded liposomes. TPB^- enhances the magnitude of the fluorescence increase produced by addition of valinomycin. The effect is transitory.

Addition of TPB^- to liposomes in the presence of DMP^+ enhanced the fluorescence of the dye in the absence of a membrane potential. Fig. 9 shows the effect of adding different concentrations of TPB^- to fixed concentrations of DMP^+. The fluorescence intensity reached a plateau level which was dependent on the amount of DMP^+ present (curves 1, 2). Curves 3 and 4 for higher levels of DMP^+ are incomplete as the fluorescence intensity became off-scale. In the absence of TPB^-, DMP^+ showed maximum fluorescence emission at about 575 nm. Addition of TPB^- caused this to shift to about 563 nm.

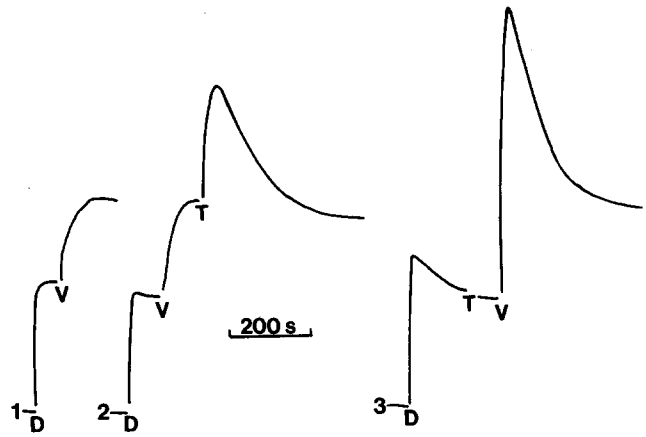

Fig. 8. Effect of TPB^- on DMP^+ fluorescence in egg PC liposomes. The reaction mixture contained 0.6 M sucrose in 10 mM Hepes (pH 7.5). T, addition of 0.125 μM TPB^-. Other symbols as in Fig. 1.

These data suggest that TPB^-, by interacting with the oppositely-charged DMP^+, might facilitate partitioning of DMP^+ into the phospholipid bilayer. This was confirmed by measuring the partition coefficient of DMP^+ in the presence of TPB^- (Table II). Further evidence was obtained using the bovine serum albumin method, mentioned before, to measure the amount of DMP^+ remaining in the medium following various

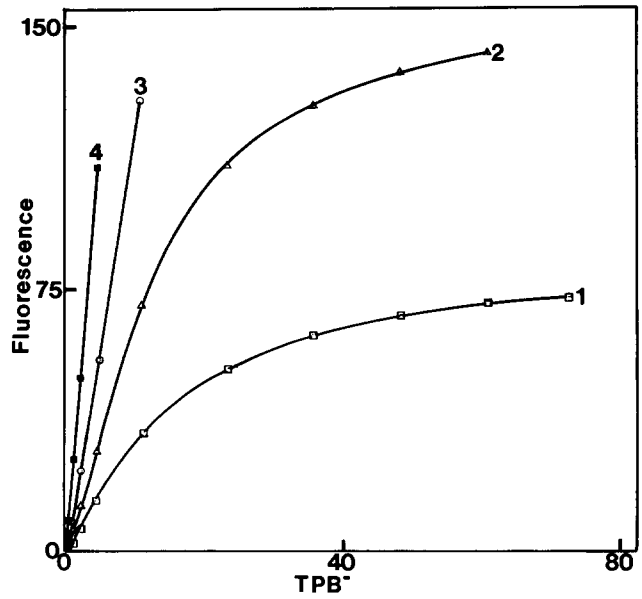

Fig. 9. Effect of increasing concentrations of TPB^- on DMP^+ fluorescence in egg PC liposomes. Curves 1–4, 0.31, 0.62, 1.25 and 2.5 μM DMP, respectively. The concentration of TPB^- is expressed as μM.

additions to the liposome suspension. In two experiments where this was examined, 90% of the DMP^+ was not associated with the liposomes following its addition to the suspension. This decreased to 78% when 25 μM TPB^- was added. Only 10% of the DMP^+ was unbound after the further addition of valinomycin. Thus, TPB^- facilitates entry of DMP^+ into the phospholipid bilayer. Evidence for the formation of a complex of the two dyes is given in Fig. 10. Curve 2 shows the effect of adding 6.25 μM TPB^- to 1.25 μM DMP^+ (curve 1). There was little increase in fluorescence. However, addition of TPB^- to 12.5 μM resulted in the formation of a highly fluorescent complex with maximum emission at 612 nm (curve 3). (Variation in the relative amounts of the two compounds indicated that maximum fluorescence was achieved when the molar ratio was 1 : 1, indicative of a DMP^+-TPB^- complex) (results not shown). Addition of 0.3 M KCl-loaded liposomes resulted in a change in the emission maximum to 568 nm as the complex entered the phospholipid bilayer (curve 4). The fluorescence was enhanced by addition of valinomycin (curve 5) and decreased by the further addition of nigericin (curve 6), the wavelength of maximum fluorescence emission remaining the same.

The effect of higher concentrations (up to 12.5 μM) TPB^- in inducing increases in the fluorescence intensity of DMP^+ was not due to an effect of TPB^- on the KCl gradient. As shown in Fig. 11 (lower curve), addition of 12.5 μM TPB^- greatly enhanced the fluorescence of DMP^+ with KCl-loaded vesicles but had no effect in increasing the rate of loss of K^+ from the liposomes (Fig. 11, upper curve). TPB^-, like nigericin and valinomycin (Fig. 6), induced H^+ entry into pyranine- and KCl-loaded liposomes but the rate of H^+ entry was much slower than with the two ionophores. TPB^- had no effect on Cl^- movement.

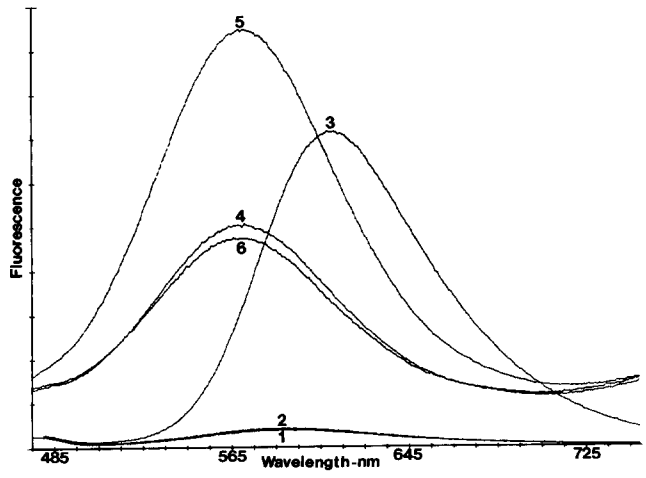

Fig. 10. Fluorescence emission spectra of DMP^+. The following sequence of additions was made to the cuvette containing buffer only: curve 1, 1.25 μM DMP^+; curve 2, 6.25 μM TPB^-; curve 3, 6.25 μM TPB^- (total $TPB^- = 12.5$ μM); curve 4, 0.050 ml liposome preparation; curve 5, 5 ng valinomycin; curve 6, 5 ng nigericin. Scans were initiated 1 min after the addition of each of the reagents.

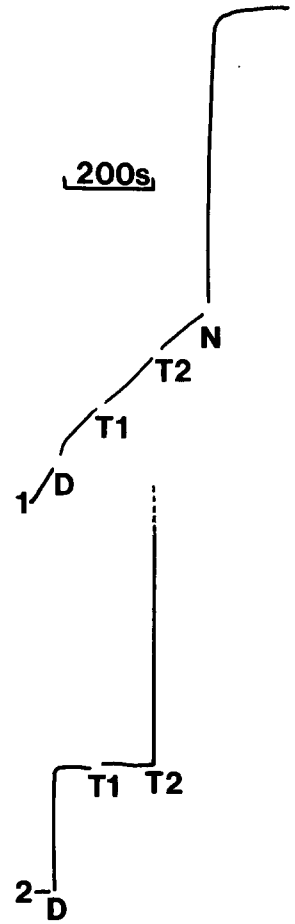

Fig. 11. (Panel 1) Potassium efflux from 0.3 M KCl-loaded egg PC liposomes. D, addition of 12.5 μM DMP^+. T1, addition of 0.125 μM TPB^-. T2, addition of 12.5 μM TPB^-. N, addition of 5 ng nigericin. (Panel 2) Changes in DMP^+ fluorescence in K^+-loaded egg PC liposomes. Additions are as indicated for panel 1. The fluorescence increased greatly to become off-scale following addition of T2.

Discussion

Addition of DMP^+ to a suspension of egg phosphatidylcholine liposomes resulted in the binding of about 10% of the dye. Uptake was greatly enhanced by the imposition of a transmembrane potential (vesicle interior negative) by valinomycin-induced K^+ diffusion down its concentration gradient. The movement of H^+ and Cl^- which occurred following addition of valinomycin were due to leakage of the ions across the phospholipid membrane either in response to the induced membrane potential (H^+) or to a concentration gradient (Cl^-). These ion movements are not considered to be related to the uptake of DMP^+ or to changes in its fluorescence.

Uptake of DMP^+ driven by the transmembrane potential is largely reversible. The ready release of the

dye on collapse of the membrane potential suggests that the bulk of it enters the external monolayer of the liposome bilayer. The fluorescence behaviour of the dye is consistent with at least a portion of the molecule entering into a nonpolar environment. The positively charged pyridinium group is presumably located in the polar headgroup region of egg phospholipids. Provision of negatively-charged headgroups by incorporation of dioleoylphosphatidic acid or dicetyl phosphate into the liposomes increased the extent of partitioning of DMP^+ into the phospholipid bilayer. Conversely, positively charged stearylamine opposed entry of the positively-charged dye into the bilayer. Imposition of a membrane potential resulted in a shift in the fluorescence emission maximum of the dye from 577 nm to 568 nm. This is consistent with further movement of DMP^+ into an environment of lower polarity. The data of Table I suggest that DMP^+ is bound initially in an environment with a polarity intermediate between that of ethanol and chloroform. On imposition of the membrane potential, the dye molecules move more deeply from the headgroup region of the bilayer into an environment with a polarity similar to that of chloroform. This presumably is the region of the fatty acyl chains of the phospholipid. We were not able to determine if any DMP^+ entered into the internal aqueous space of the liposome.

As discussed above the increase in fluorescence intensity observed on the imposition of a transmembrane potential is a consequence both of increased binding of DMP^+ and the movement of the dye into a region of lower polarity. We have not attempted to determine the conditions under which DMP^+ could be used to measure the transmembrane potential. The correlation between the maximum fluorescence intensity and the magnitude of the KCl gradient used to generate the membrane potential (Fig. 4) suggests that this is feasible. Transmembrane movement of other ions (e.g., H^+, Cl^-), although diminishing fluorescence intensity, is slower than the response time of the dye and so should not interfere with these measurements.

TPB^- facilitated the entry of DMP^+ into the phospholipid bilayer (Table II). The formation of a neutral DMP^+-TPB^- complex was observed in aqueous systems (Fig. 10). It might be expected that the entry of this complex into the nonpolar regions of the bilayer would not be opposed by the membrane dipole potential, positive inside, which accounts for the greater rates of permeation of nonpolar anions compared with nonpolar cations [17,18]. The fluorescence emission maximum of DMP^+ entering as a complex (568 nm) was the same as that observed with DMP^+ which had entered in response to the transmembrane potential, and is consistent with the complex entering the fatty acyl chain region of the bilayer. However, the fluorescence intensity of DMP^+ in the liposome was increased by concentrations of TPB^- greater than those required to form the DMP^+-TPB^- complex (Fig. 9). Clearly, there must be an additional effect of TPB^- on DMP^+ fluorescence over that accounted for by complex formation, perhaps by diminishing the opposing membrane dipole potential.

In summary, DMP^+ partitions into an egg phosphatidylcholine bilayer to a limited extent. Imposition of a transmembrane potential increases the amount of dye entering the bilayer. The dye is located initially in the headgroup region of the outer monolayer of the liposome bilayer but moves into the fatty acyl chain region in response to the transmembrane potential. It is readily released into the external medium on discharge of the transmembrane potential.

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