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PYRANINE AS A SENSITIVE pH PROBE FOR LIPOSOME INTERIORS AND SURFACES

pH GRADIENTS ACROSS PHOSPHOLIPID VESICLES

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Summary

Pyranine is shown to be a convenient and sensitive probe for reporting pH values, pH_i, at the interior of anionic and at the outer surface of cationic liposomes. It is well shielded from the phospholipid headgroups by water molecules in the interior of anionic liposomes, but it is bound to the surface of cationic liposomes. Hydrogen ion concentrations outside the liposomes, 'bulk pH values', pH_o, were measured by a combination electrode. While $pH_i = pH_o$ for neutral, $pH_i < pH_o$ for anionic and $pH_i > pH_o$ for cationic liposomes prepared in $5.0 \cdot 10^{-3}$ M phosphate buffers. pK_a values for the ionization of pyranine were 7.22 ± 0.04 and 6.00 ± 0.05 in water and at the external surface of cationic liposomes. The surface potential for cationic liposomes containing dipalmitoyl-DL-α-phosphatidylcholine, cholesterol and octadecylamine in the molar ratio of 1.00 : 0.634 : 1.01, were calculated to be +72.2 mV. Proton permeabilities were measured for single and multicompartment anionic liposomes. Transfer of anionic liposomes prepared at a given pH to a solution of different pH resulted in a pH gradient if sodium phosphate or borate were used as buffers. In the presence of sodium acetate proton equilibration is promptly established.

Introduction

Determination of pH in the interiors of cells, membranes and membrane models is of fundamental importance. Although direct measurement of intracellular pH by microelectrodes is feasible, information is more commonly obtained from distribution of indicators which are weak acids or weak bases [1]. A fundamental requirement of the latter method is that only the neutral species permeate the membrane freely. If this requirement is met, the concentration of the ionized species will be solely dependent on the pH and the

apparent dissociation constant of the indicator on each side of the membrane. Fluorescence spectroscopy is an often used and sensitive analytical technique [2—5]. Quenching of 9-aminoacridine fluorescence, for example, has provided information on proton gradients across liposomes [6], chloroplasts [7], and membranes from photosynthetic bacteria [8] as well as on proton translocation in vesicles derived from a cytochrome-deficient mutant of *Escherichia coli* [9]. A serious problem with dyes, in general, and with 9-aminoacridine in particular [10] is that they are bound to membranes to an unknown extent and thus may report false pH values. Additionally, the pH dependent alteration of fluorescence quenching is relatively small [6].

This report establishes trisodium 8-hydroxy-1,3,6-pyrene-trisulfonate, pyranine, as an extremely sensitive probe for monitoring the pH in the interiors of negatively-charged and at the outer surface of positively-charged liposomes. Since the three sulfonate groups in pyranine are completely ionized in the entire pH range, the probe is effectively repelled from the surface of the negatively-charged liposome and reports, therefore, the acidity of water molecules in liposome interiors which are not associated with the phospholipid headgroups. Conversely, anionic pyranine is attracted to the positively-charged inner and outer surface of cationic liposomes. The determined apparent dissociation constant of pyranine at the outer surface of cationic liposomes provides information on the surface charge.

Our interest in liposomes is centered on their use as drug carriers [11—14]. The proposed delivery mechanism involves the entry of the intact, drug-containing liposome into the cell, where lysosomal lipases or other factors release the drug. The very first requirement for liposome-mediated drug delivery is to ensure the entrapment of sufficient amounts of drug in the liposomes. The extent of drug encapsulation depends primarily upon its solubility in water [11]. The solubility of drugs in water, having ionizable functionalities, is pH dependent. The development of pyranine as a sensitive pH probe for liposome interiors has been prompted by the need for monitoring pH gradients across drug-carrying phospholipid vesicles.

Materials and Methods

Commercially available trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate, pyranine (Eastman), contained three fluorescent impurities (R_F = 0.13, 0.24, and 0.43, thin-layer chromatography on Merck 6-F 254 plate; n-C_4H_9OH/H_2O, 6 : 1 as eluent). Crude pyranine was repeatedly recrystallized from aqueous acetone, using charcoal. The recrystallized product did not show any impurities and had only one spot, with R_F = 0.021 corresponding to pyranine in the above thin-layer chromatography system. Cholesterol (MCB) was recrystallized twice from ethanol. Sodium dicetyl phosphate (Sigma), octadecylamine (Aldrich), and synthetic dipalmitoyl-DL-α-phosphatidylcholine (Sigma, grade I) were used without further purification.

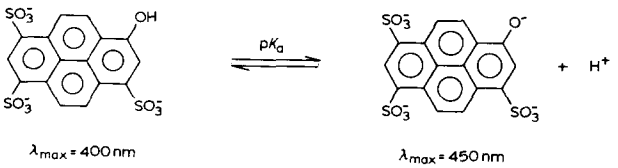
Stock solutions of synthetic dipalmitoyl-DL-α-phosphatidylcholine (12.4 mg/ml), cholesterol (1.0 mg/ml), sodium dicetyl phosphate (1.5 mg/ml) or octadecylamine (1.5 mg/ml) in chloroform were freshly prepared every two weeks and stored in the refrigerator. In a typical experiment, 2.5 ml synthetic

dipalmitoyl-DL-α-phosphatidylcholine, 2.0 ml cholesterol and 1.0 ml sodium dicetyl phosphate stock solutions were mixed in a 100-ml round bottom flask and the chloroform was evaporated under reduced pressure. The remaining thin film of lipid mixture was dried over silica gel in vacuo. Dispersion of the thin film in the appropriate buffer solution resulted in the formation of multicompartment liposomes. Typically, 2.0 ml of the aqueous solution contained $5.0 \cdot 10^{-3}$ M sodium phosphate buffer, adjusted to pH 7.00, 0.10 M NaCl, and $1.0 \cdot 10^{-4}$ M (for anionic and non-ionic liposomes) or $5.0 \cdot 10^{-4}$ M (for cationic liposomes) pyranine. Dispersion was carried out at 55—60°C (gel to liquid crystalline phase transition temperature of synthetic dipalmitoyl-DL-α-phosphatidylcholine is 41°C [15]). The milky suspension of multicompartment liposomes was passed through a Sephadex G-50 column (20—80 μm, 16 × 178 mm) using $5.0 \cdot 10^{-3}$ M sodium phosphate buffer at pH 7.0 adjusted to an ionic strength of 0.10 M with NaCl. Multicompartment liposomes containing pyranine were obtained from the first 10—15 ml eluent fractions. Single compartment liposomes were formed upon sonication under nitrogen at 50—55°C and 70 W for 15 min with a Braunsonic 1510 sonifier. Passing the liposomes through a Sephadex G-50 column separated the free pyranine from that entrapped in the vesicles.

Excitation and emission parameters were determined in the thermostated (25.0 ± 0.1°C) compartment of a SPEX Fluorolog spectrofluorimeter using 10.0 nm bandpaths and 2.5 mm slits on both the excitation and emission sides. The concentration of pyranine was, in all cases, adjusted to be less than $1 \cdot 10^{-6}$ M by diluting liposome solution with the appropriate buffers. The pH of the buffer solutions was adjusted and monitored by means of a Radiometer pHM-26 pH meter.

Results

Fig. 1 shows the excitation and emission spectra of $1.0 \cdot 10^{-6}$ M pyranine in aqueous, buffered solutions at pH 4.00, 7.00 and 10.00. There is only one emission maximum of pyranine fluorescence (at 510 nm) in this pH range. The excitation spectra, taken at 510 nm, consisted of maxima at 380, 400 and 450 nm. Intensities of the maxima at 400 and 450 nm were strongly dependent on the hydrogen ion concentration. At pH 4.00 the relative intensity of the 450 nm peak is negligible. Conversely, at pH 10.00, there is a strong maximum at this wavelength. This result implies the ionization of the 8-hydroxy group in pyranine at higher pH values and that the maxima at 400 and 450 nm in the excitation spectra, λ_{max}, are due to the unionized and ionized forms of pyranine respectively:



(1)

pH titrations of relative fluorescence intensities of pyranine (insert in Fig. 2) resulted in a pK_a value of 7.22 ± 0.04 for the ionization of pyranine. This value

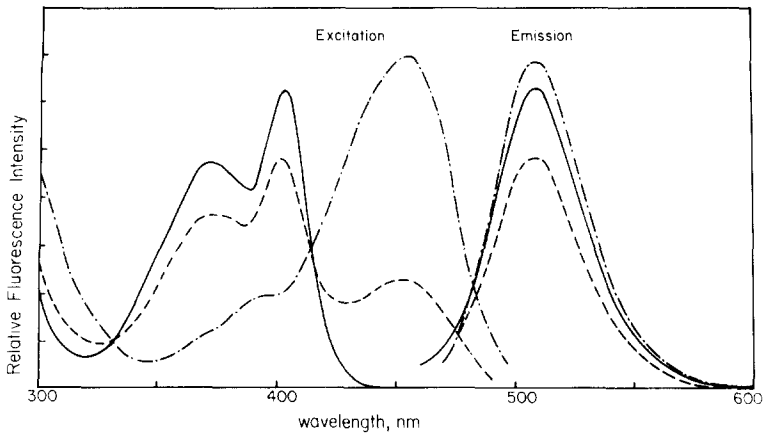


Fig. 1. Excitation and emission spectra of $1.0 \cdot 10^{-6}$ M air-saturated pyranine in aqueous buffer solutions at pH 4.00 (———), 7.00 (- - - - -), and 10.00 (- · - · -) at 25.0°C. The excitation spectra were followed at an emission wavelength of 510 nm and the emission spectra were taken at an excitation wavelength of 400 nm.

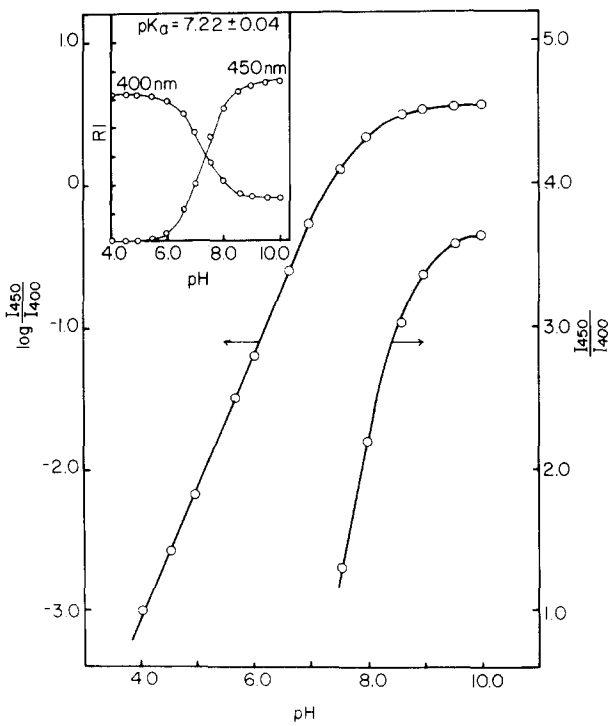


Fig. 2. Rations of relative intensities in the excitation spectra at 450 and 400 nm, observed at an emission of 510 nm, in $3.3 \cdot 10^{-7}$ M aqueous air-saturated pyranine as a function of pH at 25.0°C. $5.0 \cdot 10^{-3}$ M buffers (sodium acetate in pH 4.0—5.7, sodium phosphate in pH 6.0—8.0, and sodium borate in pH 8.6—10.0) were used at a constant ionic strength of 0.10 M (NaCl). The insert shows the titration curves of pyranine in the same aqueous buffers.

is in good agreement with that reported previously (7.2) [16]. The emission maximum of pyranine at 510 nm is due to the ionized form since the pK_a of the excited singlet state is 1.38 [16]. Ratios of the emission intensities at 510 nm upon exciting pyranine at 450 and 400 nm, $I_{450} : I_{400}$, were related to the hydrogen ion concentration (Fig. 2). There was a 5000-fold change in $I_{450} : I_{400}$ in the pH range of 4.0—10.0. Relative intensities of the maxima in the excitation spectra at given pH values were found to be independent on the presence of oxygen (approx. 10^{-3} M), chloride (approx. 1.0 M), bromide (approx. 1.0 M) and iodide (approx. 1.0 M) ions. Furthermore, intensities of the excitation bands at 400 and 450 nm (using 90° illumination) increased linearly with increasing pyranine concentration up to $2.0 \cdot 10^{-6}$ M. All of these properties make pyranine, in concentrations less than $2.0 \cdot 10^{-6}$ M, a sensitive probe for measuring the pH of its surroundings. The apparent pH values inside the vesicles were obtained from Fig. 2 and the concentrations of pyranine incorporated in the vesicles were estimated from the linear calibration plots of emission intensities vs. concentration (not shown). The concentration of pyranine did not exceed $1 \cdot 10^{-6}$ M. Table I shows the apparent pH values in liposomes as monitored by pyranine, the amounts of probe incorporated, and the pH outside the liposomes, the 'bulk pH', pH_o, as determined by a glass combination electrode.

TABLE I

pH VALUES IN BULK AND IN LIPOSOME-ENTRAPPED WATER AND FLUORESCENCE POLARIZATION OF PYRANINE IN LIPOSOMES

Liposomes: The symbols S and M indicate single and multicompartment liposomes; 0, — and + refer to neutral, anionic and cationic liposomes. Neutral liposomes were prepared from 6.0 mg (8.2 μmol) dipalmitoyl-D,L-α-phosphatidylcholine and 2.0 mg (5.2 μmol) cholesterol. Anionic or cationic liposomes additionally contained 1.5 mg (2.7 μmol) sodium dicetylphosphate or 2.25 mg (8.3 μmol) octadecylamine unless stated otherwise. All liposomes were formed in 5.0 mM sodium phosphate in the presence of 0.10 M NaCl unless stated otherwise. Mole ratio: dipalmitoyl-D,L-α-phosphatidylcholine: cholesterol: sodium dicetyl phosphate or octadecylamine. $pH_p = pH_o$: pH_p is pH of which the liposomes are prepared; pH_o is outside pH, determined by a combination electrode. pH_o remains unaltered by passing the vesicle through the Sephadex column. pH_{i45min}, pH_{i24h}: inside pH, determined by pyranine, 45 min and 24 h subsequent to the formation of liposomes. P: Fluorescence polarization of pyranine. Pyranine was excited at 400 nm and fluorescence intensities were followed at 510 nm. The degrees of polarization were calculated by [25,26]: $P = [I_{\parallel\parallel} - I_{\perp\perp}(I_{\perp\parallel}/I_{\perp\perp})]/[I_{\parallel\parallel} + I_{\perp\perp}(I_{\perp\parallel}/I_{\perp\perp})]$ where $(I_{\perp\perp}, I_{\parallel\parallel})$ and $(I_{\perp\parallel}, I_{\parallel\perp})$ means fluorescence intensities at parallel and crossed positions of the polarizers, respectively.

Liposome	Mole ratio	Pyranine incorporated (%)	$pH_p = pH_o$	pH_{i45min}	pH_{i24h}	$10^2 P$
S 0	1 : 0.634 : 0	0.55	7.0	6.9	7.0	4.86
S —	1 : 0.634 : 0.329	0.60	7.0	6.5	7.0	1.14
S — *	1 : 0.634 : 0.171	0.70	7.0	6.8	7.0	—
S — **	1 : 0.634 : 0.671	0.75	7.0	6.0	6.9	—
M —	1 : 0.634 : 0.329	4.80	7.0	6.3	6.4	0.83
S +	1 : 0.634 : 1.012	59.00	7.0	7.6	7.6	10.68
M +	1 : 0.634 : 1.012	87.00	7.0	7.9	7.9	9.29
S — ***	1 : 0.634 : 0.329	0.88	10.0	8.8	9.0	—
M — ***	1 : 0.634 : 0.329	5.75	10.0	8.2	8.9	—

* Sodium dicetyl phosphate, 1.4 μmol.

** Sodium dicetyl phosphate, 5.5 μmol.

*** $5.0 \cdot 10^{-3}$ M borate buffer containing 0.1 M NaCl was used to adjust pH_p and pH_o.

Pyranine solubilization sites were established by fluorescence polarization measurements. Table I also includes data for the fluorescence polarization (P) of pyranine in the different liposomes. The polarization of pyranine in water was found to be 0.00603. This value is close to those determined in anionic single and multicompartment liposomes. Conversely, in cationic and, to a lesser extent in uncharged liposomes, the polarization of pyranine is appreciable. These results indicate that pyranine is solubilized in the water pools in the anionic liposomes where it is effectively shielded from the negatively-charged lipid headgroups by water molecules. Pyranine is likely, however, to be electrostatically bound to the positively-charged groups both on the inside and outside of cationic liposomes. The high values for pyranine entrapment in cationic, as opposed to anionic, liposomes (Table I) support with this postulate. The site of interaction in neutral liposomes is not obvious to assess. Some degree of restricted motion is indicated by the observed fluorescence polarization (Table I).

Once entrapped, pyranine cannot readily diffuse through the phospholipid bilayer. Typically, the rate of leakage from anionic single compartment liposome was determined to be less than 1% per day.

After separating the free pyranine from that entrapped in liposomes, pH values outside the liposomes, pH_o, were determined by a combination electrode, while those in the interior or at the surface of the vesicles were calculated from fluorescence measurements. The validity of this technique was repeatedly substantiated by the identity of pH, determined either by the combination electrode or by the pyranine probe, subsequent to lysing the liposomes with Triton X-100.

Table I compares 'bulk pH values' with those determined in liposome-entrapped water by pyranine in freshly prepared solutions. 'Bulk pH values' were found to remain constant during experiments. In neutral liposomes, the outside pH was found to be identical, within experimental error, to the inside pH. In freshly prepared anionic liposomes, the inside pH (pH_{i45min}) was lower, while in cationic liposomes it was higher than that outside the vesicles. This apparent pH gradient across anionic liposomes was found to be dependent upon the concentration of added dicetyl phosphate, increasing concentrations of which increased the pH gradient. At high pH, some residual pH gradient remained even after 24 h (Table I). Apparently, for anionic liposomes, the capacity of the buffer is insufficient to compensate for the electrostatic binding of protons to the negatively-charged dicetyl phosphate (see Discussion).

Fig. 3 illustrates pH titration curves for pyranine fluorescence in water and in cationic liposomes. In water, the pK_a for pyranine was determined to be 7.22 ± 0.04. Two types of pyranine-containing cationic liposomes were prepared. Type I cationic liposomes were prepared in the usual manner by dispersing the lipid film with aqueous solutions of pyranine. Subsequent to sonication, free pyranine was removed from that bound to the liposome by gel filtration on Sephadex G-50. Here pyranine was bound electrostatically both at the outside and inside to the cationic vesicle. Since, at the time of the measurements (45 min subsequent to preparation), there was a pH gradient across the liposome, the fluorescence intensities are related to mean pH values on either side of the bilayer. The titration curve for type I cationic liposomes gave an

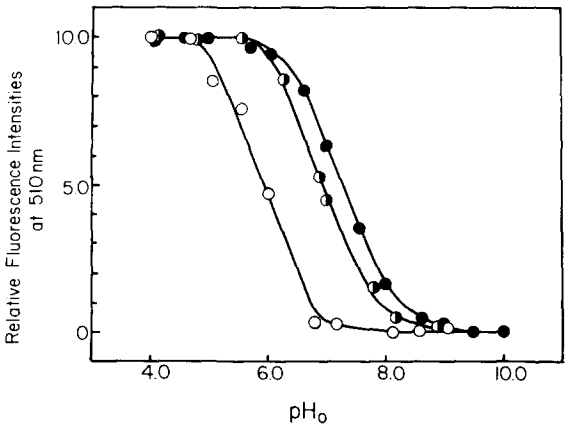


Fig. 3. pH titration curves of pyranine in water (●), at the inner and outer surface, type I liposomes (◑), and at only the outer surface of cationic single compartment liposomes, type II liposomes (○). Both liposomes were prepared at pH 7.00 using $5.0 \cdot 10^{-3}$ M phosphate buffers at an ionic strength of 0.10 M (NaCl). Type I liposome was diluted 150-fold, and Type II liposome 60-fold by $5.0 \cdot 10^{-3}$ M buffers (sodium acetate in the pH 4.0—5.7, sodium phosphate in pH 6.0—8.0, and sodium borate in pH 8.6—10.0) adjusted to appropriate pH values at constant ionic strength of 0.10 M (NaCl). Plotted are the relative fluorescence intensities at 510 nm (excitation wavelength 400 nm) against bulk pH values.

TABLE II

pH GRADIENTS ACROSS NEGATIVELY-CHARGED LIPOSOMES

Liposomes: the symbols S and M indicate single snd multicompartment liposomes. They were prepared from 6.0 mg (8.2 μmol) dipalmitoyl-D,L-α-phosphatidylcholine, 2.0 mg (5.2 μmol) cholesterol and 1.5 mg (2.7 μmol) sodium dicetyl phosphate. Buffer: $5.0 \cdot 10^{-3}$ M buffer in the presence of 0.10 M NaCl. B = sodium borate, P = sodium phosphate, A = sodium acetate, HCl = hydrochloric acid. pH_p : pH of the initially prepared liposome. pH_o of bulk buffer into which liposomes were injected 24 h subsequent to their preparation. Generally, 0.20 ml single or 0.020 ml multi-compartment liposome solution was diluted to 3.0 ml in the appropriate buffer. pH'_{i45min}: pH inside the liposomes, as reported by pyranine, 45 min subsequent to injecting concentrated liposomes, prepared 24 h previously, to appropriate buffer. pH'_{i24h}: pH inside the liposome, as reported by pyranine, 24 h subsequent to injecting concentrated liposomes, prepared 24 h previously, to the appropriate buffer.

Liposomes	Buffer	pH_p	pH_o	pH'_{i45min}	pH'_{i24h}
S	B	7.00	9.87	8.1	8.9
S	B	7.00	8.95	7.9	8.5
S	B	7.00	7.87	7.6	7.6
S	P	7.00	7.63	7.4	7.5
S	P	7.00	7.00	7.0	7.0
S	P	7.00	6.21	6.7	6.4
S	A	7.00	5.32	5.4	5.4
S	A	7.00	4.10	4.3	4.6
M	B	7.00	9.70	7.0	8.2
M	B	7.00	9.02	6.9	7.5
M	B	7.00	7.99	6.7	6.9
M	P	7.00	7.68	6.7	6.9
M	P	7.00	7.03	6.6	6.6
M	P	7.00	6.16	6.4	6.2
M	A	7.00	5.26	5.0	5.1
M	A	7.00	4.05	4.2	4.2
S	P	10.00	7.12	8.3	7.3
M	P	10.00	7.12	8.2	7.3
M	HCl	6.41	2.00	6.4	4.1 *

* pH inside the liposome, as reported by pyranine, 4 h subsequent to injecting concentrated liposomes, prepared 24 h previously, to the appropriate buffer.

apparent pK_a of 6.90 ± 0.05. In type II liposomes, 0.10 ml of $1.0 \cdot 10^{-3}$ M pyranine in $5.0 \cdot 10^{-3}$ M phosphate buffer at pH 7.00 and an ionic strength of 0.10 M was added to 2.00 ml of the positively-charged single compartment vesicles after their formation in $5.0 \cdot 10^{-3}$ M phosphate buffer, at pH 7.00, containing 0.10 M NaCl. After 5 min incubation, the free pyranine was separated from that bound to the liposome by gel filtration on a Sephadex G-50 colum. Here pyranine is only bound to the outer surface of the cationic vesicle. The apparent pK_a value at the outer surface of cationic liposomes was determined to be 6.00 ± 0.05. This value is substantially smaller than that obtained in water (7.22 ± 0.04). Similar lowering of pK_a values have been found for positively charged monomolecular films [18,19] and liposomes [20].

Since pyranine is localized well inside the interiors of negatively-charged liposomes, it can be used profitably for the investigation of pH gradients. In these experiments, pyranine-carrying liposomes, prepared at a given pH, were transferred into a large excess of a buffer adjusted to a different pH. Table II summarizes the data for these pH shocks. The pH of the initially prepared liposome, pH_p, that of the new buffer into which the liposome is transferred, pH_o, and that inside the liposome 45 min, pH'_{i45min}, and 24 h, pH'_{i24h}, subsequent to the transfer are reported. The proton permeability depends very much on the buffer used (Fig. 4). In the presence of sodium acetate there was a prompt equilibration of H^+ concentration across the liposome. Substantial

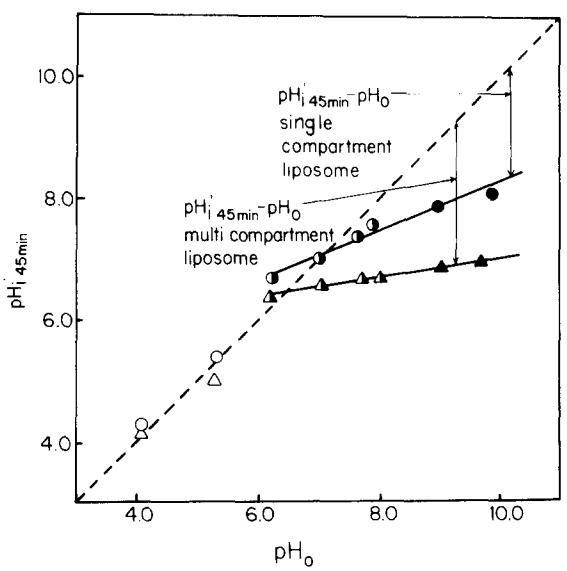


Fig. 4. pH gradient across anionic single (circles) and multi- (triangles) compartment liposomes. All liposomes were prepared at pH 7.0, using $5.0 \cdot 10^{-3}$ M phosphate buffer adjusted to an ionic strength of 0.10 M (NaCl). 24 h subsequent to their preparation 0.20 ml single or 0.020 ml multicompartment liposomes solutions were diluted into 3 ml of the appropriate buffer. pH values inside the liposomes, as reported by pyranine, were observed within 45 min of this dilution (pH'_{i45min}). Plotted are the pH'_{i45min} values against the pH of the bulk buffer, pH_o, into which the liposomes were injected. The pH of the bulk solution was maintained by $5.0 \cdot 10^{-3}$ M acetate (○△), phosphate (●▲) and borate (●▲) buffers and were adjusted to 0.10 M ionic strength by NaCl. The broken line indicates the hypothetical situation when $pH'_{i45min} = pH_o$ and deviations from this line correspond to the pH gradients across the liposome.

pH gradient is maintained, however, in the presence of phosphate and borate buffers, and the greater the pH difference, the greater the gradient.

Discussion

Pyranine is a sensitive and convenient probe for measuring the pH of its surroundings. It has several advantages: it is readily available, easy to purify and has well characterized spectral properties. It has a high fluorescence quantum yield and a large Stokes shift. Using a spectrofluorimeter of even modest resolution, a concentration of $1 \cdot 10^{-7}$ M pyranine is readily detected by measuring fluorescence spectra. Observing the excitation spectra at an emission wavelength of 510 nm, maxima at 400 and 450 nm are attributable to the non-ionized and ionized forms of trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate. The intensities of these maxima are linearly related to pyranine concentration up to $2.0 \cdot 10^{-6}$ M and their ratio changes 5000-fold in the pH 4.0—10.0 region. Most important, quenchers, usually present in biological media, do not affect the intensity ratios in the excitation spectra at 400 and 450 nm, and hence the reported pH values. The fluorescence lifetime of pyranine is sufficiently short, 5.5 ± 0.3 ns (Kano, K. and Fendler, J.H., unpublished results), to prevent extensive movement during relaxation of the excited state. Polarization of the pyranine fluorescence is a good indicator, therefore, of the microviscosity it experiences.

Ionic additives profoundly affect the pH of liposome interiors. In the presence of sodium dicetyl phosphate, $pH_i < pH_o$ for anionic liposomes (Table I). This proton gradient is explicable in terms of the weak buffer capacity of $5.0 \cdot 10^{-3}$ M phosphate ions and the poor proton permeability across the negatively-charged phospholipid bilayer. Assuming 150 Å and 38 Å for the radius of the liposome and for the bilayer thickness, respectively [21], we calculate the internal volume of a liposome to be $5.9 \cdot 10^{-18}$ cm^3, the ratio of internal surface area to the external surface area to be 0.558 and $1.3 \cdot 10^{14}$ vesicles per μmol of phospholipid. These parameters allow the calculation of the number of dicetyl phosphate molecules in a vesicle and which are localized at the inner surface, if a geometrical packing is assumed. For example, in the case of liposomes prepared in the ratio of synthetic dipalmitoyl-DL-α-phosphatidylcholine/cholesterol/sodium dicetyl phosphate, 1.00 : 0.634 : 0.171 (see Table I), each vesicle contains a total of $1.3 \cdot 10^{-21}$ mol sodium dicetyl phosphate, of which $4.7 \cdot 10^{-22}$ mol are located at the inner surface. Taking into consideration the buffer capacity of 1 l of $5.0 \cdot 10^{-3}$ M phosphate buffer, a lowering of pH from 7.0 to 6.8 is expected upon the addition of $5.1 \cdot 10^{-4}$ mol of proton. Since internal volume of a vesicle is $5.9 \cdot 10^{-18}$ cm^3, the internal pH of the vesicle should be reduced from 7.0 to 6.8 upon incorporating $3.0 \cdot 10^{-24}$ mol of proton in a vesicle. It implies that, under the present experimental conditions, if one proton is associated with approx. 100 mol sodium dicetyl phosphate (compare $4.7 \cdot 10^{-22}$ mol sodium dicetyl phosphate with $3.0 \cdot 10^{-24}$ mol proton), at the interior of the liposomes there is a pH change from 7.0 to 6.8. Although this calculation allows the prediction of the expected pH gradient as a function of added sodium dicetyl phosphate, the questionable validity of the assumption involved should be kept in mind. Particularly, gross oversimplifications are the assumed uniformity of the vesicles, the statistical

distribution of sodium dicetyl phosphate and lack of proton permeabilities during the measurements.

Pyranine is electrostatically bound to the cationic headgroups of octadecylamine, localized at the lipid-water interfaces in the outside of the positively-charged type II liposome. Pyranine indicates, therefore, the pH at the outer surface of single and multicompartment positively-charged liposomes. Differences between surface and bulk pH can be treated according to Gouy's theory [22] by Eqn. 2:

$$pK_a^s = pK_a^\circ - \frac{\psi}{59.15} \quad (2)$$

where pK_a^s and pK_a° are related to the dissociation of pyranine (Eqn. 1) at the outer surface of cationic liposomes and in 'bulk' water. Using values of pK_a^s 6.00 ± 0.05 and pK_a° 7.22 ± 0.04 (see Results) the surface potential for single compartment liposomes has been calculated to be +72.2 mV for cationic liposomes containing dipalmitoyl-DL-α-phosphatidylcholine, cholesterol and octadecylamine in the molar ratio of 1.00 : 0.634 : 1.01. Surface potentials in micelles [23], monomolecular films [19] and sonicated vesicles [20] have been determined analogously.

Substantial pH gradients can be developed and maintained for some time in anionic liposomes in the presence of phosphate and borate buffers (Table II and Fig. 4). Significantly, there is an immeasurably fast (under our experimental conditions) proton permeability in the presence of sodium acetate. Although the mechanism of sodium acetate-mediated proton transfer across anionic liposomes is not yet established, acetate can be assumed to be transported by diffusion of the undissociated form through the vesicles [24]. Apparent proton transfer rates are substantially greater for single than for multicompartment liposomes (Table II and Fig. 4). However, in the latter case, the proton has to diffuse across several layers. Comparison of proton permeabilities between single and multicompartment liposomes is not warranted. pH gradients are not maintained for longer than 24 h if liposomes are transferred from pH 10.00 to pH 7.12. Conversely, pH gradients can be maintained in transferring liposomes from pH 7.00 to pH 2.00 or pH 9.87. Judicious manipulation of experimental conditions, enabling pH gradients to be maintained, will increase the usefulness of liposomes as drug carriers.

The present work has demonstrated the advantages of pyranine as a pH probe in liposomes. This technique can be extended to the measurement of hydrogen ion concentrations at enzyme active sites and at hydrophilic protein surfaces. The only requirement is knowledge of the location of the probe.

Acknowledgements

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References

1 Waddell, W.J. and Bates, R.G. (1969) Physiol. Rev. 49, 285—329
2 Guilbault, G. (1967) Fluorescence. Theory Instrumentation and Practice, Marcel Dekker, New York

3 Radda, G.K. (1975) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 4, pp. 97—118, Plenum Press, New York
4 Wehry, E.L. (1976) Modern Fluorescence Spectroscopy, Plenum Press, New York
5 Chen, R.F. and Edelhoch, H. (1975) Biochemical Fluorescence: Concepts., Marcel Dekker, New York
6 Deamer, D., Prince, R.C. and Crofts, A.R. (1972) Biochim. Biophys. Acta 274, 323—335
7 Schuldinger, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64—70
8 Casadio, R., Baccarini-Melandri, A. and Melandri, B.A. (1974) Eur. J. Biochem. 47, 121—128
9 Singh, A.P. and Bragg, P.D. (1977) Biochim. Biophys. Acta 464, 562—570
10 Fiolet, J.W.T., Bakker, E.P. and van Dam, K. (1974) Biochim. Biophys. Acta 368, 432—445
11 Fendler, J.H. and Romero, A. (1977) Life Sci. 20, 1109—1120
12 Gregoriadis, G. (1976) N. Engl. J. Med. 295, 704—710
13 Gregoriadis, G. (1976) N. Engl. J. Med. 295, 765—770
14 Tyrrell, D.A., Heath, T.D., Colley, C.M. and Ryman, B.E. (1976) Biochim. Biophys. Acta 457, 259—302
15 Inoue, K. (1976) Biochim. Biophys. Acta 339, 390—402
16 Forster, T. (1950) Z. Electrochem. 54, 531—535
17 Weller, A. (1958) Z. Physik. Chem. Neue Folge 17, 224—245
18 Fromherz, P. (1973) Biochim. Biophys. Acta 323, 326—334
19 Fromherz, P. and Masters, B. (1976) Biochim. Biophys. Acta 356, 270—275
20 Montal, M. and Gitler, C. (1973) Bioenerg. 4, 363—382
21 Johnson, S.M. (1973) Biochim. Biophys. Acta 307, 27—41
22 Davies, J.T. and Rideal, E.K. (1963) Interfacial Phenomena, pp. 56—107, Academic Press, London
23 Mukerjee, P. and Banerjee, K. (1964) J. Phys. Chem. 68, 3567—3574
24 Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) Eur. J. Biochem. 17, 230—238
25 Price, J.M., Kaihara, M. and Howerton, H.K. (1962) Appl. Opt. 1, 521—533
26 Shinitzky, M., Dianoux, A.C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106—2113