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# Uptake of safranine and other lipophilic cations into model membrane systems in response to a membrane potential

M.B. Bally, M.J. Hope, C.J.A. Van Echteld and P.R. Cullis

*Department of Biochemistry, University of British Columbia, Vancouver, B.C., V6T 1W5 (Canada)*

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**Lipophilic cations such as safranine and methyltriphenylphosphonium ($MTPP^+$) are commonly employed to obtain measures of the membrane potential ($\Delta\psi$) exhibited by energized biological membrane systems. These probes reflect the presence of $\Delta\psi$ (inside negative) by accumulating in the interior of the membrane bound system to achieve transmembrane distributions dictated by the Nernst equation. In this work, we characterize the ability of model membrane large unilamellar vesicle systems to accumulate safranine and other biologically active lipophilic cations in response to a $K^+$ diffusion potential (interior negative) across the large unilamellar vesicle membrane. We show that safranine, $MTPP^+$, chlorpromazine and vinblastine can be rapidly accumulated to achieve interior lipophilic cation concentrations which may be more than two orders of magnitude higher than exterior concentrations. In the case of safranine, for example, incubation of 2 mM safranine with large unilamellar vesicle systems exhibiting a $\Delta\psi$ of $-100$ mV or more can lead to interior safranine concentrations in excess of 120 mM. This accumulation appears to proceed as an antiport $K^+$-safranine exchange process, and the optical 'safranine response' observed can be attributed to precipitation of the dye inside the vesicle as the interior concentrations of safranine exceeds its solubility (96 mM). These observations are discussed in terms of the utility of probes such as safranine and $MTPP^+$ for determinations of $\Delta\psi$ as well as their implications for the equilibrium transbilayer distributions of biologically active lipophilic cations in vivo.**

## Introduction

Membrane permeable lipophilic cations can be accumulated by cells or organelles exhibiting a membrane potential ($\Delta\psi$) and the subsequent determination of the interior and exterior concentrations of these agents can allow estimates of $\Delta\psi$ to be made. Examples of such probe molecules include those which exhibit optical and fluorescent responses (such as safranine O) when accumulated into the interior of the energized system, as well as radiolabelled probes (such as [$^3$H]methyltriphenylphosphonium, [$^3$H]$MTPP^+$). These agents have been utilized to determine $\Delta\psi$ in mitochondria and chloroplasts [1,2], vesicles derived from prokaryotic and eukaryotic membranes [3,4] as well as a variety of intact cells [5–7].

Our interest in this uptake of lipophilic cations was stimulated by two observations. First, the accumulation of these components by energized systems can result in substantial differences between the concentrations of the probes in the external and internal compartments. A membrane potential of $-100$ mV as detected by $MTPP^+$, for example, reflects an interior probe concentration which is 50-times higher than in the external en-

Abbreviation: $MTPP^+$, methyltriphenylphosphonium cation.

vironment. As the redistributions of the probes across the membranes can occur within minutes, it is clear that accumulation of lipophilic cations in response to $\Delta\psi$ reflects a rather effective membrane transport process. Second, a large variety of biologically active agents such as certain biological amines and many drugs are essentially lipophilic cations. Thus processes involved in the uptake of lipophilic cations employed as membrane potential probes may well be of more general significance to metabolite and drug distributions in vivo.

In this work we have characterized the ability of large unilamellar vesicle systems to accumulate selected lipophilic cations in response to a membrane potential, with three objectives in mind. First, we wished to show that the large changes in absorbance observed on accumulation of optical probes such as safranine in biological preparations can also be observed in model systems exhibiting a $\Delta\psi$, and to characterize the mechanisms involved. Second, as usually employed, probes of membrane potential are present at as low levels as possible to avoid perturbing the electrochemical gradient giving rise to $\Delta\psi$. From the transport point of view, it is of interest to determine whether higher exterior concentrations can lead to high absolute concentrations of probe accumulated in the vesicle interior. Finally, we wished to determine whether representative drugs can also be accumulated by vesicles exhibiting a membrane potential, which may provide information regarding the nonspecific uptake and distribution of these agents in vivo.

We show that the safranine optical response can be observed for egg phosphatidylcholine (egg PC) vesicle systems exhibiting a $K^+$ diffusion potential, and that the marked absorbance changes observed appear to correspond to precipitation of the dye in the vesicle interior. Further, both safranine and $MTPP^+$ can be concentrated to high levels ($> 75$ mM) in the vesicle interior for initial exterior concentrations in the range 1–2 mM. Finally, it is shown that a representative local anaesthetic (chlorpromazine) and an anticancer drug (vinblastine) can also be accumulated into model membrane vesicle systems in response to $\Delta\psi$.

## Materials and Methods

Egg phosphatidylcholine (egg PC) and soya PC were isolated from hen egg yolk and crude soya lecithin (Sigma), respectively, employing established procedures. Egg phosphatidylserine (egg PS) was obtained from egg PC utilizing the headgroup exchange capacity of phospholipase D [8]. Cholesterol, MTPPBr, chlorpromazine, vinblastine, valinomycin and Hepes buffer were obtained from Sigma. Safranine O was obtained from MCB whereas methoxy[$^{14}$C]inulin and [$^3$H]-MTPPI were obtained from NEN and $^{42}$KCl from Amersham. For ease of reference, the structures of safranine, $MTPP^+$, chlorpromazine and vinblastine are given in Fig. 1. All lipids employed were more than 99% pure as determined by thin-layer chromatography, and all other reagents were employed without further purification.

The potassium glutamate and NaCl buffers contained 20 mM Hepes adjusted to pH 7.5 employing NaOH (final $Na^+$ concentration 10 mM) and were adjusted to a common osmolarity of 310 mosM/kg (NaCl concentration 150 mM, potassium glutamate concentration 169 mM). Vesicles were prepared according to the LUVET procedure detailed in the preceding paper [9] which involved

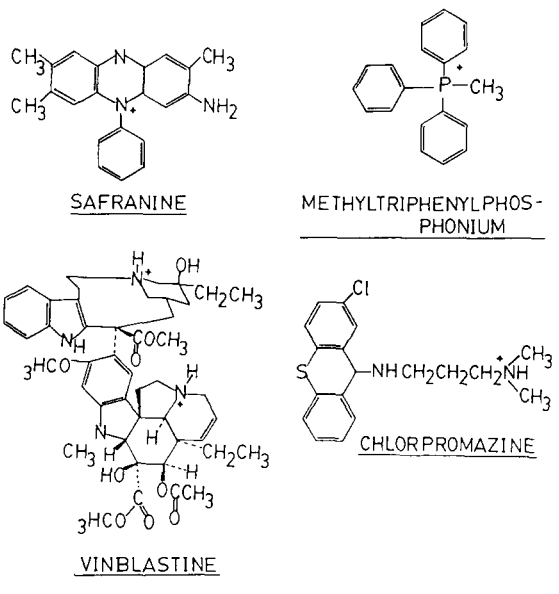


Fig. 1. Structures of safranine, methyltriphenylphosphonium ($MTPP^+$), chlorpromazine and vinblastine.

hydration of a dry lipid film to produce large multilamellar vesicles (50–100 μmol phospholipid/ml) which were subsequently extruded ten times through two (stacked) polycarbonate filters with 100 nm pore size (Nuclepore). $^{31}$P-NMR studies on the resulting vesicles showed that 50 ± 2% of the phospholipid was available to $Mn^{2+}$ (see Ref. 9) indicating a largely unilamellar nature. These LUVET's had an average diameter of 70 nm as determined by freeze-fracture and negative staining and exhibited trapped volumes in the range 1.0–1.2 μl/μmol phospholipid as determined by trapping [$^{14}$C]inulin or $^{22}$Na [9].

Transmembrane $Na^+$-$K^+$ chemical gradients ($K^+$ inside) were generated by forming LUVET's in the potassium glutamate buffer and subsequently exchanging the untrapped potassium glutamate for NaCl employing a Sephadex G-50 column. Defined $K^+$ gradients were generated by pre-equilibrating the G-50 columns with isoosmotic NaCl buffers containing the appropriate concentration of potassium glutamate. Where employed, the $K^+$ ionophore valinomycin was added to achieve a concentration of 0.5 μg/μmol lipid.

The uptake of safranine was (qualitatively) monitored spectrophotometrically over the interval 460–560 nm employing a Pye Unicam SP8-200 spectrophotometer (see Fig. 2 and related text). The actual amount of safranine accumulated was determined quantitatively by adding safranine from a saturated (96.8 mM at 20°C) stock solution to a LUVET dispersion (2–10 μmol lipid/ml) to achieve a 2 mM safranine concentration. Subsequently, at appropriate time intervals, the unsequestered dye was removed by passing aliquots of the vesicles through small (1 ml) Sephadex G-50 columns, and aliquots of the effluent were mixed with 0.5% (wt/vol. $H_2O$) Triton X-100 to disrupt the vesicles and release sequestered safranine. Safranine concentrations were then determined from the absorbance at 516 nm, and the phospholipid phosphorus assayed by standard techniques.

Membrane potentials were determined employing [$^3$H]MTPP$^+$ and the efflux of $K^+$ measured employing $^{42}$K in combination with the ultrafiltration technique described in the preceding study [9].

Uptake of chlorpromazine and vinblastine was determined quantitatively employing similar procedures as for safranine. Accumulation of chlorpromazine was monitored in a system containing 200 μM chlorpromazine (containing 2 μCi/ml [$^3$H]chlorpromazine) which was added to egg PC LUVET's (1 μmol phospholipid/ml). After various incubation times, the vesicles were separated from unsequestered chlorpromazine (employing the 1 ml Sephadex G-50 columns). The effluent was counted to assay for chlorpromazine and assayed for phospholipid employing the assay of Ames et al. [10]. In the case of vinblastine, egg PC LUVET's (10 μmol phospholipid/ml) were incubated in the presence of 1 mM vinblastine, the vesicles separated on the G-50 column and LUVET-associated vinblastine assayed (after disruption with 0.5% Triton X-100) at 265 nm.

## Results

Large absorbance changes can occur when safranine is incubated in the presence of energized biological systems such as mitochondria [11] and vesicles derived from *E. coli* membranes [3]. Such a 'safranine response' has been correlated with the presence of a membrane potential $\Delta\psi$. This response can also be observed for egg PC LUVET systems experiencing a $K^+$ diffusion potential induced by valinomycin as illustrated in Fig. 2. The addition of valinomycin to LUVET's prepared with asymmetric $Na^+$-$K^+$ transmembrane distributions ($K^+$ inside) and incubated in the presence of safranine results in a marked time dependent decrease in absorption in the region of 516 nm, which is combined with a shift in the absorbance maximum ($\lambda_{max}$) from 516 to 472 nm. The change in the absorbance at 516 nm ($\Delta A_{516}$) is essentially complete after 20 min and the situation is then relatively stable as $\Delta A_{516}$ decreases by less than 20% over a 24 h time-course (results not shown).

As may be expected, the extent of the absorbance changes associated with this safranine response were found to be sensitive to the vesicle and safranine concentrations employed. In order to determine optimum conditions, the (normalized) absorbance changes were monitored as a function of safranine concentration for a fixed vesicle concentration corresponding to 0.5 mM phospholipid. As indicated in Fig. 3, the normal-

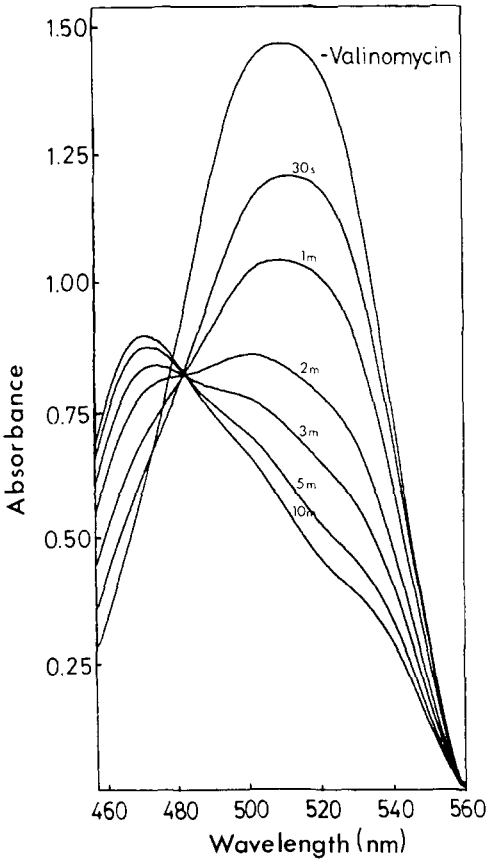


Fig. 2. Spectrophotometric response observed for safranine incubated at 20°C in the presence of egg PC LUVET's exhibiting an electrochemical $Na^+/K^+$ gradient in the presence of valinomycin. The LUVET's were prepared in the potassium glutamate buffer, and the untrapped buffer exchanged for the NaCl buffer as described in Materials and Methods. Subsequently the vesicles were diluted to achieve a concentration of 0.5 mM phospholipid in 3 ml of the NaCl buffer which contained 60 $\mu$M safranine. Spectra were taken before (upper trace) and at various times after the addition of valinomycin (0.5 $\mu$g/$\mu$mol phospholipid). s, seconds; m, minutes.

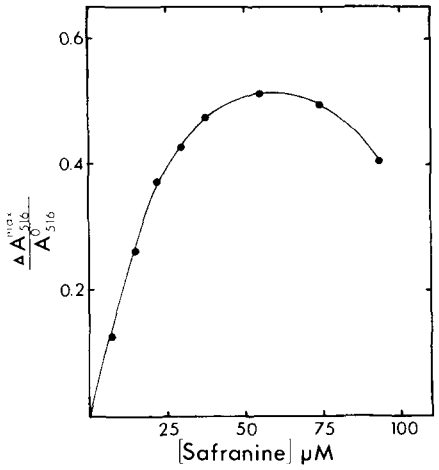


Fig. 3. Influence of increasing safranine concentrations on the (normalized) safranine response obtained in the presence of egg PC LUVET systems (0.5 mM phospholipid). The LUVET systems were prepared with $Na^+/K^+$ electrochemical gradients as indicated in the legend to Fig. 2 (and Materials and Methods) and the normalized safranine response ($\Delta A_{516}^{max}/A_{516}^{0}$) is measured as the difference between the initial absorbance ($A_{516}^{0}$) at 516 nm (before the addition of valinomycin) and the absorbance observed 20 min after the addition of valinomycin, divided by $A_{516}^{0}$.

ized optical response then exhibits a maximum in the region of 60 $\mu$M safranine. Other investigators (for review, see ref. 12) have suggested that the safranine response arises from active uptake of safranine followed by a membrane associated 'stacking' phenomenon, which gives rise to the observed absorbance changes. This process (which, as indicated below, appears to correspond to precipitation of safranine in the vesicle interior) could explain the behaviour noted in Fig. 3. At low safranine concentrations the dye concentrations achieved in the vesicle interior may not be sufficient to result in precipitation or 'stacking', whereas at higher exterior safranine levels, the spectral response for accumulated material will be swamped by the absorption from excess exterior safranine.

The results of Figs. 2 and 3 are consistent with an accumulation of safranine into the egg EPC LUVET system which is driven by a $K^+$ diffusion potential. It is of interest to quantify the extent of safranine uptake in response to a given $\Delta\psi$. Two situations were investigated; first, where the safranine was in excess so that the maximum levels of accumulated safranine could be characterized, and second, where the safranine was limiting, therefore allowing a determination of the efficiency of the trapping process. Briefly, LUVET's were prepared with varying transbilayer $K^+$ concentration gradients which give rise to corresponding variations in the $\Delta\psi$ obtained on addition of valinomycin (see Fig. 10). These vesicles were incubated for 30 min in the presence of valinomycin and 0.05 $\mu$mol safranine per $\mu$mol

phospholipid (safranine limiting) or 0.2 μmol safranine per μmol phospholipid (safranine in excess). The untrapped safranine was then removed by passing the vesicles over a Sephadex G-50 column (see Methods). Determination of the vesicle-associated safranine per μmol phospholipid resulted in the data shown in Fig. 4, which leads to two conclusions. First, when the amount of safranine is limiting, vesicle-associated safranine reaches levels in excess of 0.04 μmol safranine per μmol phospholipid for $|\Delta\psi| > 80$ mV indicating trapping efficiencies in excess of 80%. Second, when safranine is in excess, extremely high levels of accumulated safranine can be achieved (> 0.12 μmol safranine per μmol phospholipid). Given the measured trapped volumes (1.15 μl/μmol phospholipid) of these LUVET systems, this indicates interior safranine concentrations of 100 mM or more. As a saturated solution of safranine at 20°C is 96.2 mM, this suggests that the marked changes in safranine absorption result from precipitation of the dye in the vesicle interior. In order to determine whether this may be the case, the safranine response ($\Delta A_{516}^{max}$) was measured for LUVETS with various transmembrane $K^+$ gradients (as for Fig. 4, 0.2 μmol safranine/μmol phospholipid) allowing a correlation to be obtained between $\Delta A_{516}^{max}$ and the amount of safranine accumulated. As indicated in Fig. 5A, a dramatic increase in $\Delta A_{516}^{max}$ occurs for amounts of vesicle-associated safranine corresponding to interior concentrations of approx. 100 mM or more. This is clearly consistent with the proposal that the safranine response reflects a precipitation of

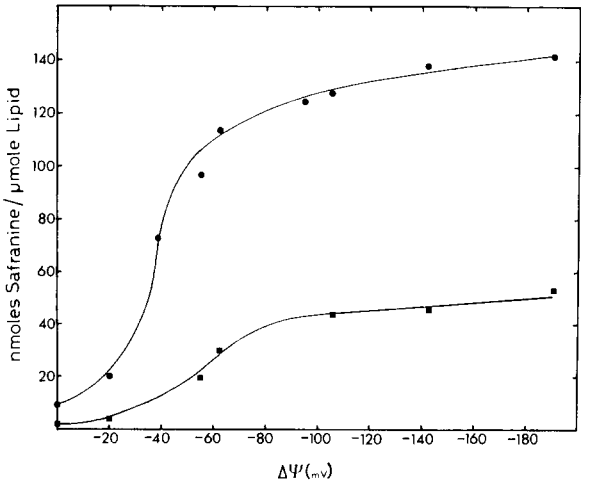


Fig. 4. Levels of LUVET associated safranine obtained as a function of (initial) transmembrane potential $\Delta\psi$. The vesicles were prepared from egg PC dispersed in the potassium glutamate buffer, and the external medium was replaced by an NaCl buffer containing various concentrations of potassium glutamate (see Methods) to establish a range of $K^+$ gradients. The membrane potential $\Delta\psi$ developed in the presence of valinomycin was assayed employing [$^3$H]MTPP$^+$ (see Materials and Methods and Ref. 9). Subsequently, the amount of safranine accumulated by the LUVET's 30 min after incubation in 0.2 μmol safranine/μmol lipid (●) and 0.05 μmol safranine/μmol lipid (■) in the presence of valinomycin was monitored as described in Materials and Methods. In the case of the system containing 0.2 μmol safranine per μmol lipid the safranine was in excess (not all of it could be accumulated by the LUVET systems) whereas at 0.05 μmol safranine per μmol phospholipid the amount of safranine available limited the uptake.

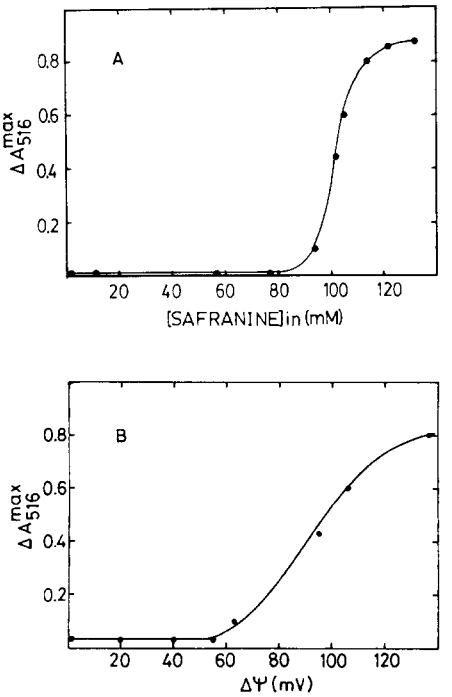


Fig. 5. Magnitude of the maximum safranine response ($\Delta A_{516}^{max}$) as: (A) a function of the internal concentration of safranine inside egg PC LUVET's and (B) as a function of the membrane potential measured from a known $K^+$ diffusion potential (see Fig. 10). The system of Fig. 4 containing 0.2 μmol safranine per μmol phospholipid was employed in both cases and the $\Delta A_{516}^{max}$ determined (see text) 30 min after addition of valinomycin. The safranine concentration inside the vesicles was calculated from the results of Fig. 4 employing a measured trapped volume of 1.15 μl/μmol phospholipid.

safranine inside the vesicles, and results in a non-linear relation between $\Delta A_{516}$ and $\Delta\psi$ (Fig. 5B).

It is useful to characterize the stability of the vesicle systems containing high levels of safranine. A LUVET preparation ($K^+$ inside) was incubated in the presence of safranine (0.2 $\mu$mol safranine/$\mu$mol phospholipid) and valinomycin, and the amount of vesicle associated safranine determined at various time intervals. As shown in Fig. 6, the safranine accumulated in the presence of valinomycin reaches a plateau after about 2 h and remains constant at 0.14 $\mu$mol safranine per $\mu$mol phospholipid for 8 h or more. It is interesting to note that significant safranine uptake is achieved, albeit at a much slower rate, in the absence of valinomycin. Accumulation of lipophilic cations such as safranine likely proceeds in exchange for an efflux of $K^+$ [13], and thus the slow valinomycin-independent uptake may arise in response to passive efflux of $K^+$.

To further characterize the relation between safranine uptake and $K^+$ efflux, the release of $K^+$ from vesicles on uptake of safranine was monitored employing the radioisotope $^{42}K$. As illustrated in Fig. 7, limited release of $K^+$ is observed on addition of valinomycin which arises in response to valinomycin-facilitated influx of $Na^+$ [9]. The subsequent addition of safranine results in a rapid release of the remaining entrapped $K^+$, and the time course of this release is similar to that observed for safranine uptake (Fig. 6). These observations are consistent with a safranine uptake mechanism which involves an electroneutral safranine-$K^+$ exchange process.

The results presented to this stage establish that egg PC LUVET's exhibiting a $K^+$ diffusion potential accumulate the lipophilic cation safranine in a manner which is consistent with a safranine-$K^+$ transmembrane exchange mechanism. In order for this to occur the safranine and the $K^+$-valinomycin complex must traverse the hydrocarbon region, suggesting that such transport should be sensitive to the acyl chain composition and 'order' in the acyl chain region. That this is the case is illustrated in Fig. 8 for LUVET's composed of soya PC and egg PC in the presence of varying amounts of cholesterol. Uptake into the relatively unsaturated soya PC system is faster than for the more saturated egg PC LUVET system (for a comparison of egg

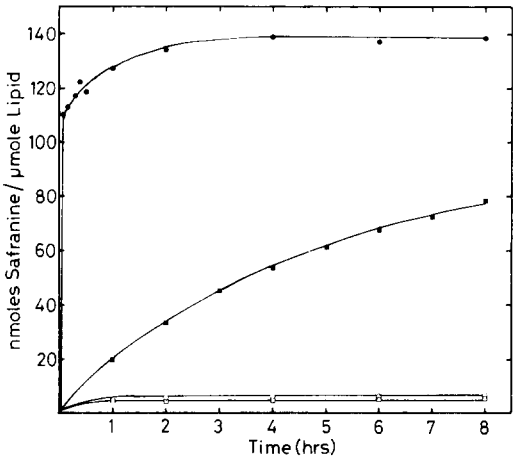


Fig. 6. Time-course for accumulation of safranine by egg PC LUVET systems experiencing a $Na^+/K^+$ transmembrane electrochemical gradient (prepared as described in the legend of Fig. 2) in the presence (●) and absence (■) of valinomycin (0.5 $\mu$g/$\mu$mol phospholipid). The safranine taken up into the vesicles was determined by removing untrapped safranine on a gel filtration column (see Materials and Methods). The open symbols indicate background uptake in the absence of an electrochemical gradient (potassium glutamate buffer on both sides of the membrane) and in the presence (○) and absence (□) of valinomycin.

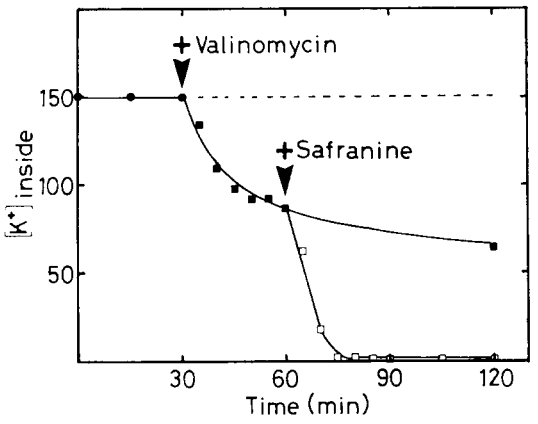


Fig. 7. Demonstration of $K^+$ release from egg PC LUVET's on addition on safranine. The LUVET's were prepared in the presence of $^{42}KCl$ where the untrapped buffer was replaced by the NaCl buffer, and were subsequently incubated in an Amicon ultrafiltration cell in the presence of valinomycin as indicated in Materials and Methods. The $^{42}K^+$ released from the vesicles was determined in the filtered eluate from the ultrafiltration cell by scintillation counting. This eluate did not contain measurable levels of phospholipid.

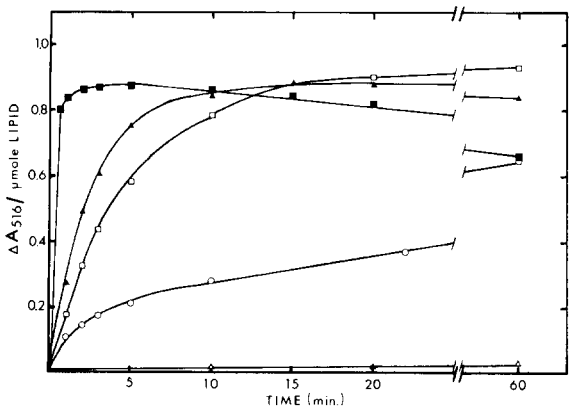


Fig. 8. Influence of lipid composition on the safranine response ($\Delta A_{516}^{max}$) measured as the difference in absorbance at 516 nm between the spectrum obtained at zero time and the spectrum obtained at various times after addition of valinomycin. All vesicle systems were prepared and spectra recorded under conditions similar to those indicated for Fig. 2. The various LUVET systems employed were composed of egg PC (▲); soya SPC (■); egg PC containing 10 mol% cholesterol (□), 25 mol% cholesterol (○) and 50 mol% cholesterol (△).

PC and soya PC acyl chain composition, see Ref. 14) and the additional presence of cholesterol results in a progressive inhibition of safranine accumulation. At equimolar cholesterol levels little or no uptake occurs within 1 h. This inhibition could result from a decreased permeability of safranine or a reduced effectiveness of the $K^+$ ionophore in less fluid membranes.

*Uptake of methyltriphenylphosphonium ($MTPP^+$)*

The results obtained for safranine uptake into model membrane systems exhibiting a $K^+$ diffusion potential show that safranine can be efficiently accumulated to high levels into the vesicle interior. It is important to determine how general these findings are, and we have therefore characterized the $\Delta\psi$-dependent uptake of a variety of other lipophilic cations. We chose initially to investigate $MTPP^+$ because transmembrane redistributions of this agent in radiolabelled form ([$^3$H]$MTPP^+$) can be detected for very low concentrations ($2 \cdot 10^{-8}$ M). This leads to minimal perturbations of the electrochemical gradients giving rise to $\Delta\psi$, and has therefore been utilized to obtain quantitative measures of $\Delta\psi$ in energized membrane systems [3,15].

The uptake of [$^3$H]$MTPP^+$ into egg PC LUVET systems in response to a valinomycin-induced $K^+$ diffusion potential, employing an (initial) exterior concentration of $2 \cdot 10^{-8}$ M [$^3$H]$MTPP^+$ is illustrated in Fig. 9. Equilibrium is achieved after approx. 2 h. It is interesting to note that a slower valinomycin independent uptake of $MTPP^+$ also occurs, presumably by a mechanism involving passive $K^+$ efflux similar to the valinomycin-independent accumulation of safranine (Fig. 6). Again, the uptake process is markedly sensitive to lipid composition and is inhibited by equimolar levels of cholesterol (results not shown).

The [$^3$H]$MTPP^+$ can be used to accurately determine the valinomycin induced membrane potential exhibited by the egg PC LUVET systems, as illustrated by the reasonably close agreement between the value of $\Delta\psi$ calculated on the basis of known trans-membrane $K^+$ distributions and the transmembrane distributions of [$^3$H]$MTPP^+$ (Fig. 10). The error at high absolute values of $\Delta\psi$ ($|\Delta\psi| > 100$ mV) likely arises from a limited release of entrapped [$^3$H]$MTPP^+$ during

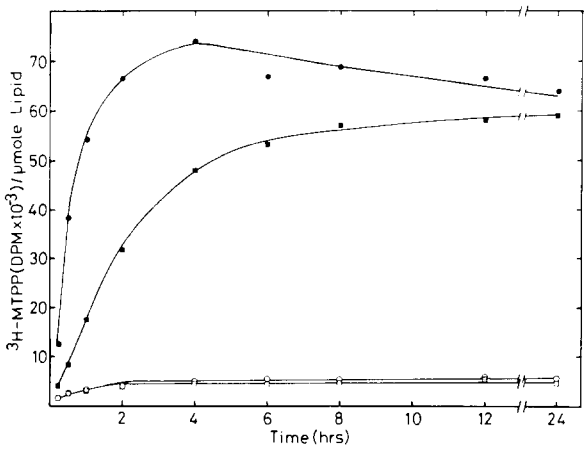


Fig. 9. Time-course of the accumulation of $^3$H-labelled methyltriphenylphosphonium ([$^3$H]$MTPP^+$) by egg PC LUVET's prepared as indicated in Materials and Methods with potassium glutamate in and NaCl out. The [$^3$H]$MTPP^+$ (50 Ci/mmol) was added to achieve a concentration of $2 \cdot 10^{-8}$ M (1 μCi/ml) of $MTPP^+$ in a dispersion of egg PC LUVET's (10 μmol phospholipid/ml). The accumulation of labelled $MTPP^+$ was monitored in the presence (●) and absence (■) of valinomycin as described in Materials and Methods. The open symbols indicate the uptake in the absence of an electrochemical $Na^+/K^+$ gradient.

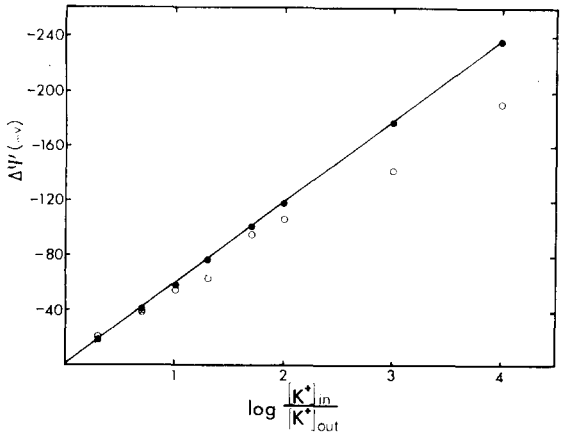


Fig. 10. Comparison between the membrane potentials obtained for various transmembrane $K^+$ chemical gradients as detected by [$^3$H]MTPP$^+$ (○) and the theoretical potentials (●) predicted by the Nernst equation. Egg PC LUVET's were prepared in the potassium glutamate buffer, and the untrapped (exterior) buffer replaced by an isoosmotic NaCl buffer containing various amounts of potassium glutamate. The membrane potential $\Delta\psi$ was determined employing [$^3$H]MTPP$^+$ (see Materials and Methods) in the presence of valinomycin after a 6 h incubation at 20°C to ensure an equilibrium transmembrane distribution of MTPP$^+$. The solid line indicates the theoretical potential given by the Nernst equation: $\Delta\psi = -59 \log([K]_i/[K]_o)$.

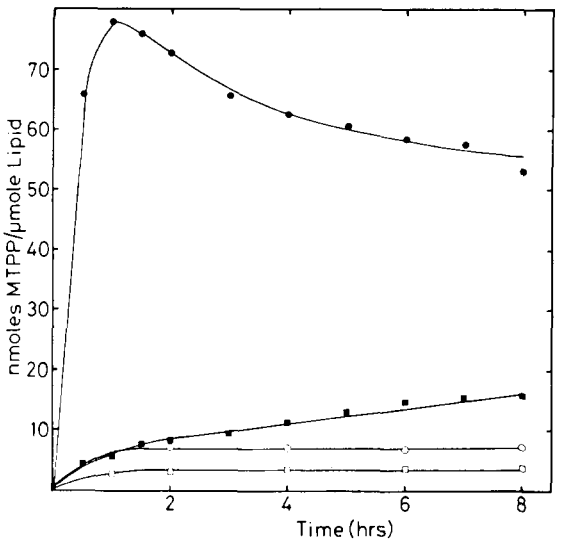


Fig. 11. Time-course of the accumulation of methyltriphenylphosphonium (MTPP$^+$) into egg PC LUVET's under the conditions of Fig. 9 but where the initial exterior concentration of MTPP$^+$ was 2 mM. The labelled [$^3$H]MTPP$^+$ was diluted into unlabelled MTPP$^+$ to achieve radioisotope levels corresponding to 1 μCi/ml. The uptake was determined in the presence (●) and absence (■) of valinomycin. The open symbols indicate the uptake obtained in the absence of an $Na^+/K^+$ electrochemical gradient.

vesicle isolation on the Sephadex G-50 columns.

These MTPP$^+$ uptake studies were extended to determine the levels of accumulated MTPP$^+$ obtained for higher (initial) external MTPP$^+$ concentrations to ascertain whether massive uptake (similar to that observed for safranine) could be achieved. As shown in Fig. 11, the presence of 2 mM external MTPP$^+$ results in valinomycin-induced accumulation of MTPP$^+$ to levels which correspond to concentrations in the vesicle interior which approach 75 mM.

*Uptake of chlorpromazine and vinblastine*

It is of interest to extend these uptake studies to include lipophilic cations with acknowledged biological activities. A large proportion of commonly employed drugs are lipophilic cations. This is presumably because most drugs must traverse the plasma membrane of cells in order to exert their biological effects, and in the absence of a specific transport protein this will be facilitated if the drug has lipophilic and cationic characteristics. In particular, it is possible that the membrane potential could then encourage drug accumulation in a manner similar to that observed here for safranine and MTPP$^+$.

The two representative drugs studied were chlorpromazine (a local anaesthetic with application in treatment of schizophrenia) and vinblastine (an anticancer drug). Structures are given in Fig. 1. Both of these compounds could be accumulated into LUVET systems displaying a $K^+$ diffusion potential as illustrated in Fig. 12. It is interesting to note that valinomycin was not essential for chlorpromazine uptake, which is consistent with previous observations that chlorpromazine increases the $K^+$ permeability of membranes [16]. The accumulated levels of chlorpromazine correspond to interior concentrations of up to 80 mM. In the case of vinblastine (Fig. 12B), the maximum accumulated levels were somewhat lower (30 mM) and such uptake could again proceed in the absence of valinomycin.

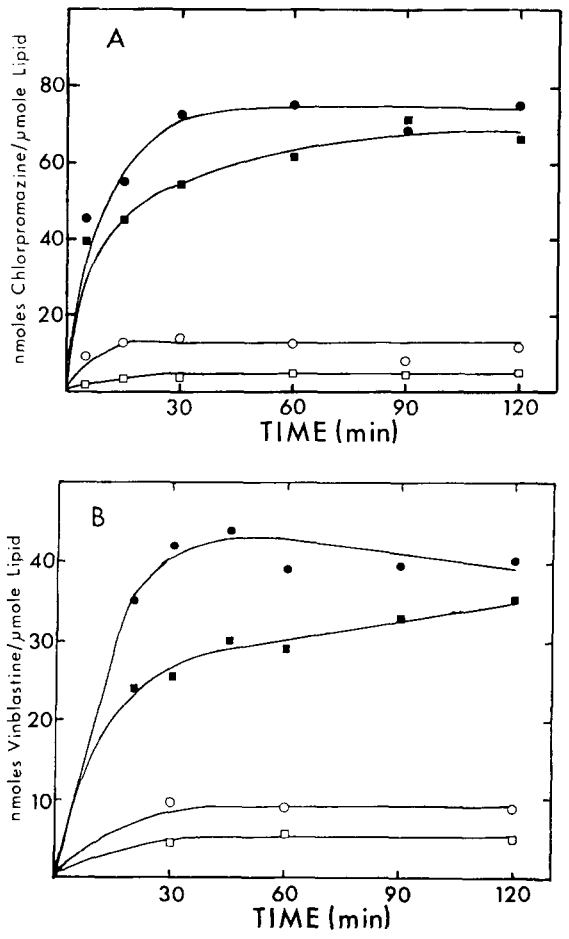


Fig. 12. Time-course of the accumulation of (A) chlorpromazine and (B) vinblastine into egg PC LUVET's experiencing a $Na^+/K^+$ electrochemical gradient. The chlorpromazine uptake was determined for LUVET's (1 μmol phospholipid/ml) incubated in the presence of 200 μM chlorpromazine (2 μCi/ml [$^3$H]chlorpromazine) and the vesicle-associated drug determined subsequently as indicated in Materials and Methods. The vinblastine uptake was determined for LUVET's (10 μmol phospholipid/ml) incubated with 1 mM vinblastine sulphate and the vesicle-associated vinblastine determined by removal of untrapped material and subsequent assay at 265 nm (see Materials and Methods). Both uptake experiments were conducted in the presence (●) and absence (■) of valinomycin (0.5 μg/μmol phospholipid). The corresponding open symbols indicate uptake observed in the absence of a $Na^+/K^+$ chemical gradient.

## Discussion

The results presented here provide new information on the mechanisms of action of indicators of membrane potential such as safranine and have important implications for the transmembrane distributions of lipophilic cationic molecules in model and biological systems. We discuss these two areas in turn.

The optical response of safranine has been employed to estimate $\Delta\psi$ in mitochondrial preparations [11–13]. These studies indicate that safranine is accumulated by an energy dependent mechanism and that the spectral changes occur as a result of a 'stacking' of the dye on the inner surface of the membrane. This stacking was proposed to involve an association with negatively charged lipids in the inner monolayer. The results presented here are consistent with the optical response of safranine to $\Delta\psi$ arising from stacking or precipitation of the dye, which may occur at the inner monolayer/water interface. However, it is clear that negatively charged lipids are not required. The limitations of the optical response of safranine as a quantitative indicator of the membrane potential are equally clear due to the inherent nonlinearity of the stacking or precipitation events giving rise to this response, which give rise to nonlinear absorbance changes as $\Delta\psi$ is increased.

The mechanism whereby safranine is accumulated by the LUV systems is of particular interest. The results presented here are consistent with an electroneutral 'antiport' $K^+$-safranine transmembrane exchange mechanism, as indicated by release of entrapped $K^+$ on safranine accumulation as well as the fact that the maximum levels of safranine accumulated are comparable to the initial levels of trapped $K^+$ ($\geq$ 100 mM as compared to 150 mM). Such a proposal is not new [13] - indeed, it is difficult to imagine any other process that could drive safranine uptake in the relatively simple model systems investigated here. The important points are that the lipophilic cation appears to 'flip' across the bilayer in a charged form in response to the $\Delta\psi$ dependent electric field gradient, and that extremely high levels of internalized safranine can be achieved. This clearly reflects a rather efficient and effective transport process which, as indicated below, may be of general significance for the distributions of lipophilic cations in vivo.

In the case of $MTPP^+$, the ability to employ

low concentrations of the radiolabelled form allows reasonable correlations to be obtained between $\Delta\psi$ calculated on the basis of experimentally determined interior and exterior concentrations of [$^3$H]MTPP$^+$ and the actual $K^+$ diffusion potential. Alternatively, higher (2 mM) initial exterior levels of MTPP$^+$ lead to accumulation of high ($\geq$ 75 mM) internal concentrations of MTPP$^+$, behaviour which corresponds to that observed for safranine.

Before discussing the similar uptake characteristics of the biologically active lipophilic cations investigated here, it is of interest to note problems that may be involved in obtaining accurate measures of $\Delta\psi$ derived from equilibrium transmembrane redistributions of lipophilic cationic probe molecules such as MTPP$^+$ or safranine. A particular difficulty concerns the length of time for equilibrium redistributions to occur, which may be on the order of hours. Further, in less fluid membranes, such as those containing cholesterol, these redistributions may be sufficiently slow as to preclude achievement of equilibrium in a reasonable time frame (see Fig. 8). This may reflect reduced partitioning of the probe molecule into the membrane or inhibition of the transmembrane 'flip' process in response to the electrochemical gradient. In any event, a requirement for an extended time course to determine whether probe uptake has achieved equilibrium is apparent, particularly for systems such as plasma membranes containing high levels of cholesterol. Gross underestimates of $\Delta\psi$ may otherwise result.

The observation that lipophilic cationic drugs such as chlorpromazine and vinblastine can be accumulated to high levels within large unilamellar vesicle systems exhibiting a membrane potential has far reaching implications in four areas. First, chlorpromazine is a local anaesthetic. A fundamental problem in understanding the mechanism whereby local anaesthetics induce their effects has been that clinical concentrations of anaesthetics have little influence on the physical properties of lipid systems, even though available evidence suggests that these agents exert their effects via the lipid component of membranes [17]. The results presented here suggest that the presence of a membrane potential could lead to local anaesthetic concentrations on the interior of a nerve membrane, for example, which are more than two orders of magnitude higher than plasma concentrations. Similar considerations apply to uptake of naturally occurring lipophilic cations, such as biological amines, in vivo. It has, for example, already been demonstrated [18] that catecholamines can be accumulated into vesicles in response to a pH gradient (low pH inside). By analogy with our results, such accumulation would be expected to proceed in association with $H^+$ efflux, consistent with an in vivo uptake mechanism which does not require a specific transport protein. The third and fourth areas concern drug design and efficient loading of liposomal drug carrier systems. Model systems such as those employed here clearly provide convenient systems for evaluating the possible influence of a given structural alteration on non-specific uptake into cells in response to a membrane potential. Alternatively, large unilamellar vesicle systems containing high levels of lipophilic cationic drugs concentrated in response to $\Delta\psi$ may well have application as vehicles for drug delivery. Reasons for this include the high drug trapping efficiencies that are possible. Preliminary studies employing the anticancer drugs, vinblastine and adriamycin, for example, show that trapping efficiencies in excess of 95% can easily be achieved.

In summary, the studies presented here reveal a remarkable ability of large unilamellar vesicle systems to accumulate safranine and other lipophilic cations in response to a $K^+$ diffusion potential. This ability may have important and general implications for the equilibrium transmembrane distributions of biologically active lipophilic cations in vivo.

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