The Behavior of 9-Aminoacridine as an Indicator of Transmembrane pH Difference in Liposomes of Natural Bacterial Phospholipids

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Abstract

The behavior of 9-aminoacridine as an indicator of pH differences artificially set across a membrane has been reexamined in liposomes prepared from bacterial phospholipids extracted from chromatophores of *Rhodopseudomonas capsulata* grown photohetero-trophically. The dye behaves as an ideal indicator for pH differences lower than about three units; at higher pH's the expected linear dependence of $Q/(100-Q)$ vs. pH is no longer strictly observed. Similarly a linear dependence upon the volume of the liposomes added has been verified. The amine ceases to respond to pH changes when the pH of the external medium exceeds the value of 10, cor-responding to the pK_a of 9-aminoacridine. The apparent volume of the inner phase of liposomes, as calculated from fluorescence quenching, but not the slope of dependence of fluorescence on pH, appears to be affected by several factors, including the ionic composition, the osmolarity of the external medium, and the microscopic structure of the liposomes. Millimolar concentrations of earth-alkaline cations diminish the apparent internal volume of liposomes, in agreement with the complexing effect of these ions on phospholipid bilayers. The osmotic response of the apparent inner volume has also been verified; this parameter decreases linearly with the reciprocal of the external osmolarity, as expected from the van't Hoff relation; an osmolarity exceeding 0.3 M is, however, necessary in order to observe this effect.

Introduction

The use of fluorescent amines, and in particular of 9-aminoacridine for the indirect determination of transmembrane pH differences in energy transducing membranes, is one of the most useful methods so far proposed, owing to its simplicity and adaptability to different experimental conditions [1]. According to the assumptions implicit in the method [2], amines in their unprotonated form should be rapidly permeable through the membrane and should distribute between the inner and outer compartment at concentrations related to the pH of the two compartments according to their protonation constant. Compartmentation of the amines can be evaluated by their fluorescence, since it is assumed that molecules entrapped in the inner compartment possess a negligibly small fluorescence.

Data confirming this behavior in macroscopic model systems [3], in liposomes [4], and in photosynthetic organelles [2, 5, 6] have been presented; the method has been also extensively used to measure the energization level of membranes, especially in photosynthetic systems [6, 7, 8].

Recently however the validity of this method, at least in some systems, has been challenged by Fiolet et al. [9], who presented evidence that in chloroplasts and in lecithin liposomes considerable deviation from ideal behavior could be observed. In particular these authors showed that in liposomes, alteration of the surface charge, obtained by addition of cationic or anionic amphipatic molecules, can induce large perturbation of the pH-dependent fluorescence quenching.

In view of these conflicting results [4, 9], the response of 9-aminoacridine to transmembrane pH differences in liposomes was reexamined. Special efforts were made in this study to mimic the phospholipid composition of natural membranes: for this reason phospholipids were extracted from bacterial chromatophores and liposomes prepared therefrom.

Materials and Methods

Rhodopseudomonas capsulata, strain St. Louis (American Type Culture Collection No. 23782) was grown photoheterotrophically in anaerobiosis in a malate containing medium as previously described [10]. Cells were harvested in the late logarithmic phase of growth and chromatophores, prepared according to procedures already reported [11], were suspended in a 50 mM Na-glycylglycine buffer, pH 7.2, at a concentration ranging from 0.5 to 1.0 mg Bchl. per ml.

Phospholipids were extracted from chromatophores following the

procedure of Rouser and Fleischer [12], modified as follows: Lipid extracts, obtained from bacterial chromatophores with standard procedures [12], were evaporated to dryness under reduced pressure and dissolved in a small volume of the same solvent. Nonlipid contaminants were removed by extracting twice with a 0.8% KCl solution. The lipid sample, evaporated to dryness, was dissolved in a small volume of ethyl ether. Polar lipids were separated by absorption on silicic acid (40 mg per ml of lipid extract) and eluted therefrom with methanol (25 ml per g of silicic acid) after two washes with ethyl ether to remove neutral lipid contaminants.

Purified phospholipids were concentrated under reduced pressure and stored under nitrogen at $-80°C$.

Separation and identification of the phospholipids was obtained by thin-layer chromatography of silica gel G plates (type 60, Merck, Darmstadt), developed with chloroform–methanol–water (65 : 25 : 4) [13]. Lipids were identified on the basis of their R_f values and of cochromatography with authentic samples. Quantitative determination of lipid phosphorus was performed on the whole extract or on individual chromatographic spots, scraped off from the plates, following the procedure of Parker and Peterson [13].

For the preparation of liposomes an adequate amount of phospholipid solution (generally corresponding to about 0.5 mg of lipid phosphorus) was evaporated to dryness under reduced pressure. The residue, dissolved in few drops of methanol, was dispersed in buffer (generally 0.1 M phosphate–pyrophosphate buffer) by sonicating under nitrogen, for variable lengths of time with a MSE 150-W sonifier, equipped with a No. 34041 exponential probe, and vibrating at an amplitude of 14 μm (peak to peak).

It was found that a sonication time of at least 20 min was necessary to obtain maximum quenching of 9-aminoacridine upon addition of a given volume of liposome suspension; electron microscopic examinations showed that only with such a long irradiation were small, double-layered liposomes of an average diameter of 300 Å obtained [cf. also Reference 14]. The liposome suspension was routinely passed through a small G 75 Sephadex column (1 × 15 cm) equilibrated with 10 mM phosphate pyrophosphate buffer containing 14% sucrose and stored in ice. Under these conditions the preparation was found to be stable for several days as far as its effect in fluorescence quenching and its ultramicroscopic structure were concerned.

Liposome preparations were observed by electron microscopy using a Siemens 1-A electron microscope, after negative staining with 1% uranyl acetate at pH 5.0, or with 2.15% ammonium molybdate, pH 5.0.

The quenching of fluorescence of 9-aminoacridine was measured with a filter fluorimeter as previously described [15]; emission and excitation spectra were recorded with the aid of a Perkin Elmer MPF4 spectrofluorimeter.

Results and Discussion

Phospholipid Composition of Bacterial Chromatophores

The typical phospholipid composition of preparations of chromatophores, isolated from cells of *Rhodopseudomonas capsulata* strain St. Louis, grown photoheterotrophically and anaerobically, are shown in Table I.

The results are in good agreement with those obtained in whole cells of the same bacterium by Steiner et al. [16], who analyzed chromatographically the deacylated phospholipids. The most noticeable feature of the analysis presented is the large amount of phosphatidylglycerol, generally from 25% to 30%, which confers a strongly acidic character to the lipid component of the membrane.

It is clear therefore that, if phospholipid liposomes are to be taken as a model of natural membranes, these liposomes must be much more acidic than those normally used in previous studies with acridine dyes [4, 9]. This condition is particularly important in view of the apparent response of 9-aminoacridine fluorescence to the acidity of liposomes [9], or more generally in consideration of the profound effects exerted by surface charges on the structure of phospholipid layers [17]. The percentage composition of the natural phospholipid mixture used for the preparation of the liposomes in this study is also reported in Table I and closely agrees with that of the native chromatophores.

TABLE I. Phospholipid composition of chromatophores from *Rhodopseudomonas capsulata* and of liposomes prepared therefrom[a]

	Chromatophores	Liposomes
Phosphatidylethanolamine	47	41
Phosphatidylcholine	27	28
Phosphatidylglycerol	26	31

[a] The data were obtained by thin-layer chromatography as described in the section Materials and Methods.

The Response of 9-Aminoacridine to pH Gradients

As reported already by other authors [4, 9], when liposomes, in which a proton gradient of at least 1.5 pH units is present across the lipid layer, are added to a buffer containing micromolar concentrations of 9-aminoacridine, a substantial decrease of fluorescence of the dye is observed. According to the model proposed by Schuldiner et al. [2] the equation relating proton concentration to amine fluorescence should be

$$\log \frac{Q}{100-Q} = \log \frac{K_a + [H^+]_{in}}{K_a + [H^+]_{out}} + \log \frac{v_i}{V_o} \tag{1}$$

where Q indicates the percentage quenching of fluorescence of the amine, v_i and V_o the volumes of the inner and outer compartment, respectively, and K_a the proton dissociation constant of the amine ($10^{-9.99}$) [2] in the case of 9-aminoacridine.

Implicit in relation (1) is the assumption that the quenching of fluorescence reflects quantitatively the distribution of the amine between the inner and outer compartment of liposomes, with the fluorescence of the molecules trapped in the inner phase completely quenched.

Excitation and emission spectra of 9-aminoacridine in the presence of internally acidic liposomes are consistent with this assumption: no distortion either of the emission or excitation spectra is caused by addition of liposomes, but rather the quenching corresponds to a general decrease of fluorescence over the whole range of wavelengths. The original fluorescence is totally restored (except for a small amount, probably due to internal filter effects of the liposomes) after destruction of membrane impermeability to protons by addition of Triton X-100 (0.04% vol/vol). Similar results have been previously presented with energized bacterial chromatophores [5].

Other properties of the phenomenon of quenching are consistent with the model: quenching is reversed by any agent dissipating the proton gradient, NH_4Cl, K^+ plus nigericin or detergents. The quenching is practically independent of the concentration of the amine used, at least for concentrations ranging between 2 and 15 µM, and of the nature of the buffer used.

A first property of this model, which is at the very basis of its use as a ΔpH indicator, is that the degree of the quenching of fluorescence should be related only to the difference in pH of the internal and external phases. From Equation (1) it can be deduced that if one maintains constant the internal pH and the amount of liposomes added to the assay, the dependence of the log $Q/(100-Q)$ should be linear with the pH of the external medium, but this only for pH values lower than pK_a of the amine
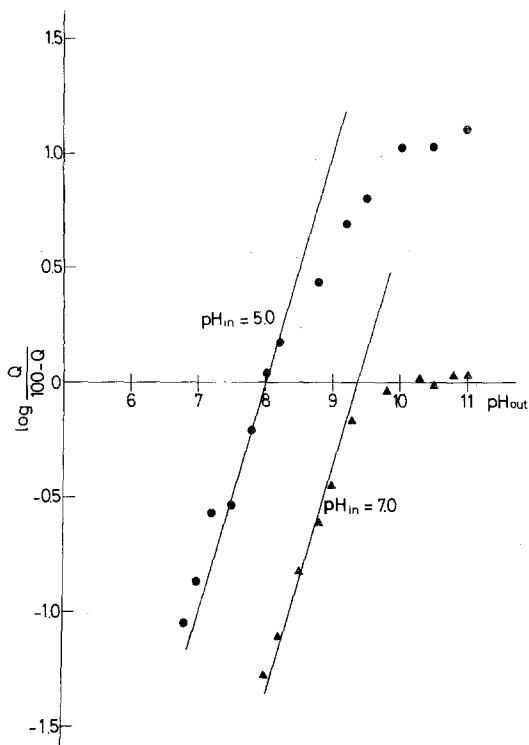
Figure 1. Relation between the fluorescence quenching of 9-aminoacridine and the pH of the external medium. The assay contained 2 ml of phosphate pyrophosphate buffer 100 mM ranging in pH from 5.0 to 11.0; 9-aminoacridine 5 μM; liposomes prepared at pH 5.0 (●) or 7.0 (▲), respectively, were added and the maximum quenching, immediately after addition, was recorded.

(9.99 for 9-aminoacridine [2]); for higher outside pH the fluorescence should be independent of proton concentration. This point is verified by the experiments shown in Fig. 1, in which two preparations of liposomes, with internal pH 5.0 and 7.0, respectively, were added to a fluorescence assay at different outside pH's. As previously shown by other authors [4], log $Q/(100-Q)$ increases linearly with outside pH, with the expected slope of 1 up to ΔpH of about 3 units; at higher outside pH the proton gradient becomes excessively large and linearity is no longer verified. This deviation from the ideal behavior may be due to an excessive accumulation of the dye in the internal phase, or to an equilibration of the dye across the membrane that is relatively slow in comparison with the dissipation of the protonic gradient. A slope equal to 1 was obtained with either of the inner pH's tested, although at the same outside pH, the absolute value of the
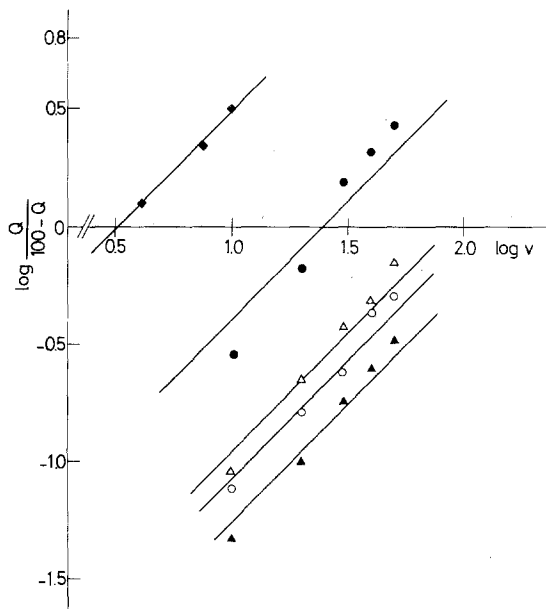
Figure 2. Dependence of the quenching of fluorescence of 9-aminoacridine upon the volume of the liposome suspension. Variable amounts of liposome suspension prepared in 100 mM phosphate pyrophosphate buffer at pH 5.0 or 7.0 were added to 2 ml of 100 mM phosphate pyrophosphate buffers at different pH's as indicated in the figure; 5 μM 9-aminoacridine was present. ◆ 225 μg P/ml, $pH_{in} = 5$, $pH_{out} = 9$, ● 40 μg P/ml, $pH_{in} = 5$, $pH_{out} = 8$; △ 15 μg P/ml, $pH_{in} = 7$, $pH_{out} = 10$; ○ 15 μg P/ml, $pH_{in} = 7$, $pH_{out} = 9.5$; ▲ 15 μg P/ml, $pH_{in} = 7$, $pH_{out} = 9$.

quenching is lower at higher inner pH and vanishes gradually when the outside and inside pH become sufficiently close ($\Delta pH \leq 1$).

Our data therefore demonstrate that an absolute requirement for obtaining quenching of fluorescence is the presence of a transmembrane pH gradient and that the absolute value of the inner or outer pH has a minor importance, if any, as compared to the transmembrane difference.

At about $pH_{out} = 10$, however, *and irrespective of the value of the inside pH,* fluorescence becomes insensitive to external pH, as anticipated by the model (Fig. 1).

It is important to underline that this transition-pH in the behavior of 9-aminoacridine corresponds exactly to the pK_a of the dye and has no relation to the protonation equilibria of negatively charged groups of the phospholipids, which in principle could bind the dye and thus contribute to its fluorescence response. These results therefore indicate that direct interactions between acridines and phospholipids, electrostatic or hydrophobic in nature, although theoretically possible and experimentally

proved in some systems [18, 19], play a negligible role in the phenomenon of fluorescence quenching of 9-aminoacridine in comparison with the pH-driven distribution of the dye into the acid phase of the vesicles.

Another prediction of the model is that, all other conditions kept constant, $Q/(100-Q)$ should be linear with the volume of the inner phase of the particles added to the assay. Since the absolute value of this volume cannot be easily evaluated, this property can be tested by plotting log $Q/(100-Q)$ against the logarithm of the volume of the liposome suspension added to the assay, which is proportional to the actual internal volume (and therefore corresponds to a constant shift on the abscissa in a double logarithmic plot). A series of such experiments performed at different values of inside pH and at different ΔpH are shown in Fig. 2. The expected slope of 1 in the double logarithmic plot is verified with sufficient accuracy irrespective of the absolute values of pH_{in} or ΔpH [in agreement with References 4, 9].

Effect of Ions

The effect of salts on the liposome-induced attenuation of the fluorescence of 9-aminoacridine was also examined. In these experiments the concentration of the phosphate pyrophosphate buffer was routinely lowered to 30 mM, in order to decrease the ionic strength and consequently enhance electrostatic interactions. Under these conditions earth-alkaline cations (added as chloride salts) at mM concentrations were found to decrease significantly the extent of quenching in comparison to controls. This effect increased with increasing concentration of the added salt.

A closer examination of this phenomenon indicated that the decrease in quenching did not affect at all the ideal slope in the log $Q/(100-Q)$ vs ΔpH plot, but was only reflected in the intercept on the ordinate axis at ΔpH $= 0$, a measure, according to Equation (1), of log v_i/V_0. According to this criterion, for example, the general decrease of the level of quenching upon addition of 5 mM $MgCl_2$ corresponded approximatively to a 1.5-fold decrease of the apparent internal volume.

These results are consistent, therefore, with the suggested action of Mg^{++} or Ca^{++} on the structure of the phospholipid bilayers, which are shown, by different techniques, to become more rigid and stable in the presence of these divalent cations [17, 20].

Additions of EDTA or EGTA at equimolar concentrations were not able to reverse the effect of Mg^{++}, probably owing to the high chelating power of the strongly acid liposomes used in these experiments [by contrast, compare Reference 21].

Salts of monovalent cations (KCl, NaCl, KNO_3) had no effect at all up to

concentrations of 50 mM; at higher concentration a small decrease of fluorescence was observed, probably due to a non-specific osmotic effect of the added salt. Equally without effect was the addition of 5 mM putrescine-Cl_2.

The Osmotic Response of the Apparent Internal Volume

A very basic feature of the model of Schuldiner et al. is that the internal volume of liposomes, as calculated from Equation (1), should be responsive to changes in the osmotic pressure of the external medium. We have tested this point extensively by adding an osmotically active solute (sucrose) to assays, under controlled conditions of ΔpH. Generally it was observed, as expected, that an increase of the osmolarity of the external medium corresponded to a decrease of the extent of the quenching; a slowing of the response of the fluorescence changes upon addition of liposomes was also observed, which was presumably due to the increased viscosity of the medium.
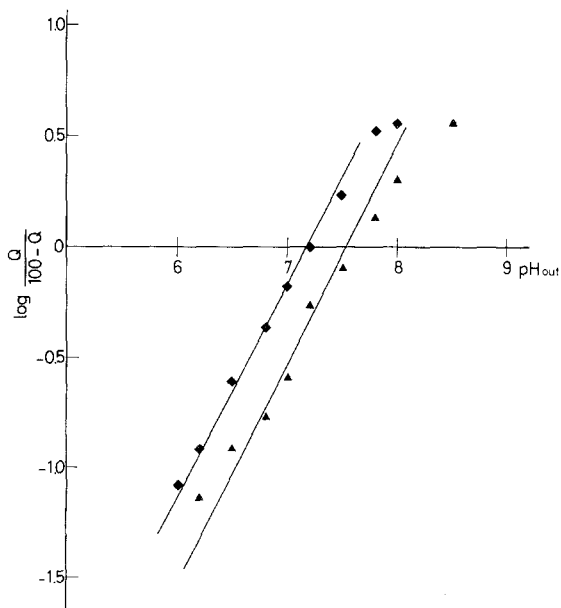

Figure 3. Effect of increased osmolarity on the fluorescence response of 9-aminoacridine to transmembrane pH difference. The conditions for the assay were: 2 ml of phosphate pyrophosphate buffer 30 mM ranging from pH 6.0 to 9.0, 5 μM 9-aminoacridine, liposomes (pH_{in}=5.0) corresponding to 50 nmoles of phosphorus per ml. 1 M sucrose was added to the external medium. (♦) In absence of sucrose. (▲) In presence of sucrose.

The response of fluorescence to changes in transmembrane pH difference in control experiments and after addition of 1 M sucrose is shown in Fig. 3: these results confirm that the effect of sucrose is only on the apparent internal volume as predicted by the model. In this experiment addition of 1 M sucrose to an assay, in which the osmolarity of the inner and outer phase of liposomes was otherwise identical, caused the decrease of v_i by about 3 times.

The effect of sucrose concentration on the value of v_i, calculated from the value of $Q/(100-Q)$ obtained in the single experiments at a known ΔpH of 2.5 units, is shown in Fig. 4. The internal volume decreases linearly with 1/(sucrose) as expected for an ideal osmotic behavior of the liposome vesicles. At concentration of sucrose lower than 0.25–0.30 M, however, the fluorescence quenching, and consequently the calculated value of v_i, becomes gradually insensitive to changes in osmolarity. This probably indicates that a considerable pressure is necessary in order to counteract the electrostatic repulsions of the surface charges of the vesicles; this conclusion also would imply that strong acidic liposomes behave as a
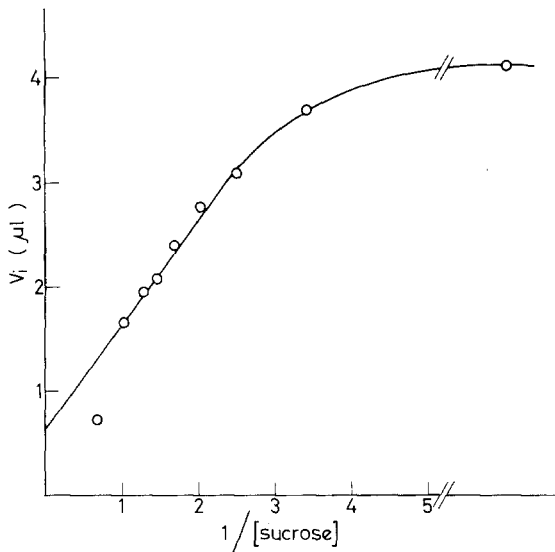

Figure 4. Dependence of internal volume, as calculated from the quenching of fluorescence of 9-aminoacridine, upon the osmolarity of the external medium. The assays contained 2 ml of phosphate pyrophosphate buffer 30 mM pH 7.5, 9-aminoacridine 5 μM, and variable concentrations of sucrose. Liposomes (pH$_i$ = 5.0) corresponding to 70 nmoles of phosphorus per ml and prepared in a buffer of osmolarity identical to that of the outer phase in the absence of sucrose were used.

rather rigid structure and are generally in a fully stretched conformation of the lipid bilayer.

The demonstration of the osmotic response of the apparent volume of the vesicles, as evaluated from fluorescence quenching, is of particular importance, since it was shown that the quenching of fluorescence by illuminated chloroplasts is unaffected by changes in the osmolarity of the external medium [9, but cf. 22]. Our data indicate, on the contrary, that this is not the case in an artificial model system, where the level of energy stored as an externally imposed pH gradient can be accurately controlled. The extrapolated volume of the inner phase of the vesicles, maximally compressed at infinite osmolarity of the external medium, corresponds to about 10 μl/μmole of phospholipids. Assuming an average area of 100 Å^2 per molecule of phospholipids [23], and taking into account the average diameter of our liposome preparation (300 nm), the inner volume of the vesicle can be calculated to be $3/n$ μl/μmole of phospholipid, where n indicates the average number of monolayers in the liposome structure. This relatively small inconsistency between these calculations and the experimental results could possibly be justified by the strong electrostatic repulsion of phospholipids, which could increase considerably the effective molecular area to be introduced in these calculations.

Conclusions

Little doubt exists, in our opinion, that 9-aminoacridine is a suitable quantitative indicator of transmembrane ΔpH in liposomes of strongly acidic bacterial phospholipids. Deviations from the behavior predicted by the model were presented by other authors in less acidic liposomes, in which, however, nonphysiological charged amphipatic molecules (dicetylphosphate and hexadecyl trimethylammonium) had been introduced into the lipid mixture [9]. Some of the discrepancies between those results and the ones presented here are, however, unexplained, since the ideal response of 9-aminoacridine to ΔpH was also verified by us in completely neutral liposomes formed from synthetic dipalmitoyl-lecithin.

In this study, the effects on 9-aminoacridine fluorescence of such factors as the ionic composition of the assay, the time of sonication, the microscopic structure of liposome, and the osmolarity of the medium were examined in detail. All these factors were shown to affect directly only the apparent inner volume of liposomes and not the ideal behavior of 9-aminoacridine as judged from its fluoresence response to ΔpH. The most noticeable inconsistency between the experimental results and the theoretical calculations emerges from the comparison of the inner calculated volumes and the experimental values obtained, which appear to

be overestimated. This point should therefore be carefully tested in employing 9-aminoacridine for determining ΔpH across natural membranes. A direct comparison of the osmotic volumes obtained by trace techniques [5, 24] and the apparent volume obtained empirically from 9-aminoacridine experiments would be very useful in this respect.

It should be underlined, moreover, that phospholipids are present in photosynthetic membranes from *Rps. capsulata* at a concentration of about 0.55 mg per mg of intrinsic proteins and that consequently phospholipid bilayers should represent a predominant part of the area in this biomembrane [25].

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