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Transmembrane Distribution of Lipophilic Cations in Response to an Electrochemical Potential in Reconstituted Cytochrome *c* Oxidase Vesicles and in Vesicles Exhibiting a Potassium Ion Diffusion Potential

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It has been shown previously that biogenic amines and a number of pharmaceutical agents can redistribute across vesicle membranes in response to imposed potassium ion or proton gradients. Surprisingly, drug accumulation is observed for vesicles exhibiting either a pH gradient (interior acidic) or a membrane potential (interior negative), implying that these compounds can traverse the lipid bilayer as either the neutral or charged species. This interpretation, however, is complicated by the fact that vesicles exhibiting a membrane potential (interior negative) accumulate protons in response to this potential, thereby creating a pH gradient (interior acidic). This raises the possibility that in both vesicle systems drug redistribution occurs in response to the proton gradient present. We have therefore compared the uptake of several lipophilic cations by reconstituted cytochrome *c* oxidase vesicles and by similar vesicles exhibiting a potassium ion diffusion potential. While turnover of the oxidase generates a membrane potential of comparable magnitude to the potassium ion diffusion system, it is associated with a proton gradient of opposite polarity (interior basic). Both systems show rapid uptake of the permanently charged lipophilic cation, tetraphenylphosphonium, but only the potassium ion diffusion system accumulates the lipophilic amines doxorubicin and propranolol. This provides compelling evidence that such weak bases redistribute only in response to pH gradients and not membrane potential.

KEY WORDS: Reconstituted cytochrome oxidase; membrane potential; proton gradients; lipophilic cations.

INTRODUCTION

The electrochemical potential generated by energy transducing membranes can generally be divided into two components, a proton gradient and a membrane potential, the relative magnitudes of which vary greatly between different organelles and between different energy states for the same organelle. The influence of these two components on the transmembrane distribution of lipophilic ions is distinctive and has been used to independently estimate the magnitude of each. Observations that ammonia (Krogmann *et al.*, 1959) and other amines (Hind and Whittingham, 1963) could uncouple photophosphorylation from electron flow in chloroplasts were later shown to be concentration dependent and reflected a redistribution of the amine in response to the proton gradient (Crofts, 1967). If the assumption is made that the neutral species of the amine is membrane permeable while the charged species is impermeable, then its transmembrane distribution will vary with the proton gradient in accordance with the Henderson–Hasselbach equation. Based on this relationship, proton

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gradients have been estimated from the transmembrane distribution of radiolabelled amines (Rottenberg *et al.*, 1971), fluorescent amines (Deamer *et al.*, 1972), and spin-labeled amines (Cafiso and Hubbell, 1978). In contrast to such proton gradient probes, molecules used to estimate the membrane potential must carry a net charge and cross the membrane in this charged form. The membrane potential can then be determined from their transbilayer distribution using the Nernst equation. Examples of such membrane potential probes are ^{42}K and ^{86}Rb, which in the presence of the ionophore valinomycin are membrane permeable, and ions such as thiocyanate and tetraphenylphosphonium (Bakeeva *et al.*, 1970) which are able to cross the membrane as the result of charge delocalization and charge shielding, respectively.

In addition to such electrochemical gradient probes, it has been shown recently that a variety of compounds that are weak bases, including biogenic amines (Bally *et al.*, 1988) and a number of pharmaceutical agents (Bally *et al.*, 1985; Mayer *et al.*, 1985a, 1986, 1988; Madden *et al.*, 1990), redistribute across membranes in response to imposed potassium ion or proton gradients. In many cases high levels of drug accumulation were observed irrespective of whether the vesicles exhibited a proton gradient (interior acid) or a membrane potential (interior negative). This would imply that such compounds could cross the bilayer in either the neutral or charged (protonated) form. The assessment of these data is complicated, however, by the fact that vesicles exhibiting a membrane potential (interior negative) show transbilayer proton movement in response to this potential, thereby developing a proton gradient (interior acid) (Bally *et al.*, 1988; Mayer *et al.*, 1988; Redelmeier *et al.*, 1989). This raises the possibility that in both vesicle systems drug redistribution occurs in response to the proton gradient present.

We have therefore compared the accumulation of several lipophilic cations by reconstituted vesicles containing cytochrome *c* oxidase and by similar vesicles exhibiting a potassium ion diffusion potential. The oxidation of ferrocytochrome *c* by the oxidase generates a membrane potential (interior negative) comparable to that created by a potassium ion diffusion gradient. In the case of the oxidase system, however, protons are oxidized in the vesicle interior as well as being vectorially pumped out, thus creating a proton gradient (interior basic) in the opposite direction to that generated by the potassium ion diffusion system. A comparison of drug uptake by these two vesicle systems will therefore identify whether accumulation is in response to the proton gradient or membrane potential.

MATERIALS AND METHODS

Materials

Cytochrome *c* oxidase was prepared by the method of Kuboyama *et al.* (1972). The oxidase was finally resuspended in 1% Tween-80 and stored in liquid nitrogen at a protein concentration of approximately 50 mg/ml. Cytochrome *c* (horse heart, type VI), sodium cholate, Tween-80, carboxyl cyanide *m*-chlorophenylhydrazone (CCCP),[3] nigericin, valinomycin, ascorbic acid, and doxorubicin were purchased from Sigma (St. Louis, Missouri). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, Alabama), while 3,3′-dipropylthiodicarbocyanine [DiS-C_3-(5)] and hydroxy-1,3,6-pyrenetrisulfonate (pyranine) were purchased from Molecular Probes (Eugene, Oregon). [^{3}H]-Dipalmitoylphosphatidylcholine and [^{14}C]-tetraphenylphosphonium were obtained from New England Nuclear.

Preparation of Reconstituted Cytochrome Oxidase Vesicles

For the reconstitution experiments, 60 mg of phospholipid (DOPC : DOPE 1 : 1 molar ratio) and 30 mg of sodium cholate were hydrated in 4 ml of 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4. Unilamellar vesicles were then prepared according to the LUVET procedure (Hope *et al.*, 1985) employing an Extruder (Lipex Biomembranes, Vancouver, Canada) as described previously (Madden and Cullis, 1984). Cytochrome *c* oxidase was then added (400 μg) and the mixture dialyzed (Spectrapor 2, 10-mm-diameter dialysis tubing) for 24 h against 3 changes of 400 volumes each 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM

[3] Abbreviations: CCCP, carboxyl cyanide *m*-chlorophenylhydrazone; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DiS-C_3-(5), 3,3-dipropylthiodicarbocyanine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid; TPP^+, tetraphenylphosphonium ion.

HEPES, pH 7.4, at 4°C. The vesicles were then dialyzed for 2 h against 400 volumes 250 mM sucrose, 22.5 mM K_2SO_4, and 2.5 mM HEPES, pH 7.4. To separate vesicles containing outwardly oriented oxidase from either protein-free vesicles or those containing inwardly oriented enzyme, 1-ml aliquots of the reconstitution mixture were applied to DEAE-Sephacel columns (0.9 × 10 cm) pre-equilibrated with 250 mM sucrose, 22.5 mM K_2SO_4, and 2.5 mM HEPES, pH 7.4, and vesicles washed onto the column with a further 2 ml of the same buffer. The columns were washed with 8 ml of 200 mM sucrose, 45 mM K_2SO_4, and 5 mM HEPES, pH 7.4, eluting protein-free vesicles and those containing inwardly oriented oxidase (Madden and Cullis, 1984). Vesicles containing outwardly oriented enzyme were then eluted with 150 mM K_2SO_4 and 20 mM HEPES, pH 7.4 (Madden *et al.*, 1984; Zhang *et al.*, 1985). Fractions containing a majority of the enzyme activity were pooled and dialyzed for 12 h against 400 volumes of 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4. Aliquots of protein-free vesicles were similarly dialyzed against this buffer, and used to measure lipophilic cation uptake in response to a potassium ion diffusion potential (see below). Recent batches of DEAE-Sephacel (Pharmacia) gave poor recovery of reconstituted vesicles, and we therefore recommend the use of DEAE Sepharose 6B-CL (Pharmacia) instead. Different preparations of reconstituted vesicles used in the present study exhibited respiratory control ratios of 3.8–5.7 and specific activities (uncoupled) of 12–14 μmol ferrocytochrome *c* oxidized/min/nmol heme *a*.

Determination of the Membrane Potential Generated in Cytochrome Oxidase Vesicles

The procedure used to quantitate the membrane potential generated upon oxidation of ferrocytochrome *c* by oxidase vesicles is essentially that described by Kita *et al.* (1982) and Matsushita *et al.* (1984). The concentration-dependent quenching of 3,3′-dipropylthiodicarbocyanine [Dis-C_3-(5)] is followed fluorimetrically as it is accumulated within oxidase vesicles in response to the membrane potential. The assay medium consisted of 150 mM sucrose, 67.5 mM K_2SO_4, 100 nM DiS-C_3-(5), and 7.5 mM HEPES, pH 7.4, to which oxidase vesicles (0.35 nM aa_3 final concentration) were added. The reaction was initiated by the addition of ferrocytochrome *c* (3.3–33.3 μM final concentration). Where applicable, nigericin was present at a final concentration of 25 nM. A correction was made for the fluorescence quenching of DiS-C_3-(5) by ferrocytochrome *c* (Singh *et al.*, 1985). A calibration curve of known membrane potentials was prepared by assaying the fluorescent quenching of DiS-C_3-(5) by protein-free vesicles containing 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4 (obtained as the void volume fraction on DEAE chromatography of oxidase-reconstituted vesicles) diluted into 150 mM sucrose, 100 nM DiS-C_3-(5), and 7.5 mM HEPES, pH 7.4, containing various ratios of 67.5 mM Na_2SO_4 to 67.5 mM K_2SO_4 in the presence of valinomycin (1 μM).

Determination of Transmembrane pH Gradients

Measurements of intravesicular pH were made using the fluorescent probe pyranine as described by Clement and Gould (1981). Pyranine (1 mM) was incorporated inside the vesicles by including it in the solution (150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4) in which reconstituted cytochrome oxidase vesicles were prepared. The probe was present at the same concentration in the first two solutions against which the reconstituted vesicles were dialyzed but was not present in the third and final dialysate. Any unencapsulated pyranine remaining in the medium binds strongly to the DEAE Sephacel and can readily be separated from the protein-free and outwardly oriented oxidase-containing vesicles. These two fractions were then dialyzed overnight against 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4.

The fluorescence intensity of entrapped pyranine was determined using excitation and emission wavelengths of 460 nm (5 nm bandpass) and 520 nm (10 nm bandpass), respectively, on a SLM Aminco SPF 500C spectrofluorimeter. A standard curve was constructed by diluting vesicle aliquots (15 μl) into 3 ml of 150 mM sucrose, 60 mM K_2SO_4, 7.5 mM HEPES, 7.5 mM MES, and 7.5 mM $KHPO_4$ adjusted with sodium hydroxide to range in pH from 5.8 to 8.8 Valinomycin (0.8 μM) and nigericin (200 nM) were present to ensure transmembrane proton equilibration.

Accumulation of Lipophilic Cations by Oxidase Vesicles

Prior to uptake experiments, the oxidase vesicles were passed through a 0.4 μm polycarbonate filter

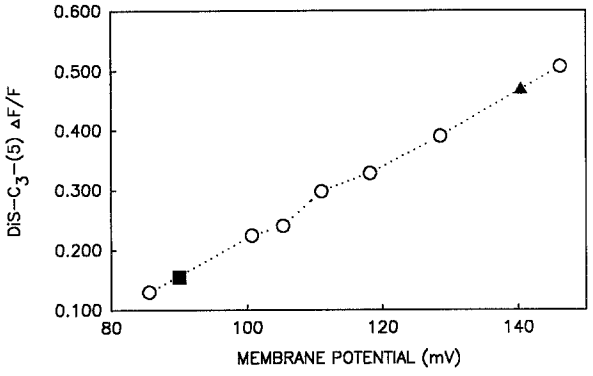

Fig. 1. Membrane potential generated by reconstituted cytochrome *c* oxidase vesicles. A calibration curve of DiS-C_3-(5) fluorescence response to vesicles exhibiting a defined membrane potential is shown (○). Probe response to reconstituted oxidase vesicles in the presence (▲) and absence (■) of nigericin (25 nM) is indicated.

(Nuclepore Corp.) at 4°C to ensure there was no bacterial contamination of the preparation. Similarly all buffers were filtered prior to use. To an aliquot of the oxidase vesicles (2.5 ml) were added sodium ascorbate (5 mM final concentration) and either doxorubicin, tetraphenylphosphonium, or propranolol (100 μM final concentration). The sample was then dialyzed against 50 ml of 150 mM sucrose, 67.5 mM K_2SO_4, 7.5 mM HEPES, and 5 mM ascorbate, pH 7.4, containing 100 μM of the same lipophilic cation for 2 h at 4°C. The dialysis bag containing the oxidase vesicles (520–570 μM lipid; $2.0–2.4 \times 10^{-2}$ μM cytochrome oxidase) was then placed in 50 ml of the same external solution rapidly stirred at 25°C, and following equilibration for 10 min the reaction was initiated by addition of cytochrome *c* (30 μM) to the dialysis bag. Control experiments verified that this protocol prevented oxygen depletion during the uptake assay. At various times aliquots (100 μL) were taken and spun on 1 mL minicolumns (Pick, 1981) containing Sephadex G-50 (medium) hydrated in 150 mM sucrose, 67.5 mM K_2SO_4, 7.5 mM HEPES, 5 mM sodium ascorbate, and 30 μM cytochrome *c*, pH 7.4, to separate oxidase vesicles from unsequestered lipophilic cation. Where the influence of nigericin (25 nM) or valinomycin (1 μM) plus CCCP (20 μM) on the uptake process was studied, the ionophore was included in the external medium as well as with the oxidase vesicles.

Uptake of Lipophilic Cations by Protein-Free Vesicles Exhibiting a Potassium Ion Diffusion Potential

To establish a potassium ion gradient, protein-free vesicles separated from oxidase vesicles by DEAE chromatography were passed down a Sephadex G-50 (fine) column (1.5 × 10 cm) pre-equilibrated with 150 mM sucrose, 67.5 mM Na_2SO_4, and 7.5 mM HEPES, pH 7.4. For uptake experiments the eluted vesicles were then diluted with the same buffer to a similar concentration (500 μM phospholipid) as the oxidase vesicles. To achieve a potassium ion diffusion-driven membrane potential of the same magnitude as the oxidase-driven potential, 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4, was added to the external medium (5 μl/ml). This gives a potassium concentration gradient of 200:1 (inside:outside) which in the presence of valinomycin will generate a membrane potential of −136 mV. Control samples (K^+ inside/K^+ outside) were prepared simply by dilution of the protein-free vesicles with 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4. Uptake of the lipophilic cations, TPP^+, doxorubicin, and propranolol (100 μM) was followed at room temperature. Four samples consisting of control vesicles (K^+ in/K^+ out) and vesicles exhibiting a potassium ion gradient (K^+ in/Na^+ out) in the presence or absence of valinomycin (0.5 μM) or valinomycin plus CCCP (10 μM) were examined.

Table I. Characterization of the Electrochemical Gradient Generated by Cytochrome *c* Oxidase Vesicles upon Ferrocytochrome *c* Oxidation and by Vesicles Exhibiting a Potassium Ion Diffusion Potential

Sample	Membrane potential ($\Delta\Psi$)		Proton gradient (ΔpH)			
	−Nigericin	+Nigericin	External pH	Internal pH	ΔpH	Internal pH + nigericin
Oxidase vesicles	−90 mV ± 4.5 mV ($n = 3$)	−140 mV ± 4.8 mV ($n = 5$)	7.40 7.01	8.10 ± 0.06 7.88 ± 0.06	0.70 ± 0.06 0.87 ± 0.06	7.31 ± 0.05 7.07 ± 0.04
Protein-free vesicles	−136 mV (applied)		7.40	< 5.80[a]	> 1.6[a]	

[a] In the presence of CCCP (10 μM).

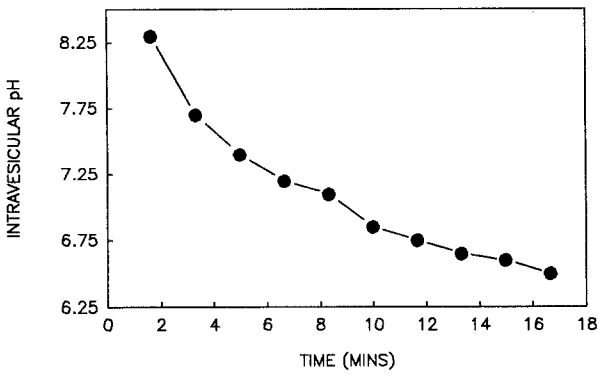

Fig. 2. Influence of a potassium ion diffusion potential on the intravesicular pH of protein-free vesicles. A membrane potential of −136 mV (interior negative) was generated across protein-free vesicles by diluting them into a potassium-free medium (150 mM sucrose, 67.5 mM Na_2SO_4, 7.5 mM HEPES pH 7.4) containing valinomycin (1 μM). The subsequent decrease in intravesicular pH was followed from the fluorescent behavior of entrapped pyranine (1 mM) as described under Materials and Methods.

Where present, the ionophores were added to a glass test tube in ethanol which was removed under a stream of nitrogen gas prior to addition of the vesicle suspension. Following incubation at room temperature for various times, aliquots were taken and vesicles separated from unentrapped lipophilic cation by centrifugation on 1 ml minicolumns as described above. The columns contained Sephadex G-50 (medium) hydrated in either 150 mM sucrose, 67.5 mM Na_2SO_4, and 7.5 mM HEPES, pH 7.4, or 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4, as appropriate. Phospholipid, doxorubicin, TPP^+, or propranolol were assayed in the eluted vesicles as described below.

Analytical Procedures

Phospholipid concentrations were determined either by phosphate analysis following perchloric acid digestion (Chen *et al.*, 1956) or using tracer levels of [^{3}H]-DPPC. Doxorubicin and propranolol were quantified by fluorimetry following solubilization of the vesicles in 30 mM octyl glucopyranoside. An SLM Amino SPF 500C instrument was used at excitation and emission wavelengths of 490 nm and 590 nm for doxorubicin and 292 nm and 337 nm for propranolol. TPP^+ was quantified by including trace levels of [^{14}C]-TPP^+ which was measured by liquid scintillation counting employing a Packard 2000 CA instrument. Vesicle sizes were determined by quasi-elastic light scattering on a Nicomp 270 submicron particle sizer (Nicomp Instruments, Goleta, California) operating at 632.8 nm and 5 mW as described previously (Madden *et al.*, 1988).

RESULTS

We have shown previously that when cytochrome oxidase is reconstituted at high lipid-to-protein ratios the resultant vesicles contain predominantly a single oxidase. Using DEAE chromatography, vesicles containing outwardly oriented protein can be separated from protein-free systems and from those where the cytochrome *c* binding site faces the vesicle interior (Madden *et al.*, 1984; Zhang *et al.*, 1985). We first sought, therefore, to determine the magnitude of the membrane potential and proton gradient established upon oxidation of ferrocytochrome *c* by vesicles containing outwardly oriented oxidase. In addition, the proton gradient generated by similar vesicles exhibiting a potassium ion diffusion potential was characterized.

A standard curve of DiS-C_3-(5) fluorescence quenching in response to protein-free vesicles exhibiting defined membrane potentials is shown in Fig. 1. From this standard curve the membrane potential generated by outwardly oriented oxidase vesicles in the presence and absence of nigericin can be determined (Fig. 1 and Table I) as originally described by Kita *et al.* (1982). The oxidation of ferrocytochrome *c* generated a membrane potential in the absence of nigericin of about −90 mV. This is in good agreement with values reported by Drachev *et al.* (1976), −100 mV; de Vrij *et al.* (1986), −100 mV; and Singh and Nicholls (1986), −90 mV. The ionophore nigericin promotes an exchange of protons for potassium ions resulting in a considerable increase in the measured membrane potential, to −140 mV, resulting from the dissipation of the proton gradient (Ramos *et al.*, 1976; Matsushita *et al.*, 1984). Using the fluorescent dye, pyranine, to measure intravesicular pH, the proton gradient generated by oxidase turnover can be determined. Measurements were made at external pH's of 7.40 and 7.01 (determined using a standard pH electrode) and indicated proton gradients of approximately 0.70 and 0.87 pH units (interior basic), respectively (Table I). As expected, the addition of nigericin abolished this gradient.

It has been shown previously that when a potassium ion diffusion potential is created across a

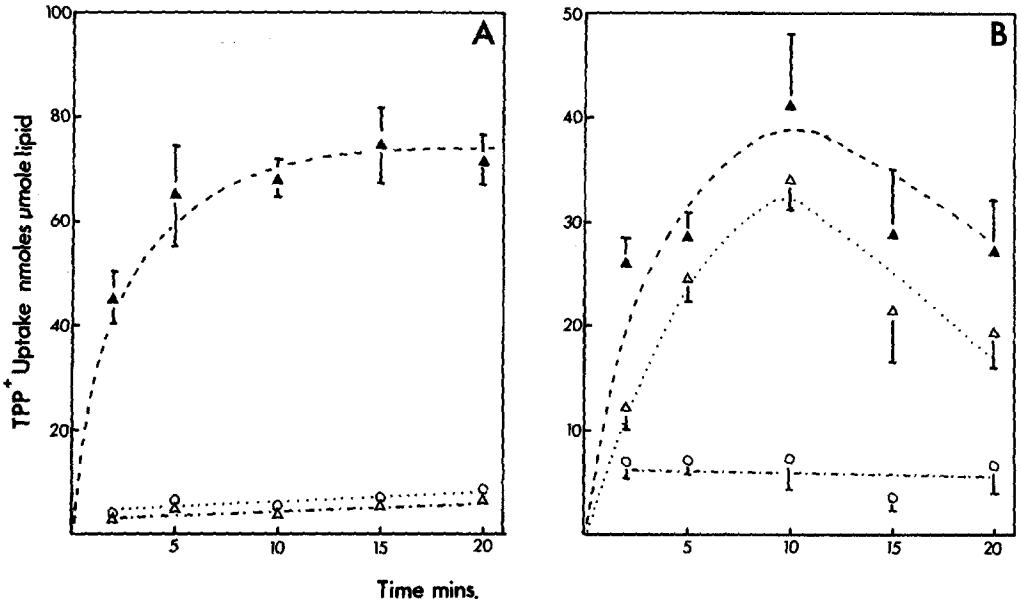

Fig. 3. Accumulation of tetraphenylphosphonium ion by reconstituted cytochrome *c* oxidase vesicles or vesicles exhibiting a potassium ion diffusion potential. A. The uptake of TPP^+ by protein-free vesicles containing 150 mM sucrose, 67.5 mM K_2SO_4, 7.5 mM HEPES, pH 7.4, diluted into potassium-free medium (150 mM sucrose, 67.5 mM Na_2SO_4, 7.5 mM HEPES, pH 7.4) in the presence, (▲) or absence, (○), of valinomycin (1 µM) was followed. Uptake by control vesicles diluted into 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, (△), is also shown. B. TPP^+ uptake was also followed into oxidase vesicles in the presence of cytochrome *c* and ascorbate. Reconstituted vesicles were incubated in the presence of no ionophores, (△), nigericin (25 nM) (▲), or valinomycin (1 µM), plus CCCP (20 µM) (○).

vesicle membrane (interior negative), protons migrate in response to this potential, creating a proton gradient (interior acid) (Bally *et al.*, 1988; Mayer *et al.*, 1988; Redelmeier *et al.*, 1989). To determine the rate of formation of this proton gradient and its magnitude in the systems used for this present study, the intravesicular pH was followed in pyranine-loaded oxidase-free vesicles following the establishment of a potassium ion diffusion potential. Surprisingly, we observed that prior to the establishment of a membrane potential the intravesicular pH is greater than 8.6 (upper limit for the pyranine assay) despite the external pH of 7.4. We have determined that this alkalinization occurs during the reconstitution process (T.E. Redelmeier, unpublished observations). It is likely that as sodium cholate is removed from the vesicles during dialysis, detergent originally present in the inner leaflet of the vesicle membrane migrates to the outer monolayer as the neutral protonated species. As cholate removal proceeds, protons are effectively lost from the intravesicular medium, resulting in the observed alkalinization. Following the establishment of a potential by the addition of valinomycin to vesicles diluted into a potassium-free medium, a slow acidification of the vesicle interior occurs such that after about 15 min the internal pH has dropped to about 6.5 (Fig. 2). This acidification is greatly accelerated by the addition of CCCP resulting in an interior pH of less than 5.8 (lower limit for the pyranine assay).

Before comparing the uptake of doxorubicin and propranolol into vesicle systems displaying similar membrane potentials but proton gradients of opposite polarity, the accumulation of tetraphenylphosphonium ion by such systems was examined. This lipophilic molecule carries a permanent positive charge and should accumulate inside vesicles in response to a membrane potential (interior negative) regardless of the presence of a proton gradient. As shown in Fig. 3, uptake is observed for both an oxidase-driven and potassium ion diffusion-driven potential. The transmembrane tetraphenylphosphonium ion concentration gradients generated (calculated based on a measured vesicle trapped volume of 1.5 µl/µmol phospholipid) indicate membrane potentials of −165 mV and −144 mV for the potassium ion diffusion- and

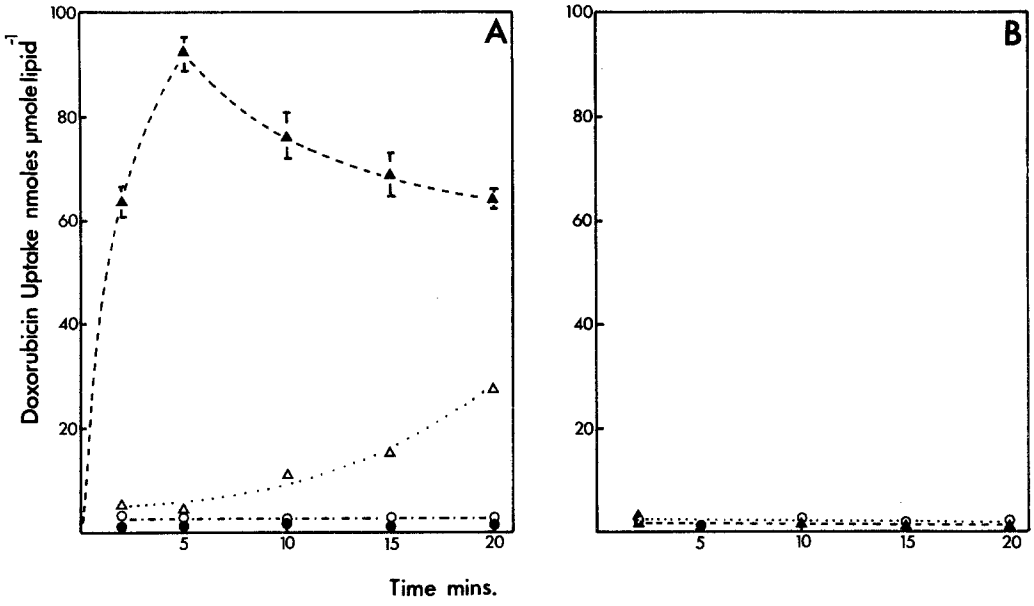

Fig. 4. Transmembrane distribution of doxoribuicin in response to vesicles exhibiting a potassium ion diffusion potential or reconstituted cytochrome *c* oxidase vesicles. A. Uptake of doxorubicin by protein-free vesicles containing 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4, diluted into potassium-free medium (150 mM sucrose, 67.5 mM Na_2SO_4, 7.5 mM HEPES, pH 7.4) and followed in the presence (△) or absence (○) of valinomycin (1 µM) or both valinomycin and CCCP (10 µM) (▲). Control vesicles were diluted into 150 mM sucrose, 67.5 mM K_2SO_4, 7.5 mM HEPES, pH 7.4 (●). B. Accumulation of doxorubicin by reconstituted oxidase vesicles in the presence of cytochrome *c* and ascorbate was examined in the presence of no ionophores (▲), nigericin (25 nM), (▲), or valinomycin (1 µM), or valinomycin (1 µM) plus CCCP (20 µM) (○).

oxidase-driven systems, respectively. These values indicate that redistribution in good agreement with theoretical expectations is observed even at relatively high concentrations of the permeant cation. As expected, lower levels of accumulation by the oxidase system are observed in the absence of nigericin. The oxidase system also exhibits some reduction in intravesicular TPP^+ concentration at the longer time points. This may reflect a decrease in enzyme activity over the time course examined. Under conditions where the electrochemical gradient generated by ferrocytochrome *c* oxidation is dissipated (valinomycin plus CCCP), only background binding of TPP^+ to the vesicles is observed.

In contrast to the rapid uptake of TPP^+ by both oxidase-driven and potassium ion diffusion driven systems, accumulation of lipophilic amines by the two systems is strikingly dissimilar. As shown in Fig. 4A, vesicles exhibiting a potassium ion diffusion potential rapidly take up doxorubicin in the presence of CCCP. In the absence of the proton ionophore, a lag period is seen prior to a much slower rate of drug accumulation. This behavior is consistent with doxorubicin redistributing in response to the induced proton gradient (interior acidic) rather than the membrane potential given the observation that intravesicular acidification proceeds relatively slowly in the absence of a proton ionophore (cf. Fig. 2). Further evidence to support this contention is provided by the observation that no doxorubicin accumulation occurs in oxidase containing vesicles upon ferrocytochrome *c* oxidation despite the fact that they display membrane potentials of comparable magnitude to the potassium ion diffusion system (Fig. 4B).

The uptake of a second lipophilic amine, propranolol, was also compared in the two vesicle systems. As in the case of doxorubicin, drug accumulation occurred rapidly into vesicles exhibiting a potassium ion diffusion potential in the presence of CCCP (Fig. 5A). For both propranolol and doxorubicin, the maximal level of uptake represents a theoretical intravesicular concentration of greater than 60 mM and an inside/outside drug gradient in excess of 1000 : 1. In the case of propranolol, however, uptake is not stable and virtually complete release occurs by 20 min. This suggests that the high intravesicular drug concentrations generated by

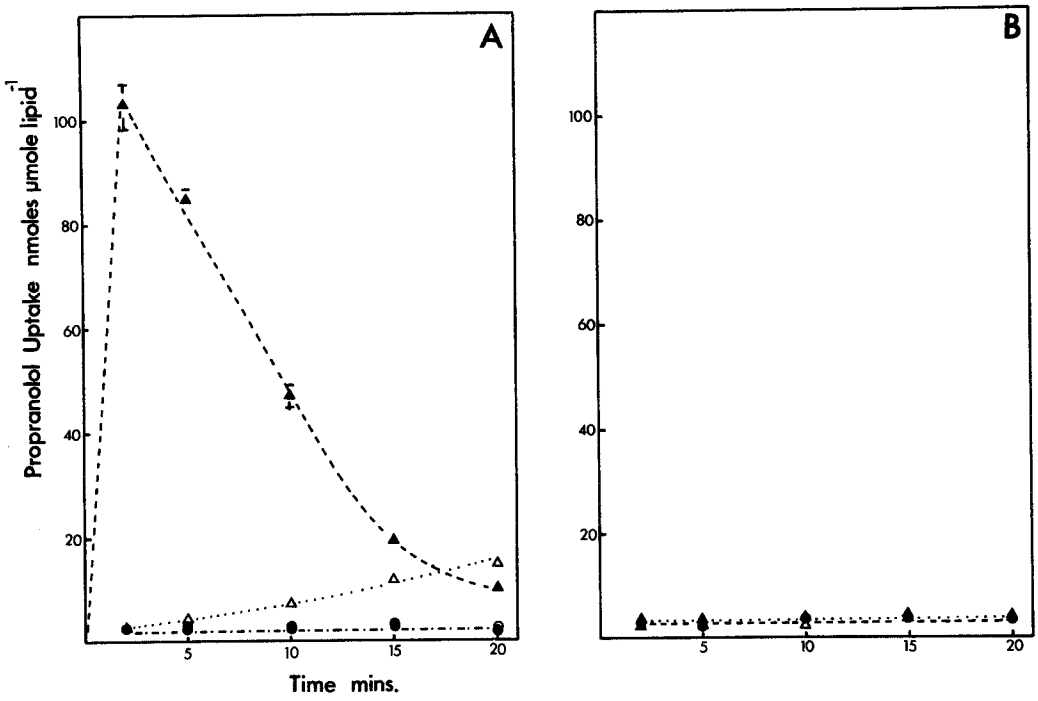

Fig. 5. Transmembrane distribution of propanolol in response to vesicles exhibiting a potassium ion diffusion potential or reconstituted cytochrome *c* oxidase vesicles. A. Uptake of propranolol by protein-free vesicles containing 150 mM sucrose, 67.5 mM K_2SO_4, and 7.5 mM HEPES, pH 7.4, diluted into potassium-free medium (150 mM sucrose, 67.5 mM Na_2SO_4, 7.5 mM HEPES, pH 7.4) in the presence (△) or absence (○) of valinomycin (1 μM) or valinomycin plus CCCP (10 μM) (▲). Control vesicles were diluted into 150 mM sucrose, 67.5 mM K_2SO_4 and 7.5 mM HEPES, pH 7.4 (●). B. Accumulation of propranolol by reconstituted oxidase vesicles in the presence of cytochrome *c* and ascorbate was examined in the presence of no ionophores (△), nigericin (25 nM), (▲), or valinomycin (1 μM), plus CCCP (20 μM) (○).

uptake increase membrane ion permeability, leading to dissipation of the induced proton gradient (Redelmeier *et al.*, 1989). Again, in the absence of a proton ionophore, this system shows slow propranolol uptake following a lag period. Again, as for doxorubicin, no uptake is observed by oxidase-containing vesicles upon ferrocytochrome *c* oxidation (Fig. 5B).

While earlier reports had suggested that cytochrome oxidase is strongly inhibited by doxorubicin (Goormaghtigh *et al.*, 1982), later work proposed that loss of enzyme activity occurs only as the result of peroxidative damage catalyzed by a complex of this anthracycline and ferric ions (Demont and Jensen, 1983; Demont, 1983). We therefore examined the influence of doxorubicin, propranolol, and TPP^+ on both the oxidase activity and coupling ratio of reconstituted vesicles. As shown in Table II, these lipophilic cations produce only a small inhibition of oxidase activity when assayed in the presence of valinomycin and CCCP. No significant influence on enzyme turnover was seen for the coupled system. The modest reduction in respiratory control arises, therefore, solely from this decrease in maximal enzyme activity. The inability of reconstituted oxidase vesicles to accumulate doxorubicin and propranolol, therefore, cannot be ascribed to a dissipation of the electrochemical gradient. These observations are consistent with earlier work by Garlid and Nakashima (1983) who examined the ability of various amine local anesthetics to uncouple mitochondria. Unlike classical protonophores such as CCCP, hydrophobic amines such as chlorpromazine, dibucaine, propranolol, and others were observed to produce uncoupling only in the presence of lipophilic anions such as thiocyanate and tetraphenylboron. These authors proposed that the protonated amine was membrane impermeable and that protonophoretic ability resulted from transmembrane cycling of neutral amine, charged lipophilic anion, and a neutral complex of the anion and protonated amine (Garlid and Nakashima, 1983).

Table II. Influence of Various Lipophilic Cations on Reconstituted Cytochrome Oxidase Vesicles

Sample	Inhibition of oxidase activity[a]	Respiratory control ratio
Control		3.85
DiS-C_3-(5) (100 nM)	0%	3.81
TPP^+ (0.1 mM)	6%	3.81
Propranolol (0.1 mM)	7%	3.61
Control[b]		5.37
Doxorubicin (0.1 mM)	5%	5.00

[a] The influence of the various lipophilic cations on cytochrome oxidase activity was determined in the presence of valinomycin (1 μM) plus CCCP (20 μM).
[b] The influence of doxorubicin was determined using a second vesicle preparation.

This hypothesis is consistent with our observations that doxorubicin and propranolol do not redistribute in response to a membrane potential which would require permeation of the charged protonated species.

DISCUSSION

The use of liposomes as delivery systems for therapeutic agents (for a review, see Ostro and Cullis, 1989) has led to the development of techniques to efficiently entrap pharmaceuticals into preformed vesicles. Earlier studies indicated that large concentration gradients and high intravesicular concentrations of chlorpromazine, vinblastine (Bally *et al.*, 1985), doxorubicin (Mayer *et al.*, 1985a), and dibucaine (Mayer *et al.*, 1985b) could be obtained in vesicles exhibiting a potassium ion diffusion potential (interior negative) as well as for systems exhibiting a proton gradient (interior acidic) (Nichols and Deamer, 1976; Mayer *et al.*, 1986). It is generally accepted that weak bases can redistribute across a membrane in response to a proton gradient via permeation of the neutral (deprotonated) species. As a result, drug redistribution can occur in response to a transmembrane pH gradient in accordance with the Henderson–Hasselbach equation. Where drug redistribution was observed in response to an applied potassium ion concentration gradient, however, this was suggested to reflect transmembrane migration of the charged (protonated) species. While it was recognized that an applied membrane potential (interior negative) resulted in proton flux into the vesicles, the measured pH gradient was considered too small to account for the drug concentration gradients achieved. Further, when the induced ΔpH was modulated by altering the intravesicular buffering capacity, no significant difference in drug uptake was observed (Bally *et al.*, 1988; Mayer *et al.*, 1988). This interpretation is at odds, however, with the observation that such amines do not act as classical uncouplers (Garlid and Nakashima, 1983). The present research was undertaken, therefore, to try and resolve this issue by comparing accumulation of lipophilic amines into vesicles exhibiting similar membrane potentials but proton gradients of opposite polarity.

Vesicles exhibiting a potassium ion diffusion potential accumulate doxorubicin and propranolol; however, this uptake appears to be dependent upon acidification of the vesicle interior. The proton ionophore, CCCP, induces a rapid reduction in intravesicular pH in this system while at the same time greatly accelerating the uptake of doxorubicin and propranolol. Further evidence that drug redistribution is in response to the induced proton gradient is provided by the oxidase-containing systems which show no drug accumulation despite exhibiting membrane potentials of equal magnitude to the potassium ion diffusion system. Taken together, these two observations provide compelling evidence for drug redistribution via the neutral species in response to a proton gradient only. We are still left, however, with the earlier observation that drug concentration gradients generated are greatly in excess of those predicted by the Henderson–Hasselbach equation. Two factors may contribute to this phenomenon. First, if the charged (protonated) drug partitions into the inner monolayer of the vesicle membrane, this will effectively lower the intravesicular free drug concentration, allowing further accumulation (Harrigan *et al.*, 1993). An alternative possibility concerns the observation that the theoretical intravesicular concentrations of many drugs following uptake exceeds their solubility limit (Madden *et al.*, 1990). This raises the possibility that precipitation

within the vesicle may occur, again lowering the concentration of free drug in solution and driving further accumulation. Depending upon the particular compound, either or both of these mechanisms may contribute to the observed excess accumulation over theoretical predictions.

Finally, it should be noted that while the present results indicate that doxorubicin and propranolol do not redistribute in response to a membrane potential, it would be premature to extrapolate these data to all other weak bases. The barrier to redistribution relates to insertion of the charged (protonated) species into the hydrophobic bilayer interior. It is conceivable, therefore, that compounds in which the amine function is shielded by hydrophobic regions may behave in a manner more analogous to membrane potential probes such as tetraphenylphosphonium ion.

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REFERENCES

Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kuliene, V. V., Levisky, D. O., Liberman, E. A., Severina, I. I., and Skulachev, V. P. (1970). *Biochim. Biophys. Acta* **216**, 13–22.

Bally, M. B., Hope, M. J., Van Echteld, C. J. A., and Cullis, P. R. (1985). *Biochim. Biophys. Acta* **812**, 66–76.

Bally, M. B., Mayer, L. D., Loughrey, H., Redelmeier, T., Madden, T. D., Wong, K., Harrigan, P. R., Hope, M. J., and Cullis, P. R. (1988). *Chem. Phys. Lipids* **47**, 97–107.

Cafiso, D. S., and Hubbell, W. L. (1978). *Biochemistry* **17**, 3871–3877.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956). *Anal. Chem.* **28**, 1756–1758.

Clement, N. R., and Gould, J. M. (1981). *Biochemistry* **20**, 1534–1538.

Crofts, A. R. (1967). *J. Biol. Chem.* **242**, 3352–3359.

Deamer, D. W., Prince, R. C., and Crofts, A. R. (1972). *Biochim. Biophys. Acta* **274**, 323–335.

Demont, E. J. F. (1983). *Eur. J. Biochem.* **137**, 113–118.

Demont, E. J. F., and Jensen, P. K. (1983). *Eur. J. Biochem.* **132**, 551–565.

De Vrij, W., Dreissen, A. J. M., Hellingwerf, K. J., and Konings, W. N. (1986). *Eur. J. Biochem.* **156**, 431–440.

Drachev, L. A., Jasaitis, A. A., Kaulen, A. D., Kondrashin, A. A., Chu, L. V., Semenov, A. Y., Severina, I. I., and Skulachev, V. P. (1976). *J. Biol. Chem.* **251**, 7072–7076.

Garlid, K. D., and Nakashima, R. A. (1983). *J. Biol. Chem.* **258**, 7974–7980.

Goormaghtigh, E., Brasseur, R., and Ruysschaert, J.-M. (1982). *Biochem. Biophys. Res. Commun.* **104**, 314–320.

Harrigan, P. R., Wong, K. F., Redelmeier, T. E., Wheeler, J. J., and Cullis, P. R. (1993). *Biochim. Biophys. Acta* **1149** 329–338.

Hind, G., and Whittingham, C. P. (1963). *Biochim. Biophys. Acta* **75**, 194–202.

Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985). *Biochim. Biophys. Acta* **812**, 55–65.

Kita, K., Kasahara, M., and Anraku, Y. (1982). *J. Biol. Chem.* **257**, 7933–7935.

Krogmann, D. W., Jagendorf, A. T., and Avron, M. (1959). *Plant Physiol.* **34**, 272–284.

Kuboyama, M., Yong, F. C., and King, T. E. (1972). *J. Biol. Chem.* **247**, 6365–6383.

Madden, T. D., and Cullis, P. R. (1984). *J. Biol. Chem.* **259**, 7655–7658.

Madden, T. D., Hope, M. J., and Cullis, P. R. (1984). *Biochemistry* **23**, 1413–1418.

Madden, T. D., Tilcock, C. P. S., Wong, K., and Cullis, P. R. (1988). *Biochemistry* **27**, 8724–8730.

Madden, T. D., Harrigan, P. R., Tai, L. C. L., Bally, M. B., Mayer, L. D., Redelmeier, T. E. Loughrey, H. C., Tilcock, C. P. S., Reinish, L. W., and Cullis, P. R. (1990). *Chem. Phys. Lipids* **53**, 37–46.

Matsushita, K., Patel, L., and Kaback, H. R. (1984). *Biochemistry* **23**, 4703–4713.

Mayer, L. D., Bally, M. B., Hope, M. J., and Cullis, P. R. (1985a). *Biochim. Biophys. Acta* **816**, 294–302.

Mayer, L. D., Bally, M. B., Hope, M. J., and Cullis, P. R. (1985b). *J. Biol. Chem.* **260**, 802–808.

Mayer, L. D., Bally, M. B., and Cullis, P. R. (1986). *Biochim. Biophys. Acta* **857**, 123–126.

Mayer, L. D., Wong, K. F., Menon, K., Chong, C., Harrigan, P. R., and Cullis, P. R. (1988). *Biochemistry* **27**, 2053–2060.

Nichols, J. W., and Deamer, D. W. (1976). *Biochim. Biophys. Acta* **455**, 269–271.

Ostro, M. J., and Cullis, P. R. (1989). *Am. J. Hosp. Pharm.* **46**, 1576–1587.

Pick, U. (1981). *Arch. Biochem. Biophys.* **212**, 186–19.

Ramos, S., Schuldiner, S., and Kaback, H. R. (1976). *Proc. Natl. Acad. Sci. USA* **73**, 1892–1896.

Redelmeier, T. E., Bally, M. B., Mayer, L., Wong, K., and Cullis, P. R. (1989). *Biophys. J.* **56**, 385–393.

Rottenberg, H., Grunwald, T., and Avron, M. (1971). *FEBS Lett.* **13**, 41–44.

Singh, A. P., and Nicholls, P. (1986). *Arch. Biochem. Biophys.* **245**, 436–445.

Singh, A. P., Chanady, G. A., and Nicholls, P. (1985). *J. Membr. Biol.* **84**, 183–190.

Zhang, Y.-Z., Capaldi, R. A., Cullis, P. R., and Madden, T. D. (1985). *Biochim. Biophys. Acta* **808**, 209–211.