New pH-Sensitive Aminoxyls: Application to the Study of Biomembrane Transport Processes

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An EPR method for studying transmembrane proton transport using pH-sensitive aminoxyl radicals has been developed for applications in the pH region 4–7. To this end pH-sensitive spin-labelled dextran (pK = 6.1) and sulfonic acid (pK = 5.25), which do not penetrate phospholipid membranes, were synthesized. Using EPR spectroscopy these probes were used to measure the kinetics of pH variations inside large unilamellar phospholipid vesicles after creation of a transmembrane proton gradient. Membrane permeability coefficients for H^+ (5×10^{-4} cm s^{-1}), Cl^- (10^{-10} cm s^{-1}) and Tl^+ (10^{-8} cm s^{-1}) were estimated.

pH-sensitive aminoxyl radicals are promising for pH studies in biological systems, including membranes and proteins.[1-3] Among the spin probes reviewed in ref. 4 imidazoline radicals have the most sensitive EPR spectral parameters owing to protonation of the N3 atom of the radical heterocycle.

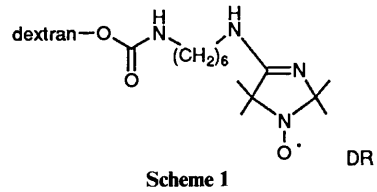
Here we report the synthesis and application of hydrophilic pH-sensitive amidine derivatives of imidazoline aminoxyls used in the study of transmembrane transport processes influenced by transmembrane proton transport. Proton transfer across biological membranes is of great importance for bioenergetics and in the functional regulation of cell processes.[5-7] Many significant results in the study of transmembrane proton transport obtained in recent years are connected with measurements of the relaxation of the pH gradient across cellular and model membranes using molecular pH probes such as fluorescence,[8,9] NMR[10,11] and EPR.[12,13] However, the application of fluorescent pH probes is restricted by the requirement of optical transparency of samples and the high and often unpredictable sensitivity of the fluorescence intensity to probe–membrane interactions. NMR application is limited owing to the low sensitivity of the method. The use of spin-labelled amphiphiles[12,13] is restricted to model membrane systems, in which calibration of the distribution sensitivity of the spin probes between water and lipid phases to transmembrane pH gradient can be performed.

Previously we proposed a new method for studying transmembrane proton transport using pH-sensitive spin probes.[2] This method seems promising because of the high sensitivity of EPR, the high state of development of EPR theory[14] and the progress in synthesis and applications of stable aminoxyl radicals as indicators of medium pH.[1-4,15] However, the proposed spin pH probe[2] allows one to study transmembrane proton transport only in the acidic pH region 1.5–4.5, essentially limiting its application.

To develop the previously proposed method for higher pH, pH-sensitive amidines covalently bound to hydrophilic sulfonic acid (pK = 5.25) and dextran (pK = 6.1) were synthesized. These probes were used to follow by EPR the kinetics of pH variations inside large unilamellar phospholipid vesicles after the creation of a transmembrane proton gradient. The fast kinetics of intraliposomal pH change due to electrically uncompensated proton transport results in the transmembrane proton gradient being equilibrated by the establishment of a transmembrane electric potential. The relaxation of this transmembrane electric potential due to transport of Cl^-, K^+ (in the presence of K valinomycin) and Tl^+ ions was studied.

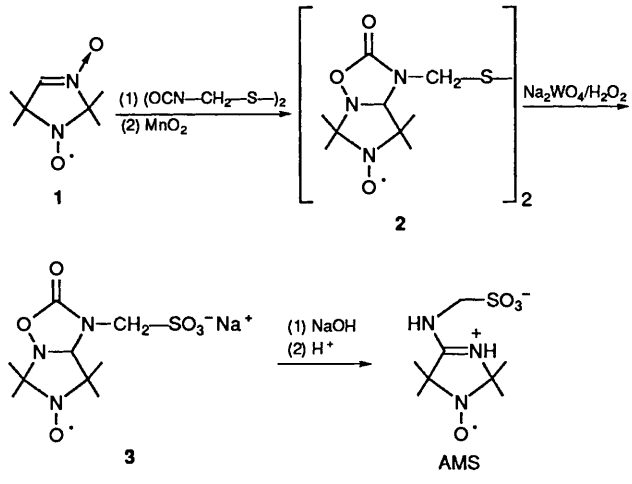
Experimental

The synthesis of spin-labelled dextran, DR follows ref. 15 using dextran from Fluka (40 kD).



Scheme 1

The synthesis of the pH-sensitive hydrophilic derivative of sulfonic acid, AMS, followed Scheme 2.



Scheme 2

Synthesis of Bis(5,5,7,7-*tetramethyl*-2-*oxo*-6-*ylooxy*-1,2,5,6,-7,7a-*hexahydroimidazo*[1,5-b][1,2,4]*oxadiazol*-1-*ylmethyl*) *Disulfide* **2**.—A solution of the dithiodiglycolic acid diazide (prepared from the corresponding dihydrazide according to ref. 16) (3.5 g, 15 mmol) in dry $CHCl_3$ (100 cm^3) was refluxed for 2 h and the solvent was distilled off to leave a residual volume of 15 cm^3. This was added dropwise to a stirred solution of the *N*-oxide **1** (6.3 g, 40 mmol, excess) in dry $CHCl_3$ (35 cm^3) at 0 °C. The mixture was allowed to stand overnight at room temperature after which the solution was cooled to 0 °C and MnO_2 (5 g) was added with stirring. After 10 min the mixture was filtered, evaporated and chromatographed (silica gel;

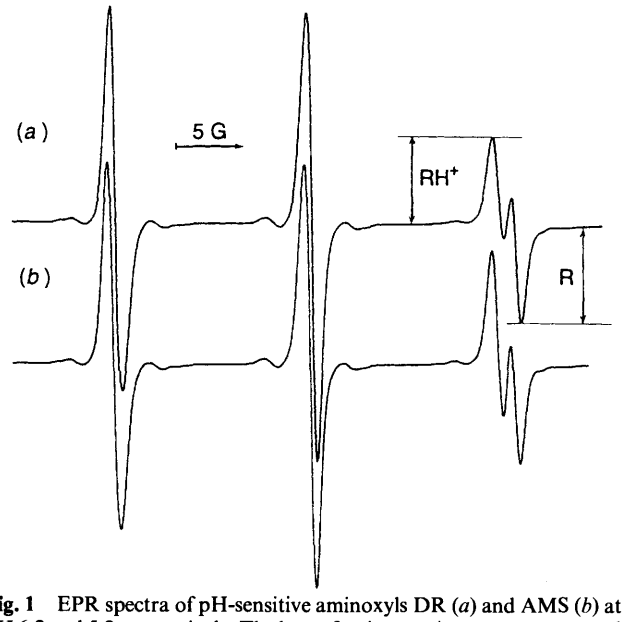


Fig. 1 EPR spectra of pH-sensitive aminoxyls DR (*a*) and AMS (*b*) at pH 6.2 and 5.2, respectively. The hyperfine interaction constant, a_N, and intensity ratio, $I_R/(I_R + I_{RH^+})$, are convenient parameters for pH measurement by these pH probes. Note that the intensity ratio I_R/I_{RH^+} is slightly different from the concentration ratio $[R]/[RH^+]$ owing to the overlap of the EPR signal. However, the pH dependence of I_R/I_{RH^+} is a convenient calibration curve for the measurement of aqueous pH and for the determination of the radical's pK [f(pK) = 0.5] due to the negligible difference in the spectral linewidth of the R and RH^+ forms.[4]

$CHCl_3$). The biradical fraction was collected and evaporated under vacuum, yield 4.8 g (65% from diazide). Recrystallization from MeOH gave compound **2** with m.p. = 157–159 °C.

Synthesis of Sodium 5,5,7,7-*Tetramethyl*-2-*oxo*-6-*ylooxy*-1,2,5,6,7,7a-*hexahydroimidazo*[1,5-b][1,2,4]*oxadiazol*-1-*ylmethanesulfonate* **3**.—To a suspension of **2** (1.5 g, 3 mmol) in an aqueous solution (10 cm^3) of Na_2WO_4 (0.8 g), $NaHCO_3$ (1 g) and disodium salt of ethylenediamine tetraacetic acid (EDTA) (0.35 g), was added dropwise a 30% aqueous solution of H_2O_2 (2 cm^3) (over about 20 min) at 0 °C. The mixture was stirred for a further 2 h at 0 °C after which a catalytic amount of MnO_2 was added to remove unchanged H_2O_2. After 10 min the mixture was filtered, washed with $CHCl_3$ (2 × 5 cm^3) and evaporated under vacuum (T < 50 °C). The solid residue was extracted with hot C_2H_5OH (2 × 15 cm^3), and the extract was evaporated to give 1 g of the sodium sulfonate **3** (53%). Recrystallization of this from MeOH gave analytically pure samples.

Synthesis of (2,2,5,5-*Tetramethyl*-1-*ylooxy*-2,5-*dihydro*-1H-*imidazol*-3-*ium*-4-*yl*)*aminomethanesulfonic Acid, AMS*.—To a saturated solution of **3** in water was added a 2 molar excess of NaOH at room temperature. After 4 h the solution was acidified with HCl to pH 3.5–4.0 and allowed to stand overnight at 5 °C. The resulting precipitate was filtered and then washed consecutively with cold water and cold MeOH. Yield 74%. For further purification AMS was reprecipitated from saturated aqueous solution on decreasing the pH from 10.0 to 3.5–4.0. M.p. = 270–271 °C.

The structures of newly synthesized compounds **2**–**4** were confirmed by IR spectroscopy and satisfactory microanalyses (C, H, N, S ± 0.35%).

Liposome Preparation.—Egg phosphatidylcholine (PC) was isolated and purified according to the literature.[17] Large unilamellar liposomes from egg PC were prepared according to ref. 18. Liposomes with DR were prepared in 50 mmol dm^{-3} *N*′-(2-hydroxyethyl)piperazine-*N*-ethanesulfonicacid(HEPES), pH 7.1, lipid concentration 20 mg cm^{-3}, pH probe concentration, DR, 10 mg cm^{-3} (about 1 mmol dm^{-3} of radical). DR, not present in the inner liposomal volume, was removed as follows. The liposomes were centrifuged at 10 000*g* for 2 min, the supernatant was removed and the sedimented liposomes were diluted with 50 mmol dm^{-3} HEPES to the same volume. The procedure described was repeated 3–4 times, and the sedimented liposomes were diluted with buffer, containing 50 mmol dm^{-3} 2-morpholinoethanesulfonic acid (MES), pH 7.1, and 50 mmol dm^{-3} sucrose. As a result the radical concentration in the supernatant was less than 0.1 μmol dm^{-3}, while in the liposome solution it was about 0.1 mmol dm^{-3}. The liposomes with AMS were prepared in the same manner and contained 1 mmol dm^{-3} AMS, 50 mmol dm^{-3} K_2SO_4, 2 mmol dm^{-3} EDTA, 20 mmol dm^{-3} K-citrate buffer in the inner volume of liposome and 50 mmol dm^{-3} K_2SO_4, 2 mmol dm^{-3} EDTA, 20 mmol dm^{-3} K-citrate buffer in the outer volume of liposome. Note that the internal volume of the liposome at a lipid concentration of 20 mg cm^{-3} was equal to about 10% of the total volume of the liposome solution and their radius was about 2000 Å.[2] To study the influence of KCl and TlCl on the transmembrane electric potential, buffers with these salts were used. To study the influence of ion carriers on the relaxation of transmembrane proton gradient 5 μl aliquots of ethanolic solutions of proton carriers (gramicidin A, concentration 0.3 mmol dm^{-3}) and potassium (K-valinomycin, 1 mmol dm^{-3}) were added to 1 cm^3 of the buffer solutions. The transmembrane pH gradient was established by changing the pH of the outer buffer solution by addition of the same buffer at the required pH. The changes in external pH due to the relaxation of the experimental transmembrane pH gradient were < 0.1 pH unit.

EPR spectra were recorded on an ER-200D-SRC (Bruker) spectrometer in a flat cell or in a mixer of 200 μl volume. The hyperfine interaction constant, a_N, was measured as the distance between the low field and the central lines of the triplet and are accurate to within 0.02 G. pH values were measured using a pH meter OP-205/1 (Hungary) to 0.05 pH units.

Results and Discussion

Previously we have shown[2] that spin pH probes can be useful for the study of transmembrane proton transport across phospholipid vesicles. We used spin-labelled glutathione, pK = 3.0, to follow intraliposomal pH variations in the acidic pH region from 1.5 to 4.5. To develop this method for use at higher pH we synthesized hydrophilic spin probes AMS and DR (see the Experimental section). The pH dependences of the hyperfine interaction constant, a_N, are typical titration curves with pK = 5.25 for AMS and pK = 6.1 for DR (Fig. 2). The same values of pK were obtained using pH dependences of the ratio of the spectral intensities of the protonated and unprotonated forms of the radicals (data not shown; see caption to Fig. 1). When AMS and DR are located in the inner liposome volume, there is no change in the EPR spectrum intensity after addition of 0.1 mol dm^{-3} $K_3Fe(CN)_6$ even at experimental transmembrane proton gradients. Addition of 0.1 mol dm^{-3} of the detergent Triton X-100 to this liposome solution results in a *ca.* fourfold decrease in the EPR spectrum intensity of the probes because of line broadening. So we conclude that AMS and DR do not penetrate across phospholipid membranes and can be used for determining the intraliposomal pH in the range pH = 4.0–7.2 (Fig. 2).

With a pH gradient applied across the membrane of PC liposomes, we observed the kinetics of variation of a_N and I_R (data not shown). Fig. 3 demonstrates the corresponding kinetics of calculated intraliposomal pH changes calibrated against the pH-dependence of a_N (Fig. 2). The acceleration of the kinetics of intraliposomal pH changes on addition of a

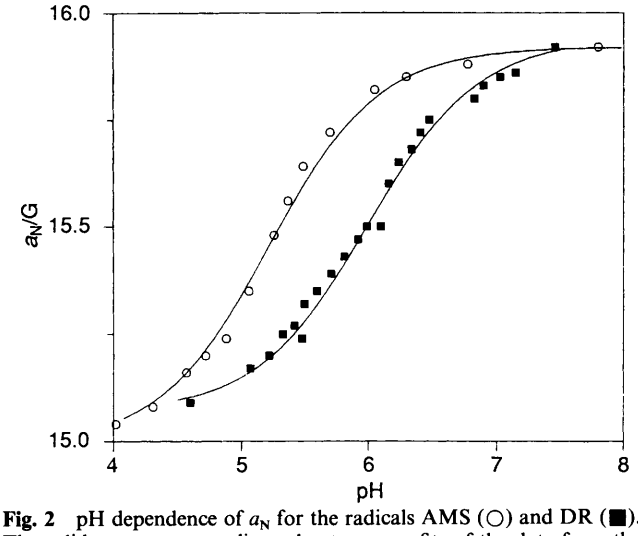


Fig. 2 pH dependence of a_N for the radicals AMS (○) and DR (■). The solid curves are non-linear least-squares fits of the data from the titration equation given in ref. 4 [$a_N(pH) = (a_R + a_{RH^+} \times 10^{pK-pH})/(1 + 10^{pK-pH})$], where the values of a_N in protonated/unprotonated forms were determined from EPR spectra at the extremes of the titration to have values of a_R = 15.92 G, a_{RH^+} = 15.00 G for AMS and a_R = 15.94 G, a_{RH^+} = 15.06 G for DR.

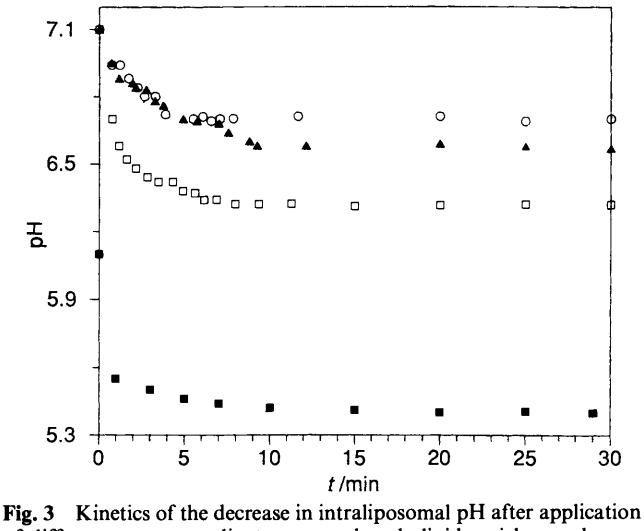


Fig. 3 Kinetics of the decrease in intraliposomal pH after application of different proton gradients across phospholipid vesicle membranes measured by means of spin probe AMS (■), ΔpH = 1.1, or DR (○) ΔpH = 0.76; (▲) ΔpH = 1.13; (□) ΔpH = 1.89. The rest pH gradients are equilibrated by the establishment of a transmembrane electric potential according to the Nernst equation[19] $\Delta\Phi \simeq 60 \log[H^+]_{out}/H^+_{in}]$, thus ΔΦ is equal to 21 mV (○), 36 mV (▲), 66 mV (□) and 24 mV (■). Note that the values of ΔΦ display a reasonably linear dependence on the changes in internal H^+ concentration. The ratio $\Delta\Phi/\Delta pH_{in} \simeq (68 \pm 15)$ mV per pH unit for DR-containing liposomes and 34 mV per pH_{in} unit for AMS-containing liposomes. The lower value for $\Delta\Phi/\Delta pH_{in}$ and faster initial kinetics for AMS-containing liposomes are in quantitative agreement with the lower buffer capacity of the sample (*ca.* a factor of two, see the Experimental section).

proton carrier (gramicidin A, data not shown) reflects the influence of the proton transfer on the internal pH. The plateau in the kinetic plot shown in Fig. 3 demonstrates the establishment of a rest transmembrane proton gradient, the value of which depends on initial pH gradient and which remains for some hours. These results can be explained in terms of a transmembrane electric potential,[19] which causes the transmembrane proton gradient to equilibrate (see Fig. 3 caption). In this case the addition of a counterion for the proton and the proton carriers (Cl^-, K^+ and K-valinomycin, Tl^+) would produce the proton influx due to a counterion-limiting

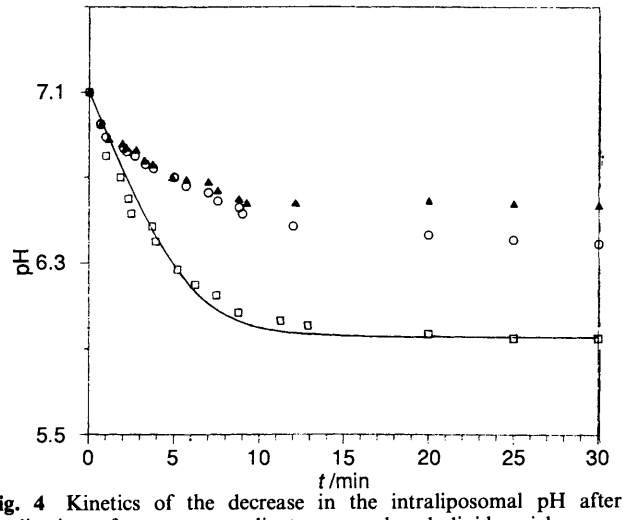


Fig. 4 Kinetics of the decrease in the intraliposomal pH after application of a proton gradient across phospholipid vesicle membranes measured by the spin probe DR: (*a*) (▲) ΔpH = 1.13; (*b*) (○) ΔpH = 1.13, 0.1 mol dm^{-3} KCl added; (*c*) (□) ΔpH = 1.13, 0.1 mol dm^{-3} KCl, 30 μmol dm^{-3} K-valinomycin. The estimation of permeability coefficient of Cl^- from the slope of the slow phase of the kinetics (○) gives a value of $(1 \pm 0.3) \times 10^{-10}$ cm s^{-1}. The solid curve is a non-linear least-squares fit of the data to the Nernst–Planck equation[19] $dpH_{in}(t)/dt = 3p_{H^+} \cdot [H]_{out} - [H]_{in})/B \cdot r$, where buffer capacity, *B*, and outer proton concentration, $[H]_{out}$, are assumed to be constant during the kinetics, B ≃ 25 = mmol dm^{-3} and $[H]_{out} \simeq 10^{-5.97}$; liposome radius $r \simeq 2000$ Å, giving $p_{H^+} = (5 \pm 1.5) \times 10^{-4}$ cm s^{-1}.

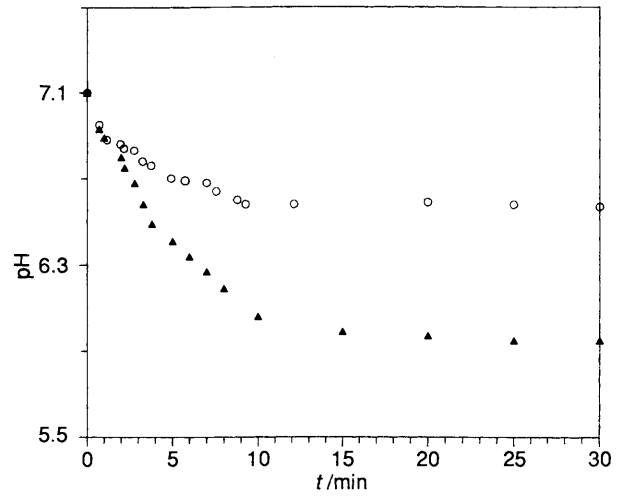


Fig. 5 Kinetics of the decrease in the intraliposomal pH after application of a proton gradient across phospholipid vesicle membranes measured by means of the spin probe DR: (○) ΔpH = 1.13, (▲) ΔpH = 1.13, 10 mmol dm^{-3} TlCl added

proton–counterion exchange. Indeed, in the presence of 0.1 mol dm^{-3} KCl we have observed biphasic kinetics of intraliposomal pH changes [Fig. 4(*b*)], which can be explained by relaxation of the transmembrane electric potential due to Cl^- transport. An estimation of the corresponding permeability coefficient gives a value of 10^{-10} cm s^{-1} in reasonable agreement with the literature data.[20] The presence of K-valinomycin accelerates this slow phase of the kinetics [Fig. 4(*c*)] due to transfer of K^+ by valinomycin. The proton permeability coefficients found from the initial part of this kinetic plot and from computer optimization of the relaxation curve (see Fig. 4 caption) are close to 5×10^{-4} cm s^{-1} and in satisfactory agreement with literature data.[20]

The dramatic influence of Tl^+ ions on the kinetics of intraliposomal pH changes (Fig. 5) merits further discussion.

There are a number of studies that conclude that Tl^+ is a 'lipid-permeable cation',[21,22] despite the fact that estimations of permeability coefficients of Tl^+ using electrical conductance measurements of planar membranes[23] give very small permeability coefficients for Tl^+ (2×10^{-10}–10^{-11} cm s^{-1}). An explanation for this disagreement based on Tl^+ transmembrane transport in the form of the neutral salt TlCl or complexes of Tl^+ with buffer molecules given in ref. 23 is not satisfactory. Indeed, Rabon and Sachs[24] found that the high permeability of Tl^+ is a process electrically coupled with proton transport and so cannot be explained in terms of transport of Tl^+ in a neutral complex. This conclusion is supported by our result (Fig. 4). The presence of Tl^+ dramatically increases the rate of proton transfer because of H^+–Tl^+ exchange. An estimation of the permeability of Tl^+ gives a value of about $(1.1 \pm 0.4) \times 10^{-8}$ cm s^{-1}. Thus the disagreement surrounding the existing data on Tl^+ membrane permeability may be connected with the specific mechanism of Tl^+ transfer, which requires further study.

Conclusions

An EPR spin probing method for measuring the rates of proton transfer across phospholipid vesicle membranes in the pH range 4–7 has been developed. A spin-labelled pH-sensitive dextran and sulfonic acid were synthesized and used as probes for measuring intraliposomal pH variations. The membrane permeability coefficients for H^+ (5×10^{-4} cm s^{-1}), Cl^- (10^{-10} cm s^{-1}) and Tl^+ (10^{-8} cm s^{-1}) were estimated.

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