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Determination of pK_a values of lipophilic fluorescein derivatives in phospholipid vesicles by fluorescence emission ratio titration

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Lipophilic xanthene dyes are promising fluorescent indicators of pH at the surfaces of living cells. For this purpose, we have prepared *N*-dichlorofluoresceinyl- and *N*-fluoresceinyl-oleylamine and characterized the effects of pH on fluorescence in phospholipid vesicles, using *N*-oleoyl-aminorhodamine B as a pH-independent standard. Fluorescence was measured at two emission wavelengths during excitation at a single wavelength, and the ratio of the intensities was calculated. The emission ratios were fitted to the Henderson-Hasselbalch equation to give the experimentally observed pK_{obs}. Both *N*-dichlorofluoresceinyl- and *N*-fluoresceinyl-oleylamine were sensitive indicators of pH with pK_{obs} values of 5.2 and 6.8, respectively, and they should be applicable to the measurement of interfacial pH values.

Introduction

Fluorescence ratio imaging microscopy provides a high resolution method of measuring the cytoplasmic pH of individual cells loaded with water-soluble fluorescein derivatives [1,2]. Membrane-bound fluoresceinyl-phosphatidylethanolamine shows a similar response to pH [3,4] and we have suggested that xanthene dye-labelled lipids may be appropriate for ratio imaging of the pH distribution at the surfaces of living cells [5]. Interfacial pH values are of great interest in the case of chondrocytes, for example, as these cells can be stimulated to resorb their extracellular matrix by inflammatory mediators such as interleukin 1 [6] and tumour necrosis factor [7]. As the resorption appears to be mediated by proteolytic enzymes [8], it is likely that the activity of these enzymes is controlled, at least in part, by the local pH [9].

The fluorescence ratio used most commonly in pH measurements is an excitation ratio, obtained by measuring the fluorescence at a single emission wavelength during excitation at two wavelengths [10], and this varies with pH according to the Henderson-Hasselbalch equation. Excitation ratio titrations of eosinyl-, dichlorofluoresceinyl- and fluoresceinyl-phosphatidylethanolamines in phospholipid vesicles gave pK_{obs} values of 3.5, 6.3 and 7.5, respectively [5], which covered the whole range of pH values likely to be encountered under physiological conditions.

When we came to use these probes in the fluorescence microscope, however, we encountered a technical obstacle. Eosin and dichlorofluorescein had spectral parameters that differed significantly from those of fluorescein, and new filter assemblies were required. Our instrument had no spare filter spaces and the exchange of filter holders required an undesirable degree of disassembly. As the microscope was fitted with narrow band-pass filters for selectively observing both fluorescein and rhodamine fluorescence, it seemed probable that these spectral limitations could be overcome by using fluorescence emission rather than excitation ratios [11,12]. For these measurements, the fluorescein indicator dye is incorporated together with a rhodamine dye whose fluorescence does not change with pH. The mixture is excited at a single wavelength and the emissions are measured at wavelengths appropriate for fluorescein and rhodamine. The ratio of the fluorescence emissions shows pH dependence [11,12]. We report here the pK_{obs} values of *N*-dichlorofluoresceinyl-oleylamine and *N*-fluoresceinyl-oleylamine in phospholipid vesicles as determined by both fluorescence emission and excitation ratio titrations.

Abbreviations: BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; Cf-18, *N*-dichlorofluoresceinyl-oleylamine; Fl-18, *N*-fluoresceinyl-oleylamine; Rh-18, *N*-oleoyl-aminorhodamine B

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Materials and Methods

5-Carboxyfluorescein, 5(and 6)-aminorhodamine B, oleic acid and dioleoylphosphatidylcholine were from Sigma. 5(and 6)-Carboxy-2′,7′-dichlorofluorescein was from Molecular Probes. Oleylamine was from Fluka. Benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was from Novabiochem. Other reagents were the purest grades commercially available.

Buffers

These were as follows: buffer A, 10 mM Tris-HCl (pH 7.3); buffer B, 20 mM Tris-maleate adjusted in the pH range 4–9 with 1 M HCl. pH values of buffer B were measured at 37°C with a Radiometer pHM83 with a combined electrode and temperature probe. All buffers contained 0.02% NaN_3 and 0.1 M NaCl, and were passed through 0.45 μm pore-size filters to remove scattering components.

Chromatography

Silica gel plates (Merck 5717) for preparative layer chromatography (PLC) were from BDH. Thin-layer chromatography (TLC) sheets (13 181) were from Kodak. The solvent mixtures used were as follows: solvent A, chloroform/methanol/acetic acid (70 : 30 : 3, v/v); solvent B, chloroform/methanol/35% ammonia (70 : 30 : 3, v/v). Sephadex LH-20 for gel chromatography was from Pharmacia.

Synthesis of N-dichlorofluoresceinyl-oleylamine (Cf-18)

5(and 6)-Carboxy-2′,7′-dichlorofluorescein (23.0 mg, 49 μmol) was dissolved in dimethylformamide (1.2 ml) and oleylamine (26.2 mg, 98 μmol), BOP (25.7 mg, 58 μmol) and diisopropylethylamine (20 μl, 116 μmol) were added. After stirring for 3 d at 21°C in the dark, TLC in solvent A showed a major band of product with R_f 0.58. The solvent was removed at 35°C in vacuo, and the residue was dissolved in chloroform/methanol (1 : 1 v/v, 1 ml) and applied to a column (1 cm × 90 cm, 71 ml) of Sephadex LH-20 eluted with chloroform/methanol/water (1 : 1 : 0.1, v/v). Fractions (3 min, 0.4 ml) comprising the leading peak were pooled and evaporated. The residue was redissolved as above, applied to a PLC sheet and eluted with chloroform/methanol/acetic acid (70 : 30 : 3, v/v). The major band was scraped off, eluted with methanol and the product run on Sephadex LH-20 in chloroform/methanol/water (1 : 1 : 0.08, v/v). Three bands were eluted from the column, the first and third of which were indistinguishable by TLC in solvents A and B. It was assumed that these bands represented tautomers of the product, and they were pooled and evaporated to yield 7 mg (9.8 μmol, 20%).

Synthesis of N-fluoresceinyl-oleylamine (Fl-18)

5-Carboxyfluorescein (13.8 mg, 36.8 μmol) was dissolved in dimethylformamide (1.2 ml) and oleylamine (19.8 mg, 74μmol), BOP (19.5 mg, 44.1 μmol) and diisopropylethylamine (20 μl, 116 μmol) were added. After stirring for 24 h at 21°C in the dark, TLC in solvent A showed a major band of product with R_f 0.59. This material was purified to homogeneity essentially as described above; chromatography on Sephadex LH-20 in chloroform/methanol/water (1 : 1 : 0.1, v/v) was follows by PLC in solvent A and another run on Sephadex LH-20 in chloroform/methanol/water (1 : 1 : 0.08, v/v). The final weight of product was 16.3 mg (26.1 μmol, 71%).

Synthesis of N-oleoyl-aminorhodamine B (Rh-18)

5(and 6)-Aminorhodamine B (21.5 mg, 46.7 μmol) in dimethylformamide (1.2 ml) was allowed to react with oleic acid (20.4 mg, 93.4 μmol), BOP (24.8 mg, 56.0 μmol) and diisopropylethylamine (50 μl, 290 μmol). After 3 d at 21°C in the dark, TLC showed the major product to have R_f 0.83 in solvent A. This material was purified by chromatography on Sephadex LH-20 in chloroform/methanol/water (1 : 1 : 0.1, v/v), PLC in solvent A and Sephadex LH-20 in chloroform/methanol/water (1 : 1 : 0.05, v/v). The final weight of product was 3.4 mg (4.8 μmol, 10%). All the fluorescent lipids were stable over several months as solutions in chloroform/methanol (1 : 1, v/v) at −20°C.

Phospholipid vesicles

Unilamellar vesicles were made by gel filtration of mixed cholate-dioleoylphosphatidylcholine micelles in buffer A, as described previously [13]. In order to minimise self-quenching of fluorescence [14], the concentrations of the probes did not exceed 0.8 mol%. Vesicles were passed through 0.22 μm pore-size filters and used within 24 h.

Fluorescence measurements

These were made at 37°C in a Perkin-Elmer LS-3 spectrofluorimeter equipped with Rank Brothers electronic stirrers. Buffer B (3 ml) at the chosen pH was allowed to warm to 37°C and blank values of the fluorescence were recorded before adding a sufficient volume of vesicles to give a probe concentration in the range 0.1 to 0.5 μM. Most emission ratio measurements were made with an excitation wavelength of 495 nm. Dichlorofluorescein fluorescence was measured at 540 nm and 570 nm and the ratio F_{540}/F_{570} was calculated. Fluorescein fluorescence was measured at 540 and 580 nm and the ratio F_{540}/F_{580} was calculated. Excitation ratio measurements were made as described [5].

Calculation of pK_{obs}

The theoretical basis of fluorescence emission ratio titrations can be derived by assuming that the fluores-

cence of the pH-responsive probe changes due to the ionization of a single group and that over this pH range the fluorescence of the reference probe does not change. If the dye concentrations are sufficiently low, the fluorescence observed at wavelengths λ_1 and λ_2 will be proportional to the concentrations of the emitting species:

$$F_1 = C_a \cdot F_{a1} + C_b \cdot F_{b1} + S_1 \quad (1a)$$

$$F_2 = C_a \cdot F_{a2} + C_b \cdot F_{b2} + S_2 \quad (1b)$$

where C_a and C_b are the concentrations of the acidic and basic forms of the fluorescein dye, respectively, and the proportionality coefficients are F_{a1} for the acidic dye at λ_1, F_{a2} for the acidic dye at λ_2, F_{b1} for the basic dye at λ_1 and F_{b2} for the basic dye at λ_2. S_1 and S_2 are the contributions of the reference probe to the fluorescence at λ_1 and λ_2, respectively. The concentrations C_a and C_b are related by the equation for acid dissociation

$$C_a = C_b \cdot [H^+]/K_{app} \quad (2)$$

where $[H^+]$ is the hydrogen ion concentration and K_{app} is the apparent dissociation constant. The total concentration of the fluorescein probe C_o, is given by

$$C_o = C_a + C_b \quad (3)$$

Thus, if the fluorescence ratio R is defined as F_1/F_2, and Eqns. 2 and 3 are substituted into Eqns. 1a and 1b, we obtain

$$R = \frac{[H^+] \cdot F_{a1} + K_a \cdot F_{b1} + S_1' \cdot (K_a + [H^+])}{[H^+] \cdot F_{a2} + K_a \cdot F_{b2} + S_2' \cdot (K_a + [H^+])} \quad (4)$$

where S_1' and S_2' are S_1/C_o and S_2/C_o, respectively. Eqn. 4 can be rearranged to

$$\frac{[H^+]}{K_a} = \frac{(F_{b1} + S_1') - R \cdot (F_{b2} + S_2')}{R \cdot (F_{a2} + S_2') - (F_{a1} + S_1')} \quad (5)$$

Inspection of Eqns. 1a and 1b reveals that in acidic and basic solutions R approaches limiting values R_a and R_b given by

$$R_a = (F_{a1} + S_1')/(F_{a2} + S_2') \quad (6)$$

$$R_b = (F_{b1} + S_1')/(F_{b2} + S_2') \quad (7)$$

Thus Eqn. 5 can be written in the form

$$R = (R_a \cdot [H^+] + R_b \cdot K_{obs})/([H^+] + K_{obs}) \quad (8)$$

where K_{obs} is the experimental dissociation constant. The experimental and apparent pK values are related as follows

$$pK_{obs} = pK_{app} - \log(F_b/F_a) \quad (9)$$

where F_b and F_a are the fluorescence values for the mixture of probes at the basic and acidic limits at the longest emission wavelength. Thus, if the pH-dependent probe makes no contribution to the fluorescence at this wavelength, $pK_{obs} = pK_{app}$. The fluorescence ratio R values were fitted to Eqn. 8 by non-linear regression [15].

Results

Synthesis of fluorescent probes

Previously, we used xanthene dyes bound to the amino group of phosphatidylethanolamine as indicators of pH in excitation ratio titrations [5]. We found some difficulty, however, in obtaining reproducible labelling of the plasma membranes of living chondrocytes in culture with these reagents (Dingle, J.T. and Knight, C.G., unpublished observations). By contrast, xanthene dye derivatives with a single octadecyl side chain are readily and stably incorporated into biological membranes [14,16]. As insertion appears to be favoured by an unsaturated anchor chain [17], we synthesised some new fluorescent amphiphiles in which an oleyl chain was attached through an amide linkage to the 5 (or 6) position of the fluorescent headgroup. BOP was an efficient coupling agent [18] and the reagents were readily purified to homogeneity by PLC and chromatography on lipophilic Sephadex.

Fluorescence ratio titrations

In theory [5], fluorescent indicators should show a response to pH in both excitation and emission ratio titrations. However, we found the fluorescein dyes to show very little pH-response in emission ratio measurements when excited at 495 nm. The probable reason for this behaviour is that light of 495 nm predominantly excites the ionized form of the dye, as the protonated form has much lower values of both the absorption coefficient and quantum yield [19]. The spectra suggested that 450 nm would be a more appropriate excitation wavelength and emission ratios determined under these conditions were indeed pH dependent with $pK_{obs} = 7.9$ (Fig. 1), but the method was not very sensitive or responsive, and all subsequent experiments were made using Rh-18 as a reference probe.

pH dependence of Rh-18 fluorescence

The emission ratio method relies on the pH independence of the fluorescence of Rh-18 and it was important to confirm this. Phospholipid vesicles containing 0.4 mol% of Rh-18 were excited at 530 nm and fluorescence was measured at 580 nm in the pH range 4.1–7.7 (data not shown). There was, as observed by others [11,12], some quenching at the lowest pH value, but the effect was small and it was assumed that Rh-18 fluorescence remained constant over the pH range of our experiments.

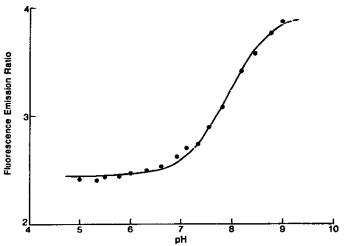

Fig. 1. Emission ratio titration of Fl-18 (0.2 mol%) in phospholipid vesicles. Fluorescence was measured at 540 nm and 580 nm during excitation at 450 nm. The line was calculated by fitting the values of the fluorescence ratio to the Henderson-Hasselbalch equation, as described in the text.

Cf-18 as a pH indicator

Vesicles containing Cf-18 (0.2 mol%) were first examined by the excitation ratio method. Fluorescence was measured at 540 nm during excitation at 450 and 520 nm and the 520/450 ratio was fitted to the Henderson-Hasselbalch equation as previously described [5]. A satisfactory fit was obtained (Fig. 2) and two independent vesicle preparations gave pK_{obs} values of 5.7 and 5.6. Next, vesicles were made with Cf-18 and Rh-18 (0.2 mol% of each), and the emission ratios were determined. These data were fitted to Eqn. 8 and values of 5.2 were obtained for pK_{obs} in duplicate experiments (Fig. 2). Thus Cf-18 was a sensitive probe of pH in both excitation and emission ratio titrations, although the latter method gave a lower pK_{obs}.

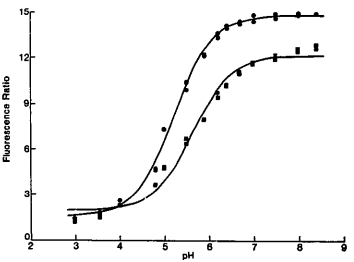

Fig. 2. Excitation and emission ratio titrations of Cf-18 (0.2 mol%) in phospholipid vesicles. Fluorescence was measured at 540 nm during excitation at 450 nm and 520 nm (■) or, in vesicles also containing Rh-18 (0.2 mol%), at 540 nm and 570 nm during excitation at 495 nm (●).

TABLE I

Effect of composition on pK_{obs} of Cf-18

Cf-18 (mol%)	Rh-18 (mol%)	pK_{obs}
0.2	0.2	5.2
0.4	0.4	5.3
0.8	0.8	5.2
0.3	0.1	4.8
0.2	0.2	5.2
0.1	0.3	5.4

Similar values of pK_{obs} were obtained when the ratio of Cf-18 to Rh-18 remained 1:1, but the total concentration of the probes was increased to 0.8 and 1.6 mol% (Table I). When the ratio of Cf-18 to Rh-18 was varied from 1:3 to 3:1 at a constant total probe

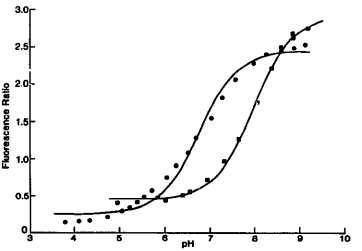

Fig. 3. Excitation and emission ratio titrations of Fl-18 (0.2 mol%) in phospholipid vesicles. Fluorescence was measured at 526 nm during excitation at 450 nm and 495 nm (■) or, in vesicles also containing Rh-18 (0.2 mol%), at 540 nm and 580 nm during excitation at 495 nm (●).

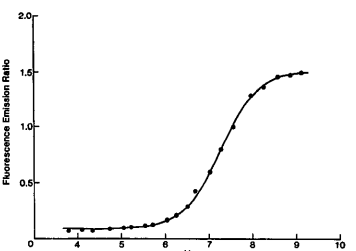

Fig. 4. Emission ratio titration of Fl-18 (0.2 mol%) in phospholipid vesicles containing Rh-18 (0.2%). Fluorescence was measured at 540 nm and 580 nm during excitation at 520 nm.

concentration of 0.4 mol%, the value of pK_{obs} increased (Table I). This behaviour is that predicted by Eqn. 9, as higher proportions of Rh-18 will mask the effects of pH on Cf-18 fluorescence and the value of $\log(F_b/F_a)$ will decrease.

These results indicated that the value of pK_{obs} was relatively insensitive to changes in the concentrations or relative proportions of the probes and all subsequent experiments were made with vesicles containing 0.2 mol% of each.

Fl-18 as a pH indicator

In experiments with phospholipid vesicles containing Fl-18 alone, the excitation ratio method give $pK_{obs} = 8.1$ (Fig. 3). As observed with Cf-18, the emission ratio titration curve was shifted towards lower pH values and this method gave $pK_{obs} = 6.8$. The titration curves, however, were noticeably different in shape (Fig. 3). The excitation ratio data fitted the Henderson-Hasselbalch equation closely, but the fit of the emission ratio results was not so good, although the probe showed a satisfactory response to pH. These deviations were observed in experiments with several vesicle preparations and could not be attributed to errors in pH.

It seemed probable that resonance energy transfer from Fl-18 to Rh-18 (cf. Ref. 16) was responsible for these effects, as this would result in the partial quenching of Fl-18 and the concomitant enhancement of Rh-18 fluorescence. As the quenching effect is critically dependent on the proximity of the energy donor and acceptor [20], dissolving quenched vesicles in detergent results in an enhancement of donor fluorescence [21]. When vesicles containing Fl-18 and Rh-18 (0.2 mol% of each) at pH 9.02 were treated with 0.25 mM cholate, the fluorescence at 540 nm increased by about 55%, while that at 580 nm fell by about 4% (data not shown), indicating that substantial energy transfer had occurred.

Energy transfer was minimised by increasing the direct excitation of Rh-18 by using an excitation wavelength of 520 nm, and measuring the fluorescence emissions at 540 and 580 nm. Under these conditions the emission ratio data showed a greatly improved fit to the Henderson-Hasselbalch equation (Fig. 4), although the value of pK_{obs} had increased to 7.3.

Discussion

As probes of pH in living cells, the xanthene dyes have major advantages. They are highly fluorescent at non-lethal wavelengths and the fluorescence changes markedly in the physiological pH range. Usually, the ratio of fluorescence emission when excited by 495 nm and 450 nm light is used as a measure of pH [10]. The ratio relationship normalizes for differences in optical pathlength, local probe concentration, illumination intensity and photobleaching [2]. By contrast, very little attention has been paid to emission ratios, which should in theory [5] also respond to pH. Such changes are seen with 1,4-dihydroxyphthalonitrile [22] and the novel indicators being developed by Molecular Probes [23], but we found the fluorescein dyes alone to show very little pH-response in emission ratio titrations (Fig. 1).

The use of rhodamine as a pH-independent standard allows far more sensitive measurements [11,12], but the theoretical basis of the method has not been presented previously. Thus, it has not always been realised that emissions from both dyes can contribute to the fluorescence at the wavelengths of measurement, provided that only one dye exhibits pH-dependence. For example, in their measurements of endosome pH in single cells by dual fluorescence flow cytometry, Murphy et al. [12] used filters to separate the fluorescein and rhodamine emissions, and corrected their data for residual spillover between the channels. Our analysis would suggest that these procedures may have been unnecessary.

The values of pK_{obs} for Cf-18 and Fl-18 found in this study are in the physiological pH range and suggest that these probes, like those based on phosphatidylethanolamine [5], are appropriate for the measurement of pH at the surfaces of living cells. It must be emphasized, however, that it will be essential to determine in situ curves of fluorescence ratio vs. pH [2,24], as the precise values of pK_{obs} will depend on the choice of excitation and emission wavelengths (cf. Figs. 3 and 4). This sensitivity of fluorescence ratios to wavelength has been seen in other studies [25] and it has usually been ascribed to differences in the molar absorptivities and quantum yields of the protonated and unprotonated species that exist in the pH range [19]. However, although fluorescence ratio titrations of xanthene dye probes can be satisfactorily characterized in terms of a single protonic equilibrium, it is much less clear whether this reflects a ground state or an excited state ionization. A priori, one expects the excited state to have a different pK_a to the ground state [26], but the actual distribution of species will depend on whether proton transfer occurs in the time scale of the excited state [27]. Although there is some evidence that proton transfer is rapid at lipid/water interfaces [4], we know of no studies of the excited-state protonation of lipophilic fluorescein probes. In the case of fluorescein, arguments have been presented for [28] and against [19] a change of pK_a in the first excited state.

In practice, we observed that the pK_{obs} of Cf-18 was unaffected by the level of labelling, but influenced by the proportion of Rh-18 in the membrane (Table I). Our previous study of xanthene-dye-labelled phosphatidylethanolamines [5] showed that pK_{obs} was relatively unaffected by the incorporation of up to 15 mol% of charged phospholipids, and we assume that the present probes will behave similarly.

An unexpected finding in this study was the distorting effect of resonance energy transfer on the emission ratio titration curves of FI-18. Athough analogous probes have been used to monitor cell and membrane fusion [14,16,21], we had assumed that energy transfer could be neglected at surface densities of 0.2 mol% of each probe. However, both theoretical [20] and experimental [21] studies have shown that this is not the case. Energy transfer does not prevent FI-18 acting as a pH indicator, but it provides another reason for in situ calibration in cellular systems.

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