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THE RESPONSE OF FLUORESCENT AMINES TO pH GRADIENTS ACROSS LIPOSOME MEMBRANES

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SUMMARY

Phospholipid liposomes were used to test atebrin and 9-aminoacridine as fluorescent probes for measuring pH gradients across membranes. Quenching of 9-aminoacridine fluorescence could be quantitatively related to the magnitude of pH gradients across liposome membranes, and the relation indicated that the distribution of this amine between inner and outer volumes in the liposome system was that of an ideal monoamine. Quenching of atebrin fluorescence could not be related to that predicted from the theoretical equation of a diamine. We conclude that 9-aminoacridine is the preferred fluorescent probe for use in the range of $\Delta pH = 2$–4 pH units.

Quenching of 9-aminoacridine fluorescence was used to measure the development of pH gradients in liposomes. In one system, an oxidation–reduction reaction mediated by a dye which accepted H^+ upon reduction, was established across liposome membranes. It was found that gradients of at least 4 pH units could develop under these conditions. In a second system, nigericin mediated the exchange of K^+ for H^+ across the liposome membranes, and it was found that the pH gradient developed depended upon the original K^+ gradient and could be at least 2.2 pH units. Liposomes offer a model membrane system in which controlled pH gradients may be established.

INTRODUCTION

Proton transport and resulting gradients of H^+ activity across membranes are now recognized to be integrally related to phosphorylation and ion transport in chloroplasts, mitochondria and other membranous systems. Present methods for estimating pH gradients across membranes involve measurements of pH changes in the external medium using a glass electrode or indicator dyes[1,2], the distribution of amines[3] or weak acids[4] in response to pH gradients, or other pH-related absorption or scattering changes[5,6]. Each of these methods has certain limitations, and a technique which more directly and rapidly measures pH gradients would be useful.

Kraayenhof[7] found that fluorescence of the diamine, atebrin, was quenched

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonic acid; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid; PMS, phenazine methosulphate.

during electron transport in chloroplast suspensions, and suggested that the quenching may be related to the high energy state of the chloroplasts. Rottenberg *et al.*[3] extended this work with atebrin and 9-aminoacridine, and proposed that the quenching may depend on inward movement of the amines in response to pH gradients which develop across chloroplast membranes during electron transport. Rottenberg *et al.* noted that fluorescence quenching may therefore be a parameter of amine uptake and calculated from the amine distribution that a pH gradient as large as 3.5 pH units could develop across the membranes of illuminated chloroplasts.

This potentially important method has two limitations. It is not known if fluorescence is completely quenched by entry into membrane enclosed volumes, and the assumption that the amines respond ideally to pH gradients across membranes is open to question. To test these points, we have studied the quenching of atebrin and 9-aminoacridine fluorescence in phospholipid liposomes[8], in which controlled pH gradients were established. In a second series of experiments, the fluorescent probes were used to determine whether pH gradients may be generated across liposome membranes by a simple electron transport system or an ion exchange mediated by the antibiotic nigericin.

METHODS

Lipids

Egg lecithin was prepared from hens eggs by column chromatography on alumina and silicic acid. The product was chromatographically pure as judged by thin-layer chromatography on Silica Gel G. Dicetyl phosphate was a kind gift of Dr A. Bangham. Bacteriochlorophyll was prepared from a methanol extract of *Rhodopseudomonas capsulata* by column chromatography on Whatman cellulose powder using 5% diethyl ether in light petroleum.

Fluorescent probes

Atebrin was purchased from Sigma, and 9-aminoacridine from Ralph N. Emanuel Ltd, Wembley, England. Both were dissolved in distilled water at a concentration of 1 mM.

Buffers

It was found that organic buffers (Tricine, 2-(*N*-morpholino)ethanesulphonic acid (MES), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)) quenched 9-aminoacridine fluorescence to variable degrees, although atebrin fluorescence was unaffected. To avoid the partial quenching, a buffer containing 0.1 M sodium phosphate and 0.1 M sodium pyrophosphate was used for the pH range 5.0 to 9.0. Since liposome membranes are relatively permeable to chloride[8,9] it was possible that any chloride in the medium could move with a proton and discharge pH gradients. Therefore, buffer pH was adjusted with H_2SO_4, rather than HCl. The pH gradients are also sensitive to amines and care was taken that no amine compounds other than the fluorescent probes were inadvertently introduced into the medium. When K^+- and Na^+-free media were required, phosphoric acid was neutralized with tetramethylammonium hydroxide.

Liposome preparations

Liposomes were produced by evaporating chloroform solutions of varying amounts of egg lecithin *plus* 5 % dicetyl phosphate under nitrogen. The lipid was sonicated for 1 min with 2 ml of the medium to be trapped inside the liposome volume. A Soniprobe 1130A (Dawe Instruments, London) was used in this step at a power setting of 50 W. During sonication, the temperature of the solution rose to approximately 40 °C and this presumably aided formation of liposome vesicles[10]. In some experiments, sonication time was varied from 0 to 30 min and the amount of trapped anion (phosphate or ferricyanide) was measured. It was found that the amount of trapped material was maximal after 30–60 s sonication. In view of the report that prolonged sonication causes some degradation of phospholipid[11] 1 min seemed a reasonable time interval. The solutions to be trapped contained either phosphate–pyrophosphate buffer at pH 5.0 to 9.0, 0.1 M $K_3Fe(CN)_6$ or varying concentrations of K_2SO_4. All solutions included 1 mM EDTA. The sonicated suspension (1.5 ml) was placed on a 15 cm × 1 cm column of Sephadex G-50, course grade[9], which was equilibrated with sucrose solution containing 10 mM buffer at the original pH. Sucrose was added to osmotically balance the vesicles during gel filtration. For instance, 0.1 M $K_3Fe(CN)_6$ would be balanced with 0.4 M sucrose. The turbid liposome suspension was collected from the column in 3–4 ml of the eluate and adjusted to a known volume (3–5 ml) with sucrose solution. Examination of the preparation by phase-contrast microscopy showed a mixed population of liposome. The major species were 0.1–1 μm vesicles apparently with single membranes, but with occasional larger multilayered liposomes. In some experiments, 2 mole % bacteriochlorophyll was included in the lipid prior to evaporation and sonication. All experiments were carried out within 6 h of sonication.

Fluorescence measurements

Fluorescence of the amines was measured in a fluorimeter with the photomultiplier at 90° to the actinic light path. The actinic beam was provided by a quartz–iodine bulb (Philips 12258/99 12 V 55 W) supplied by a stabilized power supply (Coutant Electronics Ltd, Reading, England). Blue light was selected either by a monochromator (Hilger and Watt Ltd, London, England Type D 292) or by a Wratten 36 gelatin filter (Kodak Ltd, London) together with a Corning 9782 glass filter. The photomultiplier (EMI Electronics Ltd, Hayes, Middlesex, England, Type 9601 B) was screened by a Wratten 61 filter, together with a Corning 9782 glass filter. The output was fed to a recorder (Toa Polyrecorder EPR-2TB, T.E.M. Sales Ltd, Crawley, Sussex, England) by way of a simple amplifier and backing circuit.

RESULTS

Effect of pH on intrinsic fluorescence

As noted in previous studies[7,12] atebrin fluorescence is pH dependent, and increased nearly 2-fold as pH increased from 6 to 9 (Fig. 1). This is related to the first pK of this diamine (pK = 7.9, 10.5) and the fluorescence change is half maximal at pH 7.9. There was little variation in 9-aminoacridine fluorescence (pK = 10.0) in inorganic buffers over the same pH range. It is important to note the marked quen-

ching effect of organic buffers on 9-aminoacridine fluorescence (Fig. 1). Atebrin fluorescence was not significantly affected by organic buffers.

Fluorescence quenching and enhancement dependent on pH gradients

Fig. 2 shows a typical experiment in which liposomes were added to media containing atebrin or 9-aminoacridine. When the liposomes contained buffers at pH lower than that of the medium, atebrin fluorescence was enhanced, rather than quenched (Fig. 2A). At higher lipid concentrations the enhancement may be 250%. Triton (0.1 mM) or NH_4Cl (5 mM) completely reversed the enhancement. However, if 2 mole % bacteriochlorophyll was included in the lipid phase, quenching of fluorescence occurred under the same conditions, and again the quenching was reversible with Triton or NH_4Cl (Fig. 2B). Quenching of 9-aminoacridine fluorescence occurred both with and without bacteriochlorophyll, although there was somewhat increased quenching when bacteriochlorophyll was present (Figs 2C and 2D).

The effect of a range of pH gradients on atebrin and 9-aminoacridine fluorescence quenching by liposomes is shown in Fig. 3. In both cases the inner phase was maintained at pH 5 and the external pH was varied from 5 to 9. It is apparent that quenching of the fluorescence of the amines in liposome suspensions was strongly dependent on pH gradients across the liposome membrane. In the absence of bacteriochlorophyll the enhancement of atebrin fluorescence was maximal at pH 6.2 (ΔpH = 1.2), then decreased. If bacteriochlorophyll was present, the quenching curve of

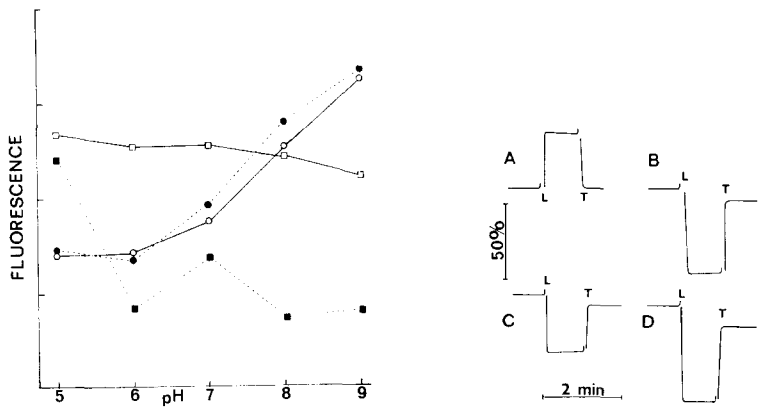

Fig. 1. Dependence of amine fluorescence on pH. The fluorescence (arbitrary units) of atebrin and 9-aminoacridine was measured in organic buffers. The organic buffers (-----) were 0.1 M MES (pH 5 and 6), 0.1 M TES (pH 7) and 0.1 M Tricine (pH 8 and 9). The inorganic buffer (—) was the phosphate–pyrophosphate buffer described in Methods. ■ and □, 4 μM 9-aminoacridine in organic and inorganic buffers, respectively; ● and ○, 4 μM atebrin in organic and inorganic buffers, respectively.

Fig. 2. Amine fluorescence changes in liposome suspensions. Liposomes (L) containing buffer at pH 5.0 were added to solutions of atebrin and 9-aminoacridine, followed by Triton X-100 (T) to a final concentration of 0.1 mM. Final lipid concentration was 0.08 mg/ml. (A) Fluorescence enhancement occurred when liposomes were added to 4 μM atebrin in inorganic buffer (pH 7.0). No bacteriochlorophyll was present. (B) Fluorescence quenching occurred under conditions for (A) but with 2 mole % bacteriochlorophyll in the liposome membranes. (C) Fluorescence quenching occurred when liposomes were added to 4 μM 9-aminoacridine in inorganic buffer (pH 8.0). No bacteriochlorophyll was present. (D) Increased fluorescence quenching occurred under conditions for (C) but with 2 mole % bacteriochlorophyll in the liposome membranes.

atebrin fluorescence was similar to that of 9-aminoacridine fluorescence, but as noted previously[3] atebrin was more responsive to pH gradients.

For monoamines it is possible to derive the relation

$$\log \frac{[H^+]_i}{[H^+]_0} = -\Delta pH_{(i-0)} = \log \frac{A_i}{A_0} + \log \frac{V_0}{V_i}$$

where V_0/V_i is the ratio of external volume to internal volume and A is the total amount of amine cation in the inner or outer volumes. Thus, $-\Delta$pH plotted against log A_i/A_0 should provide a linear relation of slope = 1.0 with an intercept equal to log V_0/V_i. When pK_a for the amine is much higher than the experimental pH, the concentration of free amine is negligible and A approximates closely to the total amine. We have treated data from the experiments using 9-aminoacridine in this form, assuming that A_i/A_0 is equivalent to $Q/(100-Q)$ where fluorescence in the absence of a pH gradient is taken as 100 and Q is the measured percent quenching. Furthermore, we varied the volume ratios since the intercept equal to the log of the volume ratios, should also vary in a predictable manner. These results are shown in Fig. 4.

In this experiment, five different liposome concentrations were used so that V_0/V_i varied in a controlled manner, and ΔpH was varied from 2 to 4 pH units. Since we do not have a precise measurement of liposome volume, we assumed that the results

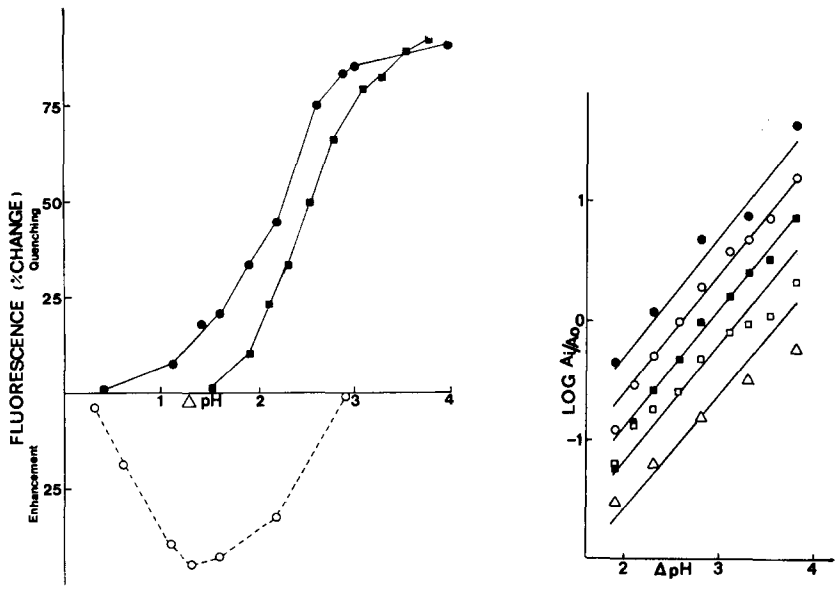

Fig. 3. Effect of varying pH gradients on fluorescence changes. ○---○, enhancement of atebrin fluorescence in presence of liposomes lacking pigment; ●—●, quenching of atebrin fluorescence by liposome containing 2 mole % bacteriochlorophyll; ■—■, quenching of 9-aminoacridine fluorescence by liposomes containing 2 mole % bacteriochlorophyll. Liposomes contained buffer at pH 5.0. Lipid concentration, 0.08 mg/ml; amine concentration, 4 μM.

Fig. 4. Effect on the distribution of 9-aminoacridine, as estimated from the quenching of fluorescence, of varying lipid concentration , and pH gradients across liposome membranes. Liposomes containing buffer (pH 5.0) were added to 2.5 ml of buffer ranging from pH 7 to 9. Bacteriochlorophyll, 2 mole %, was included in the lipid. Lipid concentrations: ●—●, 0.24 mg/ml; ○—○, 0.12 mg/ml; ■—■, 0.06 mg/ml; □—□, 0.03 mg/ml; △—△, 0.012 mg/ml.

for a liposome concentration of 0.12 mg/ml were correct. Theoretical lines with a slope of 1.0 and intercepts corresponding to the relative volume ratios were then drawn through all five sets of data. The fit of the data to the theoretical lines was quite good for the three higher liposome concentrations, but deviated to some extent from the expected lines for the lower concentrations.

A similar experiment was carried out for two concentrations of liposomes in which atebrin was the fluorescent amine. Atebrin did not behave as an ideal diamine (Fig. 5) since the results deviated considerably from the slope of the theoretical line, which was drawn by graphing the logarithmic form of the distribution equation for an ideal diamine using the values of pK_a for atebrin.

Developing pH gradients across liposome membranes

There are a number of methods by which one might expect to develop pH gradients across liposomes. Two of the most convenient are described here. In the first, an oxidation–reduction reaction was established across the liposomal membrane. This type of system has been described earlier by Hinkle[13] and Kimelberg *et al.*[14]. In the present system liposomes containing an oxidant were added to a medium containing a reductant. A lipid-soluble carrier molecule which acted as a hydrogen acceptor upon reduction was then added. The carrier would be expected to transport both protons and electrons inward and release protons inside following oxidation by the trapped couple.

A second method involved the use of the ionophoric antibiotic, nigericin, which

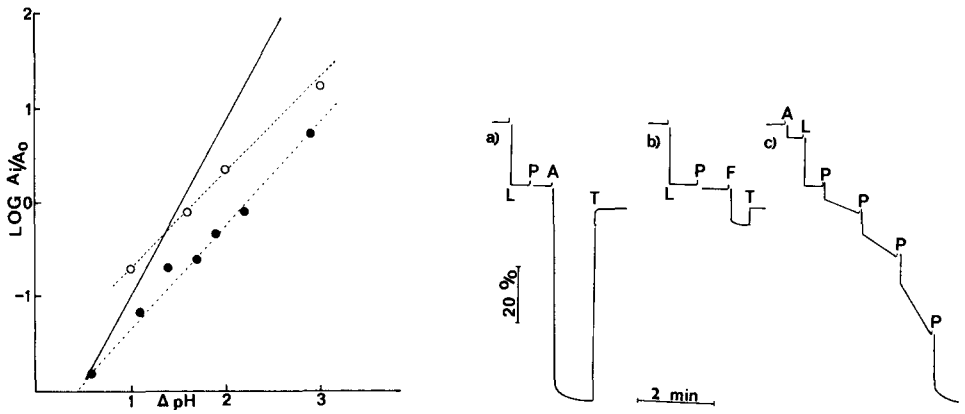

Fig. 5. Effect of varying pH gradients and lipid concentration on apparent distribution of atebrin across liposome membranes. Same conditions as Fig. 4, but buffers ranged from pH 6 to 8 and 4 μM atebrin was used. Lipid concentrations: ●---●, 0.12 mg/ml; ○---○, 0.3 mg/ml. Solid line is a theoretical line for the equation of diamine distribution assuming $V_o/V_i = 1000$. See text for details.

Fig. 6. Quenching of 9-aminoacridine fluorescence by liposomes containing $K_3Fe(CN)_6$. Liposomes (L) were added to 2.5 ml of buffer solution at pH 7.0, followed by additions of PMS (P), 2 mM ascorbic acid (A) or $K_4Fe(CN)_6$ (F), and 0.1 mM Triton X-100 (T). Lipid concentration was 0.08 mg/ml and contained 2 mole % bacteriochlorophyll. (a) If 4 μM PMS was present initially addition of ascorbic acid caused a nearly complete quenching of fluorescence which was reversible by Triton. (b) Addition of ferrocyanide under the conditions of (a) produced a much smaller quenching. (c) Addition of small increments of PMS (each addition was an increase of 0.4 μM) in the presence of ascorbic acid produced an initial rapid quenching phase, followed by a slower phase. As total PMS concentration was increased, the rate of the slower phase also increased.

mediates the exchange of K^+ and H^+ across liposome membranes[15]. If nigericin is added to a membrane system across which a K^+ gradient exists, K^+ exchanges with protons across the membranes and a pH gradient should develop. We have tested both of the above methods in the liposome system, using 9-aminoacridine to detect and measure pH gradients.

In the redox couple method ferricyanide was used as an oxidant trapped inside the liposomes, ascorbic acid or ferrocyanide was used as a reductant outside and phenazine methosulfate as a proton carrier molecule. A concentration of 0.1 M $K_3Fe(CN)_6$ was used and approximately 0.39 μmole/mg lipid could be trapped in the liposome volume. In a typical experiment (Fig. 6A) no quenching was observed until both ascorbate and phenazine methosulphate (PMS) were added, at which time a large (>90%) quenching occurred which was completely reversed upon addition of Triton or NH_4Cl. Ferrocyanide was not as effective in producing a large quenching (Fig. 6B). If the data in Fig. 4 were used to calibrate ΔpH for the lipid concentration present in the ferricyanide system, it would indicate that a ΔpH of 4.0 units developed across the liposome membranes when ascorbate was used as the reductant, and 1.6 units when ferrocyanide was used as a reductant.

The mechanism by which PMS mediated the redox reaction was of some interest. PMS could either act in a cyclic manner, in which case catalytic amounts would promote the electron transport reaction, or it could act in a non-cyclic manner, in which case the extent of the reaction would be limited by the amount of PMS present. This was tested by titrating the quenching with small additions of PMS (Fig. 6 C). It is clear that the quenching which occurs after PMS addition has both a fast and slow phase. The significance of this result will be discussed later.

Results from the second method for developing pH gradients are shown in Fig. 7. When liposomes were loaded with K_2SO_4 and placed in a K^+-free medium, addition of nigericin induced a rapid quenching of 9-aminoacridine fluorescence (not shown) similar to that seen with the redox couple system. If it is assumed that nigericin mediates the neutral exchange of one K^+ per H^+, then after addition of

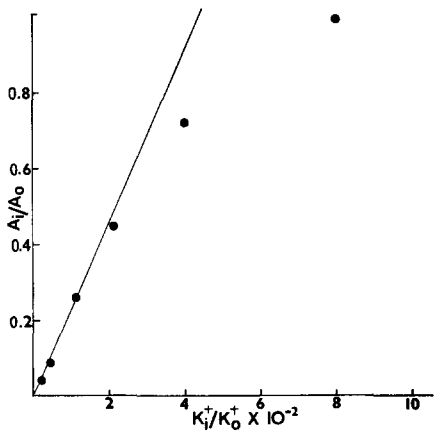

Fig. 7. Effect of nigericin and K^+ gradients on apparent 9-aminoacridine distribution. Liposomes were loaded with 0.1 M K_2SO_4 and placed in 2.5 ml solutions containing varying concentrations of K^+. Nigericin (1 μg) was then added, and A_i/A_o was calculated from the resulting quenching. The lipid (concentration, 0.08 mg/ml) contained 2 mole % bacteriochlorophyll. Solid line: theoretical curve (see text).

nigericin $A_i^+/A_0^+ = H_i^+/H_0^+ = K_i^+/K_0^+$. To test this relation, liposomes containing 0.1 M K_2SO_4 were suspended in varying concentrations of K^+ and A_i/A_0 was plotted against initial K_i^+/K_0^+. The experimental data fit the theoretical line at values of $K_i^+/K_0^+ \simeq 200$. Above this value the amount of K_i^+ which was lost to the medium in exchange for protons changed the value of K_0^+ so that the equilibrium values differed significantly from the initial values plotted and the data deviate from the theoretical line.

DISCUSSION

Fluorescent probes which respond to pH gradients across membranes may be either amines or weak acids, although none of the latter have been tested as yet. Ideally, fluorescent probes would have the following properties:

(1) They would be monoamines or monofunctional weak acids to simplify theoretical treatment.

(2) Fluorescent quenching, by whatever mechanism, would be complete when the molecule entered a membrane-enclosed volume.

(3) Intrinsic fluorescence of the probe would be independent of the composition of the external medium.

(4) The probe would be capable of rapid equilibration across membranes in response to pH gradients.

In the discussion to follow, we will compare atebrin and 9-aminoacridine in light of the above considerations.

Measurements of quenching and estimation of A_i/A_0

When atebrin and 9-aminoacridine were exposed to pigmented liposome membranes enclosing a phase at a pH lower than that of the external medium, the fluorescence of the amines was quenched. In the absence of pigment, atebrin fluorescence was enhanced. If these changes in fluorescence were related to pH gradients, it would be expected that discharging the gradient would reverse the change. Triton X-100 is a non-ionic detergent which releases ion gradients by causing membranes to become generally permeable to ions[16]. NH_4Cl is a monoamine which interacts with pH gradients according to Eqn 1 below. If present in sufficient concentration, it follows that NH_4Cl and other amines would equilibrate with a pH gradient and greatly reduce its magnitude[17]. Addition of either Triton or NH_4Cl reversed the fluorescence changes described above and we concluded that the changes were dependent on pH gradients across the liposome membranes.

The value A_i/A_0 was measured by assuming that fluorescence was completely quenched when atebrin or 9-aminoacridine entered a membrane-enclosed volume. For instance, fluorescence in the presence of liposomes might be 50 arbitrary units, and then increase to 100 units upon Triton addition. We would assume that half the amine had been in the liposomes, and that $A_i/A_0 = 1.0$. It was necessary to use the fluorescence level following Triton addition as a baseline, since some of the components of the system (bacteriochlorophyll, ascorbate, ferricyanide) could partially quench fluorescence (see Fig. 2).

Monoamines and diamines

9-Aminoacridine is a monoamine with $pK = 10.0$ (ref. 3). Ideally it should distribute across membranes[3] according to the relation

$$\frac{[A^T]_i}{[A^T]_0} = \frac{K_a + [H^+]_i}{K_a + [H^+]_0} \quad (1)$$

where $[A^T]$ is the total concentration of the amine, K_a is the dissociation constant of the amine and $[H^+]$ is the concentration of protons. When K_a is small relative to $[H^+]$ this relation may be expressed in logarithmic form as

$$\log \frac{A_i}{A_0} + \log \frac{V_0}{V_i} = -\Delta pH_{(i-0)} \quad (2)$$

where V_0/V_1 is the volume ratio of the external volume to the internal membrane enclosed volume, and A_1 and A_0 are the total amine in the internal and external volumes, respectively. We could test this relation in the liposome system, even though values of V_0/V_1 were unknown, by varying ΔpH for several lipid concentrations. The data could then be compared with theoretical lines assuming that one set of data points was correct. When this was carried out, we found that 9-aminoacridine behaved as an ideal monoamine when lipid concentration ranged from 0.06 to 0.24 mg/ml.

Atebrin is a diamine ($pK_1 = 7.9$, $pK_2 = 10.5$), which should ideally distribute across membranes according to the relation.

$$\frac{[A^T]_i}{[A^T]_0} = \frac{K_1K_2 + K_1[H^+]_i + [H^+]_i^2}{K_1K_2 + K_1[H^+]_0 + [H^+]_0^2} \quad (3)$$

For atebrin at $[H^+]_1 > 10^{-6}$, this simplifies to

$$\frac{[A^T]_i}{[A^T]_0} = \frac{[H^+]_i^2}{K_1[H^+]_0 + [H^+]_0^2} \quad (4)$$

Atebrin was tested in the liposome system by graphing the logarithmic form of Eqn 4 and comparing the resulting line with actual data. Atebrin did not behave as an ideal diamine, and the experimental data deviated considerably from the above relation (Fig. 5). The reason for this deviation is unknown, but may be related to incomplete quenching, or the fluorescence enhancement we observed under certain conditions.

Fluorescence quenching and enhancement

The mechanism by which distribution of atebrin and 9-aminoacridine across membranes alters their fluorescence is unknown. There are several possible effects which may contribute to the observed quenching:

(1) *Screening.* If a fluorescent molecule disappears from an external volume and enters a volume which is shielded by a pigment layer which in turn absorbs in the excitation or emission wavelength, it may be imagined that some quenching of fluorescence may occur. Schuldiner and Avron[18] suggested that this may be the major effect when atebrin fluorescence is quenched by illuminated chloroplasts. However, it is not clear that the very thin layer of pigment which separates the external and inter-

nal volumes in the liposome system would provide sufficient absorption to account for all the observed quenching. Furthermore, this mechanism certainly cannot account for the 9-aminoacridine quenching which does not depend on the presence of pigment.

(2) *Effect of pH on intrinsic fluorescence.* Atebrin fluorescence decreases by half as it accepts a second proton at lower pH ranges (Fig. 1) and this may account for a certain amount of quenching, as will be discussed later. Quenching of 9-aminoacridine fluorescence cannot be accounted for in this way.

(3) *Self-quenching.* Energy transfer between molecules of the same species may cause a decreased quantum yield of fluorescence at a specified emission wavelength. The extent of the self-quenching is naturally concentration dependent, and it may be imagined that when a fluorescent amine is concentrated within liposomes self-quenching may become important. It should be noted that under conditions described here, quite high concentrations of the amine would build up within the membrane enclosed volume. For instance, in Fig. 4, the apparent values for V_o/V_i obtained from the intercepts of the three largest lipid concentrations are 200, 400 and 800. The amine concentrations inside for $A_i/A_o = 1$ would therefore be 0.4, 0.8 and 1.6 mM, respectively.

(4) *Interaction with other molecules.* A fluorescent molecule may also lose its energy by interaction with other molecules. For instance, this is clearly evident in the quenching of 9-aminoacridine fluorescence by organic buffers (Fig. 1). A similar effect may contribute to quenching if the amine interacted with the components of liposomal or other membranes.

Comparison of atebrin and 9-aminoacridine

The fact that fluorescence is enhanced when atebrin is concentrated in liposomes further complicates the interpretation of results when it is used as a pH probe. Fig. 3 shows the enhancement effect over a range of pH. The enhancement, which was maximal at ΔpH of 1–2 pH units, decreased at larger pH values. This may be understood from the pH dependence of intrinsic atebrin fluorescence (Fig. 1). An atebrin molecule at pH 8 has twice the intrinsic fluorescence that it has at pH 5–6. Thus, atebrin leaving an external environment at pH 8 and entering an internal volume at pH 5 would lose half its fluorescence, and this would overcome the enhancement effect. The enhancement itself may possibly result from "excimer" formation as atebrin becomes more concentrated[19].

If bacteriochlorophyll or other pigments (chlorophyll *a*, *b* and carotene) were included in the liposome membranes, atebrin fluorescence was quenched. The quenching was maximal at 2–3 mole % bacteriochlorophyll, but it was not certain that the quenching was complete even with the pigments present. 9-Aminoacridine fluorescence, in contrast to atebrin, was quenched in liposomes lacking pigments. This quenching was still incomplete, since addition of pigment produced further quenching (Fig. 2). Quenching of 9-aminoacridine fluorescence may be best understood as a concentration-dependent self-quenching, with an additional contribution to quenching by any pigments present in the membranes. The close fit of experimental results to the theoretical lines in Fig. 4 suggests that quenching in the presence of pigment was nearly complete.

We conclude that the monoamine, 9-aminoacridine, is superior to atebrin as a fluorescent indicator for ΔpH = 2–4 units. However, its fluorescence is partially

quenched by organic buffers, and the method is only slightly responsive to ΔpH< 2 pH units at volume ratios commonly used in experimental systems. These defects are far outweighed by its behaviour as an ideal monoamine as indicated by its response in the liposome system, and 9-aminoacridine should be quite useful for estimating ΔpH in membranous systems which develop acid interiors. Atebrin, although more responsive in the 0–2 ΔpH range, does not behave as an ideal diamine and displays an anomalous fluorescence enhancement in the liposome system. In a naturally pigmented system such as chloroplasts or chromatophores with high pigment concentration, atebrin fluorescence quenching may very well be complete. In a non-pimented system, such as microsomes or mitochondria, atebrin probably could not be used effectively as a quantitative pH probe. It is interesting to note in this regard that Azzi *et al.*[10] found that atebrin fluorescence was partially quenched by submitochondrial particles during electron transport. It was suggested that the quenching may be related to an exchange of cationic atebrin for protons across the membranes. However, in view of the previous investigations with chloroplasts and the present study with liposomes, it seems more probably that atebrin was simply moving inward, with a consequent partial fluorescence quenching in response to a pH gradient developing across the submitochondrial membranes.

Estimation of V_o/V_i

Calculations of ΔpH from a theoretical consideration of amine distribution depend on knowing the ratio V_o/V_i. We were not able to make a reliable estimate of this ratio in the present liposome system. The usual method of finding sucrose or inulin impermeable space was impractical, since the liposomes did not form a centrifugal pellet under the conditions required for establishing pH gradients.

However, exact values of V_o/V_i are unnecessary if the data shown in Fig. 4 are used as a calibration curve. For any given lipid concentration, one may assume that under similar ionic and osmotic conditions, the V_o/V_i ratio does not vary appreciably. We were therefore able to estimate ΔpH in the redox couple and nigericin systems described below, since the lipid concentrations were within the range shown in Fig. 4.

Development of pH gradients across liposomal membranes

Redox couples and carrier molecules

The presumed reaction sequence for the redox reaction established across liposomal membranes is shown in Fig. 8.

The midpoint potential (E_0^1(pH 7)) for the ascorbate couple is + 60 mV, and for ferro/ferricyanide is + 430 mV. In the liposome system described here containing 1 mM ascorbate and 0.03 mM ferricyanide (total concentration), the ferricyanide would essentially be completely reduced if the reaction occurred in free solution.

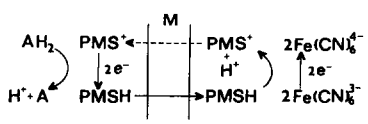

Fig. 8. Proposed reaction sequence for development of pH gradients by coupled redox reactions across liposome membranes. See text for details.

However, since the couples were separated by proton-impermeable membranes, a pH gradient would build up which would ultimately come to equilibrium with the driving force of the downhill electron transport reaction. It is difficult to calculate the relative concentrations at which this might occur. In one experiment the amount of ferricyanide reacting under the usual conditions was measured in a dual wavelength spectrophotometer, and it was found that approximately half the ferricyanide present was quickly reduced. Since the original concentration of ferricyanide in the liposomes was 0.1 M, this would suggest that about 0.05 M protons might be produced within the liposomes, equivalent to pH 1.3. At pH 7 outside, a ΔpH of 5 pH units or more would result. The 9-aminoacridine method could not accurately measure a pH gradient of this magnitude, but certainly the quenching of 9-aminoacridine fluorescence would indicate that a gradient of at least 4 pH units developed under the above conditions.

If ferrocyanide was used as a reductant, rather than ascorbate, the final equilibrium depended only on the relative concentrations of ferro- and ferricyanide in the system. Since the redox potential difference at the concentrations used was small relative to the ascorbate–ferricyanide system, one would expect a much smaller pH gradient to develop, and in fact the data for 9-aminoacridine quenching indicate a pH gradient of only 1.6 units.

The liposome system offers a potential model system for studying the mechanism of electron transport across membranes. For instance, the results with PMS (Fig. 6C) suggest that there are two steps in which PMS crosses the membranes. The first of these is the inward transport of electrons by PMS in its reduced, uncharged form, and is quite rapid. The second is the much slower, outward diffusion of PMS^+ across the membranes Thus, we only see a rapid development of a pH gradient if PMS is present in concentrations approaching the total amount of ferricyanide in the system (Fig. 6A). If smaller amounts are used, the second step becomes rate limiting (Fig. 6C). In preliminary experiments (R.C. Prince, unpublished) other carriers such as diaminodurol and dichlorophenolindophenol act at catalytic concentrations suggesting that both oxidized and reduced forms of these dyes readily cross the membrane.

Proton–K^+ exchange mediated by nigericin

The pH gradient developed when K^+ exchanges for protons in the liposome systems depends simply on the relative concentrations of K^+ across the membranes, as shown in Fig. 7. Under these controlled conditions, a ΔpH of 2.2 units as estimated from the calibration curve in Fig. 4 may be established when the expected $K_i^+/K_o^+ = 200$. The fact that 9-aminoacridine behaves ideally in the nigericin system is additional support for the conclusion that it may be used to quantitate pH gradients with considerable accuracy.

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REFERENCES

1 A. T. Jagendorf and J. Neuman, *J. Biol. Chem.*, 240 (1965) 3210.
2 B. Chance and L. Mela, *J. Biol. Chem.*, 241 (1966) 4588.
3 H. Rottenberg, T. Grunwald, S. Schuldiner and M. Avron, *Proc. 2nd Int. Congr. on Photosynthesis Research, Stresa, Italy, 1971*, in the press (also *Abstr.*, p. 19).
4 S. Addanki, R. D. Cahill and J. F. Sotos, *J. Biol. Chem.*, 243 (1968) 2337.
5 B. Rumberg and U. Siggel, *Naturwissenschaften*, 56 (1969) 130.
6 D. W. Deamer, A. R. Crofts and L. Packer, *Biochim. Biophys, Acta*, 131 (1967) 81.
7 R. Kraayenhof, *FEBS Lett.*, 6 (1970) 161.
8 A. D. Bangham, in J. A. V. Butler and D. Noble, *Progress in Biophysics and Molecular Biology*, Pergamon Press, Oxford and New York, 1968, p. 29.
9 J. D. McGivan, *The Movement of Ions across Artificial Phospholipid Membranes*, Ph. D. Thesis, The University of Bristol, 1968.
10 J. P. Reeves and R. M. Dowben, *J. Cell. Physiol.*, 73 (1969) 49.
11 H. O. Hauser, *Biochem. Biophys. Res. Commun.*, 45 (1971) 1049.
12 Z. Gromet-Elhanan, *FEBS Lett.*, 13 (1971) 124.
13 P. Hinkle, *Biochem. Biophys. Res. Commun.*, 41 (1970) 1375.
14 H. K. Kimelberg, C. P. Lee, A. Claude and E. Mrena, *J. Membrane Biol.*, 2 (1970) 235.
15 P. J. F. Henderson, J. D. McGivan and J. B. Chappell, *Biochem. J.*, 111 (1969) 521.
16 D. W. Deamer and A. R. Crofts, *J. Cell. Biol.*, 33 (1967) 395.
17 M. H. Jacobs and D. R. Stewart, *J. Cell Comp. Physiol.*, 30 (1947) 79.
18 S. Schuldiner and M. Avron, *FEBS Lett.*, 14 (1971) 233.
19 P. L. Brocklehurst, R. B. Freedman, D. J. Hancock and G. K. Radda, *Biochem. J.*, 116 (1970) 721.
20 A. Azzi, A. Fabbro, M. Santato and P. L. Gherardini, *Eur. J. Biochem.*, 21 (1971) 404.