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A NOVEL METHOD FOR MEASUREMENT OF INTRAVESICULAR pH USING FLUORESCENT PROBES

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Summary

A method for the measurement of intravesicular pH of phospholipid vesicles and gastric microsomes is described. The present method makes use of the well characterized pH-dependent shift of the emission maximum of two fluorescent amines, quinine and acridine. As the probes distribute into the intravesicular space, according to the existing pH gradient, they respond to the acidic environment and emit fluorescence at a longer wavelength than the external probes which sense the more alkaline medium. By measuring both the decrease in the alkaline fluorescence peak and the enhancement of the acidic peak, direct determination of the internal pH can be obtained. This method has the advantages of giving a positive signal (enhancement of fluorescence instead of quenching), measuring intravesicular pH directly without the need of independent measurement of the volume of the H^+ space, and also enabling the possible use of a much lower probe concentration than that required by the fluorescence-quenching method. The accuracy of the present method was quantitatively verified using phospholipid vesicles as a well defined model system. Application to biological systems was demonstrated using gastric microsomes which actively transport H^+ into the microsomal space via an H^+-K^+ exchange ATPase system. The reasoning of the present method is also extended to the monitoring of internal alkaline pH gradient by using a fluorescent weak acid, *o*-hydroxycinnamic acid. Some general criteria for the future search for better pH gradient probes are presented.

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Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

Introduction

The use of weak acids and bases to measure a pH gradient (ΔpH) across vesicular structures is a well established method. Applications using radioactive acidic [1] and basic [2,3] probes are highly sensitive, but the method is generally not suitable for rapid kinetic analysis. The introduction of the fluorescent-amine technique by Schuldiner et al. [4] provided a very convenient and continuous measurement of ΔpH. This method is based on the quenching of fluorescence intensity as the probes distribute into the intravesicular space according to the existing pH gradient. Although the exact mechanism of the quenching effect is not well understood its quantitative correlation with ΔpH was verified by Deamer et al. [5] using liposomes as a well defined model system for biological membranes. Since then the method has been applied to a wide variety of biological systems [6—8].

In this study, we present an alternative method for the direct measurement of intravesicular pH. The present method makes use of the well characterized pH-dependent shift of the emission maximum of two fluorescent amines, quinine and acridine. As the probes distribute into the intravesicular space, they respond to the acidic environment and emit fluorescence at a longer wavelength than the external probes sensing a more alkaline medium. By measuring both the decrease in the alkaline fluorescence peak and the enhancement of the acidic peak, direct determination of the internal pH can be obtained. This method has the advantages of giving a positive signal (enhancement of fluorescence instead of quenching), measuring intravesicular pH directly without the need of independent measurement of the volume of the H^+ space and also enabling the possible use of a much lower probe concentration. We will also extend this reasoning to the monitoring of an internal alkaline pH gradient by using a fluorescent acid. We will present evidence to support the concentration-dependent self-quenching proposed by Deamer et al. [5] as the most plausible mechanism for the fluorescence quenching method used previously. Finally, we will summarize the reasoning developed in the study into some general criteria for the future search for better ΔpH probes.

Material and Methods

Preparation of vesicles. Lipid vesicles were prepared from soybean phospholipids (asolectin). The crude asolectin from Sigma was partially purified by acetone extraction as described by Kagawa and Racker [9] except that the addition of antioxidant was omitted and the whole extraction procedure was carried out under N_2 instead. The composition of asolectin is reported to be 28% phosphatidylcholine, 25.6% phosphatidylethanolamine, 14.4% phosphatidylinositol, 8.8% lysophosphatidylcholine, 6.7% cardiolipin, 3.7% lysophosphatidylethanolamine, 3.6% lysophosphatidylinositol, 3% phosphatidylglycerol, 2.9% phosphatidylserine and 3.3% unidentified phospholipids [10]. The partially purified asolectin was stored as chloroform solution (106 mg/ml) at $-18°C$ under an atmosphere of N_2.

Vesicles were prepared using the sonication method essentially as described previously [11]. Partially purified asolectin (approx. 50 mg) was dried by a

stream of N_2 to evaporate the chloroform; 10 ml of 20 mM glutamate-NaOH buffer at various acidic pH values (2.5—5.0) were added to the dried lipid and the resulting suspension was sonicated in the cold under N_2 for 10 min using a Branson Sonifier (model W350). The pulsed mode of 50% duty cycle was used and the output power was set at about 75 W. All solutions were prepared with no Cl^-, e.g., pH adjustment of buffers was carried out with H_2SO_4 and NaOH. This was because it had been reported that lipid vesicles are relatively permeable to Cl^- [5], and also because the fluorescence of quinine is quenched by Cl^-.

Similar procedures were used to prepare internally alkaline vesicles except that the buffer was changed to 50 mM glycine-NaOH (pH 8.5—10.5) and the sonication time was reduced to 5 min.

Imposition of a pH gradient (ΔpH) across the vesicles and the measurement of fluorescence changes. Phospholipid vesicles prepared at a particular acidic pH were diluted by 20-fold into a fluorescence cuvette containing 2 ml sodium citrate at various pH values (4.5—6.3), thus providing an established ΔpH. The light-scattering and intrinsic fluorescence intensity of the vesicle suspension were recorded at the specific wavelength of the fluorescent probe used as a background. Probe uptake was initiated by adding a small aliquot of the probe and the resulting fluorescence change was monitored continuously. At the end of the uptake, monensin (2.5 ng/ml) was added to discharge the pH gradient and the resulting reversal of the fluorescence change was recorded.

A similar procedure was used to lower the external pH of the vesicles prepared with alkaline interiors. For this case, the external buffer was 50 mM Na^+-imidazole instead.

Preparation of gastric microsomes. Gastric microsomal vesicles were isolated from porcine fundic mucosal homogenates by differential and density gradient centrifugation procedures as previously described [6]. The pH gradient generated by the K^+-ATPase in these vesicles was measured using the 9-aminoacridine accumulation ratio [6]. The reaction medium contained 150 mM, KNO_3, 10 mM $MgSO_4$, 0.05 mM EDTA, 10 μM 9-aminoacridine and 10 mM Pipes (pH 6.7). The reaction was started by adding 0.5 mM ATP. Valinomycin (2.5 μM) was used to stimulate the formation of the pH gradient.

Miscellaneous. All fluorescence measurements were made with a Perkin Elmer MPF-44A spectrofluorimeter at room temperature (21—23°C). Quinine sulfate was obtained from Sigma Chemical Co. Acridine and *o*-hydroxycinnamic acid were from Aldrich. Monensin was a gift from Eli Lily Company and dissolved in ethanol. Wavelengths used for the fluorescence measurements were 347 → 445 nm (excitation → emission) for the acidic spectral peak of quinine and 340 → 380 nm for the alkaline peak. The acidic peak of acridine was monitored at 350 → 480 nm while the alkaline peak was measured at 350 → 430 nm. For *o*-hydroxycinnamic acid 360 → 500 nm was used.

Results

Measurement of intravesicular pH of internally acidic vesicles

The purpose of the present study was to devise a method using fluorescent probes for the direct measurement of intravesicular pH, and which gives a posi-

tive signal instead of fluorescence quenching. The approach, therefore, was to search for a probe which provides a characteristic enhancement of fluorescence when the probe senses an acidic environment. If we further require that this probe be an amine, then it will distribute according to the existing ΔpH across the membrane. Since the probe has a characteristic fluorescence in an acidic environment, the trapping and concentrating effect of the pH gradient should give rise to a large enhancement of fluorescence intensity. We examined a series of fluorescent amines and two were found to be suitable for the above requirement, they are quinine and acridine. Since, qualitatively, they behave

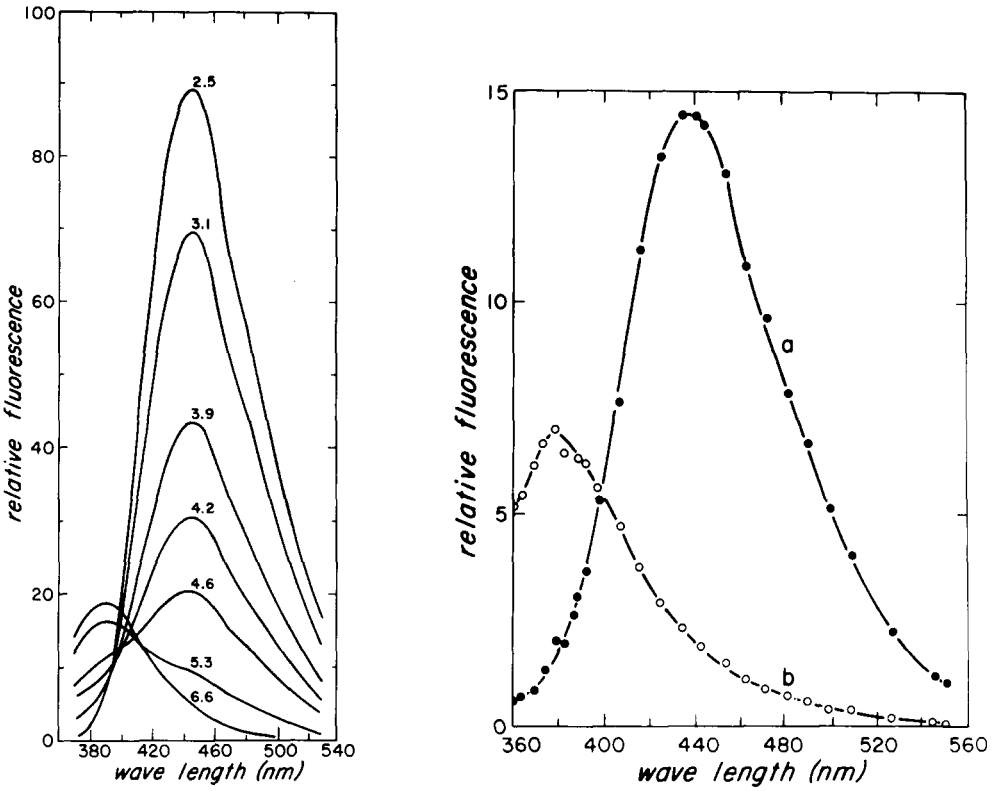

Fig. 1. Effect of pH on the fluorescence emission spectrum of quinine. 2.5 μM quinine was added to 2 ml of 20 mM citrate buffer at pH 2.5. The emission spectrum was recorded with the excitation wavelength set at 347 nm. Small aliquots of concentrated NaOH were added to titrate to the various pH values as indicated on the individual recorded spectra.

Fig. 2. Effect of ΔpH on the emission spectrum of quinine. Liposomes were prepared from asolectin as described in the text. A small aliquot of internal acidic liposomes (pH_i = 2.5) was diluted 20-fold into a medium containing 25 mM citrate at pH 6.0, thus creating a ΔpH across the liposomes. The final lipid concentration was 0.25 mg/ml. The background intensity due to light scattering and fluorescence of these liposomes was recorded with the excitation wavelength set at 340 nm. A small aliquot of quinine was added to give a final probe concentration of 0.5 μM. Curve a (•) represents the resulting fluorescence spectrum after subtracting the background intensity. The spectrum shows an emission maximum of about 445 nm, characteristic of quinine in an acidic environment. Dissipation of the ΔpH across the liposomes by the addition of 2.5 ng/ml of the ionophore, monensin, converted the emission spectrum to curve b (○). The fluorescence intensity in curve b is lower and the emission maximum is changed to about 380 nm, characteristic of quinine in a more alkaline medium.

similarly we will present a detailed characterization for only one of them, quinine.

Fig. 1 shows the emission spectrum of quinine at various pH values. It can be seen that the fluorescence intensity at 445 nm increases with decreasing pH while that at 380 nm shows the reverse. This pH-dependent spectral shift suggests a very convenient way to measure directly the intravesicular pH. Since the imposition of a ΔpH across the membrane will concentrate the probe into an intravesicular space which is more acidic than the extravesicular medium, we should measure a decrease in fluorescence intensity at 380 nm (alkaline peak) but enhancement at 445 nm (acidic peak).

In Fig. 2, the qualitative operation of the method is shown by the ΔpH-induced spectral shift from 380 to 445 nm. Phospholipid vesicles were prepared at pH 2.5 and diluted 20-fold into a medium of pH 6.0. Addition of 0.5 μM quinine resulted in an emission spectrum (curve a) characteristic of an acidic medium with an emission maximum at about 445 nm. This indicates that most of the probe was concentrated inside and therefore sensed the acidic environment. Discharging the ΔpH by adding an Na^+-H^+ exchange ionophore, monensin, should allow the probe to leave the vesicles and transform the spectrum to that characteristic of a medium of pH 6.0. This is indeed the case as shown by curve b of Fig. 2.

The time course for the changes in fluorescence intensity at 445 and 380 nm is shown in Fig. 3A and B, respectively. A detailed development of the

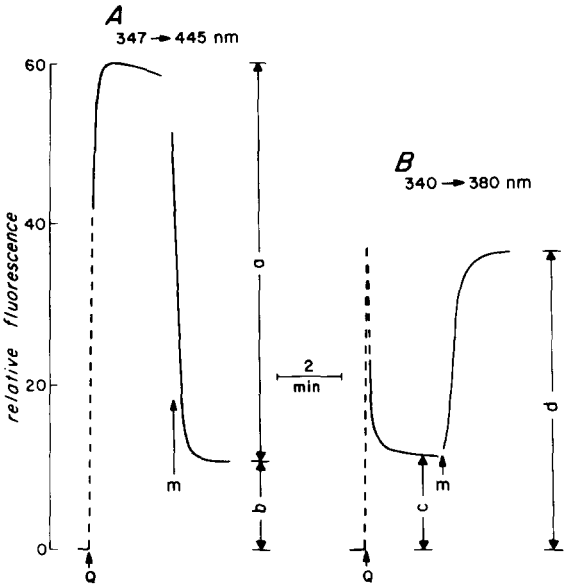

Fig. 3. Time course of the fluorescence changes induced by ΔpH. Internal acidic liposomes (pH_i = 2.5) were diluted 20-fold into a medium containing 25 mM citrate at pH 5.1 to create a ΔpH across the liposomes. Final liposome concentration was 0.25 mg/ml. A small aliquot of quinine was added to give a final concentration of 0.25 μM (indicated by the arrow labeled Q). The resulting increase in fluorescence intensity at 445 nm (excitation 347 nm) is shown in A; the concomitant decrease at 380 nm (excitation 340 nm) is shown in B. The respective reversal of fluorescence due to addition of 2.5 ng/ml of the Na^+-H^+ exchange ionophore monensin (arrow labeled m) is also shown.

method for calculating intravesicular pH from fluorescence data is presented in Appendix. The fraction of probe taken up by the vesicles is calculated from the ΔpH-induced decrease in fluorescence intensity at 380 nm. Thus, the fluorescence intensity in the presence of a pH gradient, F_{380} (c in Fig. 3B), is proportional to the amount of probe on the outside while the intensity after the addition of monensin, F'_{380} (d in figure 3B), should be proportional to total amount of probe. The fraction of the probe which remains on the outside (r_o) in the presence of a pH gradient is therefore the ratio of the two:

$$r_o = F_{380}/F'_{380} \tag{1}$$

The fraction that goes into the vesicles (r_i) should then be:

$$r_i = 1 - r_o \tag{2}$$

To calculate the intravesicular pH we need to know the fluorescence intensity due to the intravesicular probe (F_i). This can be obtained from the difference between the total fluorescence at 445 nm and that contributed by the probe in the outside bulk medium. Thus:

$$F_i = F_{445} - F'_{445} \cdot r_o \tag{3}$$

where F'_{445} is the 445 nm fluorescence in the absence of a pH gradient (b in Fig. 3A). Dividing F_i by r_i we get the total fluorescence (F_i^*) if all the probes were on the inside:

$$F_i^* = F_i/r_i = \frac{F_{445} - F'_{445} \cdot r_o}{r_i} = \frac{F_{445} - F'_{445} \cdot r_o}{1 - r_o} \tag{4}$$

By comparing F_i^* with a fluorescence vs. pH curve constructed with the same concentration of probe, the intravesicular pH can thus be obtained directly.

The accuracy of the pH thus determined requires that the fluorescence of the intravesicular probe should respond only to internal pH and not be quenched by binding or any other factors. To evaluate critically this condition, we prepared phospholipid vesicles of various internal pH while the external medium was fixed at pH 6.0. The results of such a calibration are shown in Fig. 4 where the calculated internal pH is plotted against the actual pH at which the vesicles were prepared. As can be seen the measured values fall closely along the 45° line, indicating excellent correlation.

As an alternate test, a series of measurements were made with the internal pH constant while the external pH was varied. The internal pH should be independent of external pH and therefore we expect the calculated internal pH should also demonstrate this independence. Phospholipid vesicles were prepared at pH 3.0 and the external pH varied from 4.5 to 6.3. As shown in Fig. 5, the internal pH calculated from fluorescence measurements was relatively constant over the entire range of external pH tested. The average measured internal pH of about 3.2 was slightly more alkaline than the solution in which the vesicles were prepared.

The close correlations demonstrated above strongly support the present method as an accurate and direct way to measure intravesicular pH. We therefore conclude that under appropriate conditions, quinine behaves as an ideal probe. Its distribution across the membrane is primarily determined by the

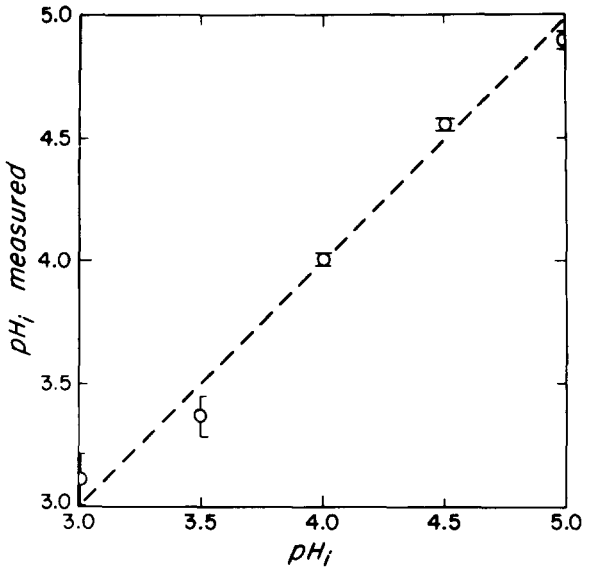

Fig. 4. Correlation between the internal pH of liposomes (pH_i) and the pH value measured by the present method (pH_i measured). Liposomes were prepared at various pH values in 20 mM glutamate according to the values represented on the horizontal axis. The liposomes were diluted 20-fold into a medium containing 25 mM citrate at pH 6.0 in order to produce the ΔpH. The final liposome concentration was 0.25 mg/ml. The measurement of internal pH (pH_i measured) was performed with 0.25 μM quinine as described in the text. The standard curve of fluorescence intensity vs. pH was constructed with the same buffer (20 mM glutamate) as that used in preparing the liposomes. The indicated values are the mean of three separate measurements on the same liposome preparation with the bars representing the standard deviation. Perfect correlation between the predicted and measured values is represented by a 45° line (dashed line).

existing pH gradient and can be measured conveniently by the decrease in fluorescence intensity at 380 nm. The intravesicular quinine responds to the acidic environment with corresponding increase in its fluorescence intensity at 445 nm.

In principle, quinine, acridine and the previously used fluorescent probe, 9-aminoacridine, are similar. They all are fluorescent amines and they respond to a pH gradient in analogous ways. Moreover, acridine and 9-aminoacridine have the same structure except for one amino group. Nonetheless, they have distinct fluorescence properties. In the case of 9-aminoacridine, the probe becomes non-fluorescent as it distributes into the intravesicular space. The exact mechanism of this quenching effect is not known; perhaps the most plausible explanation is the concentration-dependent, self-quenching mechanism suggested by Deamer et al. [5]. Thus, as the amine is accumulated by the pH gradient, the internal concentration may reach such a level that energy transfer between molecules of the same species may cause a decrease in quantum yield. If this is true, then the extent of quenching at a given ΔpH should be strongly dependent on the total probe concentration. If the total quinine concentration is increased there should be a corresponding increase in the internal concentration and thus in the fluorescence enhancement (defined as a in Fig. 3A), until the intravesicular quinine concentration reaches such a high level that self-quenching becomes important. As shown in Fig. 6, the fluorescence

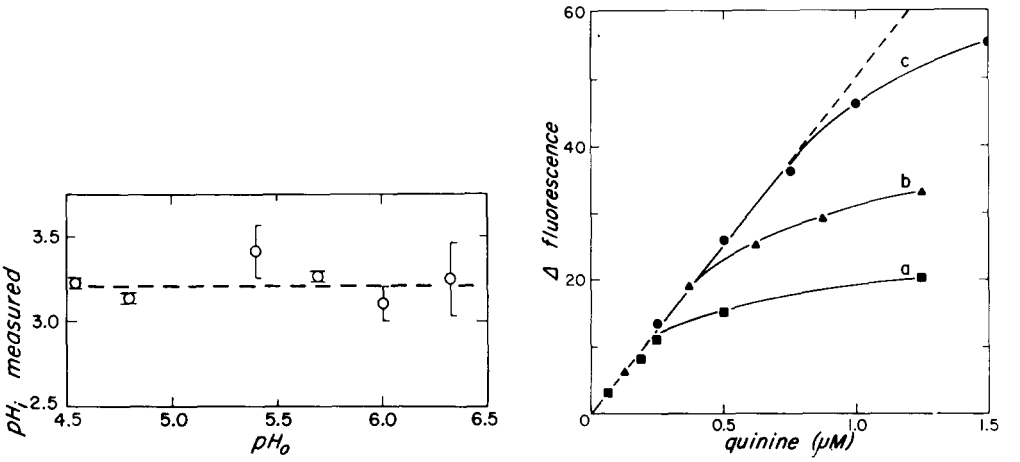

Fig. 5. Effect of variation of external pH on the measured internal pH of liposomes. Liposomes were prepared at pH 3.0 in 20 mM glutamate as described in Materials and Methods. The external pH was varied by 20-fold dilution of the liposomes into 25 mM citrate at various pH (pH_o). The internal pH was measured (pH_i measured) by using 0.25 μM quinine as described in the text. Values are given as the mean of two separate measurements at each (pH_o).

Fig. 6. Effect of quinine and liposome concentrations on the enhancement of fluorescence intensity induced by ΔpH. Liposomes were prepared at pH 4.0 and diluted into 25 mM citrate at pH 6.0. Fluorescence enhancement (Δ fluorescence) as defined in Fig. 3A was plotted against increasing concentration of quinine. The concentration of liposomes used was 0.07, 0.25 and 0.25 mg/ml in curves a, b and c, respectively.

enhancement at 445 nm (curve a) increased linearly up to 0.2 μM. However, a progressive deviation from linearity was observed at higher probe concentration. This is consistent with the self-quenching mechanism. If there exists a critical internal concentration that quinine must achieve before self-quenching becomes important, then an increase in the amount of phospholipid vesicles (intravesicular space) should increase the total probe concentration required to achieve the necessary level. This is shown in Fig. 6 (curves b and c) where an increase in the amount of vesicles also progressively increased the total quinine concentration needed to achieve self-quenching (as manifested by the deviation from linearity). It is important, therefore, to evaluate the self-quenching effect when applying the present method to other systems. Unless other factors become limiting the lowest possible concentration of probe should be used.

Application to the isolated gastric microsomes

We previously established with the use of 9-aminoacridine that isolated gastric vesicles generate an internally acidic H^+ gradient of 3 to 4 pH units when provided with ATP [6]. The molecular machinery is an H^+-K^+ exchange ATPase which transports H^+ into the intravesicular space in exchange for K^+ [6,12]. Valinomycin is thus required to provide access of K^+ to the internal site [6,13]. The time-course changes in fluorescence intensity for gastric microsomes incubated with quinine are shown in Fig. 7. Activation of the H^+-transport system by ATP produced a small increase in fluorescence intensity at 445 nm (Fig. 7A)

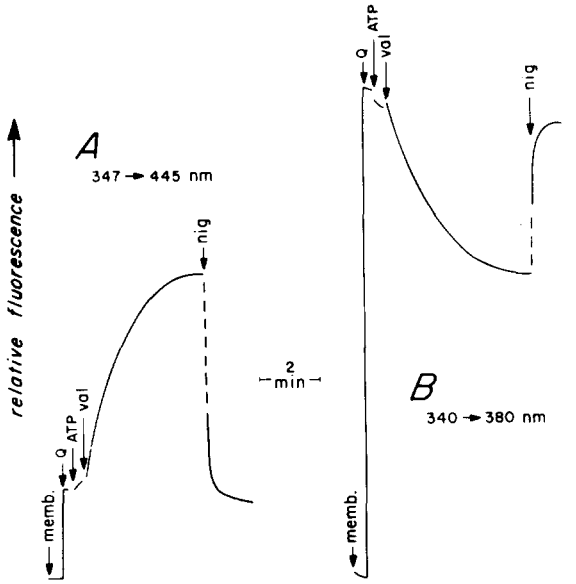

Fig. 7. Time course of fluorescence change of quinine induced by activation of the H^+-transport system in gastric microsomes. 0.5 μM quinine was added (indicated by arrows labeled Q) into a medium containing 150 mM KNO_3, 1 mM $MgSO_4$, 10 mM Pipes (pH 6.7), 0.05 mM EDTA and 0.13 mg/ml gastric microsomes. The H^+-transporting system in these microsomes was activated by the addition of 0.5 mM ATP (arrows indicated by ATP) and further stimulated by 2.5 μM valinomycin (arrows labeled val). The resulting fluorescence enhancement at 445 nm and the concomitant decrease at 380 nm are shown in A and B, respectively. The H^+ gradient was dissipated by the addition of 0.5 μg/ml nigericin (arrows labeled nig).

and a concomitant decrease in intensity at 380 nm (Fig. 7B). Addition of valinomycin greatly stimulated the formation of the pH gradient and, therefore, also the fluorescence changes as shown by the recordings. Nigericin was used to discharge the ΔpH and reverse the fluorescence change. Using the data of Fig. 7 the internal pH was calculated to be 3.8, while the external pH was fixed at pH 6.7, corresponding to a ΔpH of 2.9 pH units. An identical result was obtained using 9-aminoacridine as a ΔpH probe under the same conditions (data not shown). In the latter case, the vesicular volume of 2 μl/mg of microsomal protein [6] was assumed in order to make the calculation of ΔpH from the fluorescence-quenching data.

A qualitative probe for internal alkaline vesicles

In principle, the reasoning presented in previous sections should be equally applicable to the measurement of intravesicular pH of internally alkaline vesicles. However, a fluorescent weak acid is needed instead of an amine. This fluorescent acid should have a pK_a lower than the internal pH. Similar to the amine probe, the undissociated form of the acid should be freely permeable to the membrane and be converted to the charged form when it senses the alkaline internal pH. If the charged form is impermeable then the resulting probe distribution should be in accord with the ΔpH across the membrane. A brief survey of commonly used fluorescent indicators showed *o*-hydroxycinnamic acid may be suitable. Fig. 8 shows the effect of pH on the emission spectrum

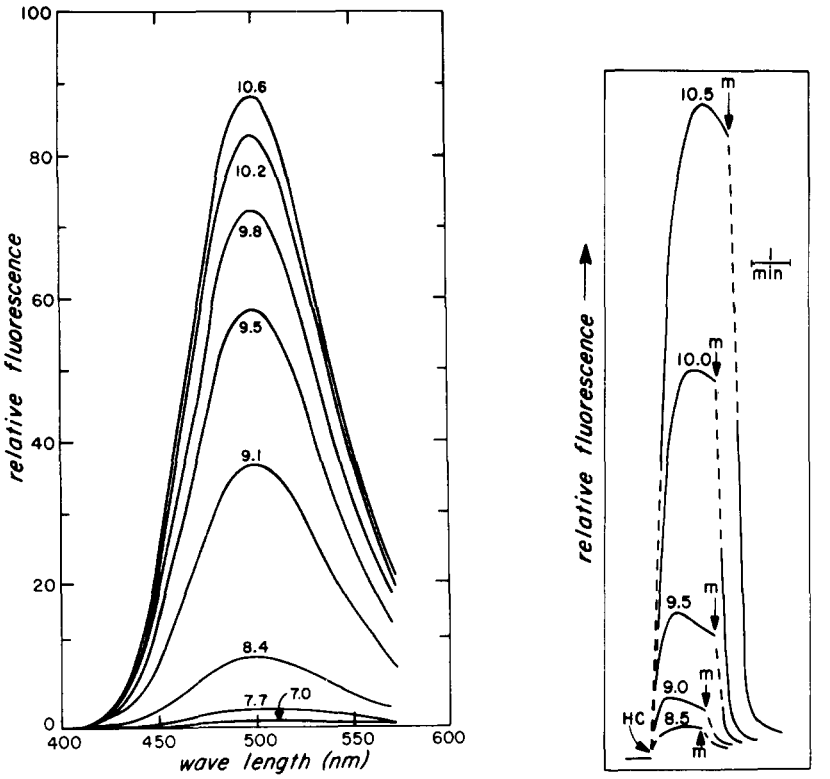

Fig. 8. Effect of pH on the fluorescence spectrum of *o*-hydroxycinnamic acid. 10 μM *o*-hydroxycinnamic acid was added to the medium containing 50 mM imidazole and 25 mM glycine, pH 7.0. The emission spectrum (excited at 360 nm) was recorded. The pH of the medium was then changed by the addition of NaOH. Spectra are shown for various pH values of the media as indicated in the graph.

Fig. 9. Time course of ΔpH-induced fluorescence enhancement of *o*-hydroxycinnamic acid. Liposomes were prepared in 50 mM glycine at the various pH values as indicated by the numbers on the figure. ΔpH was imposed by 10-fold dilution of the liposomes in a medium containing 50 mM imidazole at pH 6.5. 10 μM *o*-hydroxycinnamic acid was added (indicated by arrow labeled HC) and the resultant fluorescence enhancement recorded. Increasing internal pH resulted in increasing amount of enhancement. Addition of 2.5 ng/ml monensin (m) dissipated the ΔpH and reversed the fluorescence change.

of *o*-hydroxycinnamic acid. It is virtually non-fluorescent below pH 7.0 but the fluorescence intensity at 500 nm increases dramatically as the pH is raised to 10.6. Unfortunately, there is no pH-dependent spectral shift, thus it is not possible to calculate the internal pH without an independent estimate of probe distribution (as described in Appendix). However, it is still of interest to see whether the probe responds to ΔpH and this serves as a test for the principle.

Phospholipid vesicles with internal pH from 8.5 to 10.5 were prepared while the external pH was fixed at 6.5. Fig. 9 shows that the addition of 10 μM *o*-hydroxycinnamic to the vesicles resulted in dramatic enhancement of fluorescence intensity at 500 nm, indicating that the probe was concentrated into the intravesicular space by the pH gradient and thus sensed the alkaline environment. Discharging the ΔpH with monensin completely reversed the fluorescence enhancement, lending further support to the interpretation that ΔpH is the driving force for the enhancement. Since the external pH was fixed at 6.5,

more alkaline internal pH resulted in larger ΔpH and thus more *o*-hydroxycinnamic acid should be concentrated inside. This, together with the increase in quantum yield of the probe in alkaline medium, should give rise to a large increase in intensity. We therefore expect that the more alkaline the internal pH the more dramatic the enhancement is, as demonstrated in Fig. 9. We thus conclude that in this case the principle is at least qualitatively correct.

Discussion

In this report, we demonstrated an alternative method for the measurement of intravesicular pH. The basic principle behind the present method and the previously used fluorescence-quenching method is the same. Both make use of the ΔpH-dependent distribution of weak bases. To measure the resultant distribution of probes, the former method depends on a poorly understood mechanism of quenching of the fluorescence of an intravesicular probe. Moreover, another independent measurement of volume of the H^+ space is required in order to calculate the ΔpH from the fluorescence data [4,6,8]. In the present method improvements were made by using the pH-dependent spectral shift of the probe. Two fluorescent amines, quinine and acridine, were found to have red shifts in the emission maximum ($\Delta\lambda_{max}$ = 65 and 50 nm for quinine and acridine, respectively) and to increase in quantum yield in going from alkaline to acidic medium. By measuring both the decrease in the alkaline peak and the increase in the acidic peak, the ΔpH-induced changes in the distribution and the fluorescence enhancement of the intravesicular probes can be calculated. The internal pH can then be obtained directly by comparing with a standard curve of fluorescence vs. pH and no measurement of volume of the H^+ space is necessary. The latter advantage becomes essential when the measurement of the H^+ space is impossible, for example, if one wished to measure the pH of a small acidic compartment within a cell. In fact, from the measured pH and dye-uptake ratio, it would be possible to calculate the volume of the H^+ space. Quinine has an additional advantage as a probe for ΔpH; it is a diamine (second p*K* about 4.2) and its accumulation is much more sensitive to the pH gradient, as described by Schuldiner et al. [4]. However, a possible disadvantage in using quinine in biological systems is the extreme sensitivity to quenching by Cl^-, thus the requirement for Cl^--free solutions.

One further advantage of the general method we describe is its use of a low concentration of probe. Because of the accumulating effect of the pH gradient, the internal probe concentration can reach a very high level. This can introduce many complications. The most serious one would be the significant perturbation of intravesicular pH. In theory, the probe penetrates in its neutral form and becomes charged by accepting an H^+ from the intravesicular medium. This would alter the intravesicular pH if the probe concentration were to reach such a high level that exceeds the buffering power of the internal medium. In an effort to lower the total probe concentration using the previous 9-aminoacridine accumulation method we found that the fluorescence quenching also diminished (unpublished observation). The present method, which requires one to two orders of magnitude less probe, thus represents a significant improvement.

The applicability and accuracy of the present method lend strong support to the original interpretation of Schuldiner et al. [4] that fluorescent amines do penetrate and accumulate into intravesicular space in accordance with the existing pH gradient. It is highly unlikely that other effects can give rise to fluorescence enhancement in the present case and quenching in the former case in exact correspondence to the pH gradient. Evidence was obtained in the present study by varying vesicular volume and total probe concentration to substantiate the suggestion by Deamer et al. [5] that the high intravesicular probe concentration produces a self-quenching effect. This is further supported by our observation that the ΔpH-induced quenching effect on 9-aminoacridine fluorescence diminishes with lower probe concentration (unpublished data). Thus, it is critical to use a high probe concentration in the previous method in order to produce complete quenching, while the opposite is necessary in the present method.

It is not the purpose of the present study to argue that the particular probes used here are of universal applicability but rather to demonstrate the validity of the reasoning behind the selection of probes. In fact, any factors other than intravesicular pH that can alter the fluorescence property of the probe would affect the accuracy of the result. These factors are likely to be present and to vary with different systems. It is therefore essential to look for a suitable probe for a particular system.

Appendix

Given the system of a dye (R), which is also a weak base, distributed on two sides of a membrane, and in chemical equilibrium with H^+ in both compartments, so that:

$$\frac{R_i/V_i \cdot [H_i^+]}{RH_i/V_i} = K_a = \frac{R_o/V_o \cdot [H_o^+]}{RH_o/V_o} \tag{5,6}$$

where K_a is the equilibrium constant, the subscripts i and o, respectively, represent the inner and outer compartments, and R and RH represent the unprotonated and protonated forms of the dye in their respective compartmental volume, V. The total fluorescence measured at 445 nm (F_{445}) and 380 nm (F_{380}) can be described as the sum of the contribution of all species. Thus:

$$F_{445} = Q_{445}^{RH}(RH_i + RH_o) + Q_{445}^{R}(R_i + R_o) \tag{7}$$

$$F_{380} = Q_{380}^{RH}(RH_i + RH_o) + Q_{380}^{R}(R_i + R_o) \tag{8}$$

where Q_{445}^{RH} and Q_{380}^{RH} are the fluorescence coefficients of the acidic dye form, RH, at the respective wavelengths, and Q_{445}^{R} and Q_{380}^{R} are the respective coefficients of the R form. In the case of quinine, Q_{445}^{R} and Q_{380}^{RH} are very small (e.g., Fig. 1) and, as a good approximation, F_{445} and F_{380} can be described as the respective acidic and alkaline peaks, simplifying to:

$$F_{445} \approx Q_{445}(RH_i + RH_o) \tag{9}$$

$$F_{380} \approx Q_{380}(R_i + R_o) \tag{10}$$

In this notation, $Q_{445} \equiv Q_{445}^{RH}$ and $Q_{380} \equiv Q_{380}^{R}$. We designate the total amount of

dye, $A_t = RH_i + RH_o + R_i + R_o$. Hence the fraction of dye in the outer compartment, r_o, is:

$$r_o = \frac{RH_o + R_o}{A_t} \tag{11}$$

and the inner compartment, r_i, is:

$$r_i = \frac{RH_i + R_i}{A_t} \tag{12}$$

where $r_i + r_o = 1$. Substituting Eqns. 6 and 11 and rearranging:

$$r_o A_t = RH_o(1 + K_a/[H_o^+]) \tag{13}$$

Similarly for Eqn. 12 we have:

$$r_i A_t = RH_i(1 + K_a/[H_i^+]) \tag{14}$$

Substituting Eqns. 13 and 14 into Eqn. 9:

$$F_{445}/Q_{445} = \frac{r_o A_t}{1 + K_a/[H_o^+]} + \frac{r_i A_t}{1 + K_a/[H_i^+]} \tag{15}$$

Thus, if the relative amounts of dye in the inner and outer compartments were measured by an independent method (e.g., flow dialysis, rapid filtration, etc.) then $[H_i^+]$ could be determined from the fluorescence, since all other values are constant or predetermined by design. However, it must be pointed out that when the internal pH is more than one unit below the pK (i.e., $K_a/[H_i^+]$ approaches zero), then the actual determination of $[H_i^+]$ by this method is very sensitive to small errors of measurement.

There is an alternative method for measuring $[H_i^+]$ using an empirical calibration curve of pH vs. fluorescence and employing the simplifying assumption that intravesicular volume is much less than external volume ($V_i << V_o$). This is the method used in the main text and requires measurement of quinine fluorescence at both the alkaline and acid peaks with an existing pH gradient (F_{380} and F_{445}) and after the gradient has been dissipated by appropriate ionophores (F'_{380} and F'_{445}). First, we will use the alkaline peak as a convenient measure of the fractional amount of dye in the outer compartment, r_o.

Eqn. 10 describes F_{380} in terms of R_o and R_i in the presence of a pH gradient. Since $V_i << V_o$, we will assume $R_i << R_o$, thus:

$$F_{380}/Q_{380} \approx R_o \tag{16}$$

and substituting Eqn. 9 into the expression of total outside dye:

$$R_o + RH_o = F_{380}/Q_{380} + F_{380}/Q_{380} \cdot \frac{[H_o^+]}{K_a} \tag{17}$$

With no pH gradient:

$$F'_{380} = Q_{380}(R'_o + R'_i)$$

Since $V_i << V_o$, we will assume that $R'_i << R'_o$ and $RH'_i << RH'_o$:

$$F'_{380}/Q_{380} \approx R'_o \tag{18}$$

$$A_t \approx R'_o + RH'_o = F'_{380}/Q_{380} + F'_{380}/Q_{380} \cdot \frac{[H^+_o]'}{M} \tag{19}$$

Substituting Eqns. 17 and 19 into Eqn. 11 we have:

$$r_o = \frac{F_{380}/Q_{380} + F_{380}/Q_{380} \cdot [H^+_o]/K_a}{F'_{380}/Q_{380} + F'_{380}/Q_{380} \cdot [H^+_o]'/K_a} = \frac{F_{380}(1 + [H^+_o]/K_a)}{F'_{380}(1 + [H^+_o]'/K_a)} \tag{20}$$

when $[H^+_o] = [H^+_o]'$, e.g., as in a well buffered external medium, then:

$$r_o = \frac{F_{380}}{F'_{380}} \tag{21}$$

Thus, the ratio of the 380 nm fluorescence in the presence of a pH gradient to that after the gradient is dissipated closely approximates r_o.

The amount of fluorescence at the acidic peak in the presence of a pH gradient, F_{445}, is given by Eqn. 9: and in the absence of a pH gradient:

$$F'_{445} = Q_{445}(RH'_i + RH'_o)$$

Since $V_i << V_o$, $RH'_i << RH'_o$ and $R'_i << R'_o$, thus:

$$F'_{445} \approx Q_{445} RH'_o \tag{22}$$

and

$$A_t \approx RH'_o + R'_o \tag{23}$$

Substituting into Eqn. 11 and using Eqn. 6 we have:

$$r_o = \frac{RH_o + R_o}{RH'_o + R'_o} = \frac{RH_o(1 + [H^+_o]/K_a)}{RH'_o(1 + [H^+_o]'/K_a)} = \frac{RH_o}{RH'_o} \tag{24}$$

The fluorescence from the outside dye is:

$$Q_{445} \cdot RH_o = RH'_o \cdot Q_{445}\left(\frac{RH_o}{RH'_o}\right) = F'_{445} \cdot r_i \tag{25}$$

and by combining Eqn. 25 into Eqn. 9 we have:

$$Q_{445} \cdot RH_i = F_{445} - F'_{445} \cdot r_o \tag{3}$$

Eqn. 3 represents the 445 nm fluorescence from the intravesicular probe at a particular internal pH. We could calculate what the fluorescence would be if all of the dye were at that same internal pH by:

$$Q_{445} \cdot \frac{RH_i}{r_i} = \frac{F_{445} - F'_{445} \cdot r_o}{r_i} = \frac{F_{445} - F'_{445} \cdot r_o}{1 - r_o} \tag{4}$$

This value can be compared to a calibration curve in which the fluorescence is measured for the same total amount of dye at various pH values. Thus, the internal pH can be reliably estimated and with good sensitivity even when pH_i is more than 2 units below the pK of the dye.

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