Precipitation within Unilamellar Vesicles. Part 2.[1] Membrane Control of Ion Transport

Stephen Mann,* Matthew J. Kime, R. George Ratcliffe, and Robert J. P. Williams
Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR

Phosphorus-31 and ^1H n.m.r. spectroscopy have been used to investigate the control of intravesicular pH (pH_{in}) by lipid membranes. The presence of encapsulated permeable anions is crucial in the intravesicular precipitation of hydroxides and oxides. In the absence of permeable anions no change in pH_{in} has been observed on increasing extravesicular pH (pH_{out}) above 12.0. In the presence of permeable NO_3^- ions, pH_{in} rises only when pH_{out} is increased above a value of 11.0. These observations are described in terms of the Goldman–Katz–Hodgkin equation for ion fluxes across a membrane permeable to more than one ion.

In a previous paper[1] the precipitation of silver(I) oxide (Ag_2O) within phosphatidylcholine (pc) vesicles was described as a model system for biological precipitation in confined volumes. An observation from this work was that intravesicular precipitation initiates at an extravesicular pH (pH_{out}) of *ca.* 11.0, whereas precipitation of Ag_2O under similar conditions but in the absence of vesicles occurred at a pH of *ca.* 7.0. Thus, the vesicle membrane is a critical factor in the apparent inhibition of intravesicular precipitation through the control of intravesicular pH (pH_{in}) as pH_{out} is raised and such processes may be important in the biological control of intracellular precipitation. In the present work we describe ^1H and ^{31}P n.m.r. experiments for investigating this effect and show that the presence of different encapsulated anions markedly affects the steady-state pH gradient across the vesicle membrane.

The process of intravesicular precipitation can be followed by ^1H n.m.r. spectroscopy by precipitating a paramagnetic ion within the vesicles. The change of phase on precipitation eliminates the binding of the ion to the inner surface of the membrane and thus causes a change in the spectrum. The Co^{II} ion has been found to be a suitable probe for this purpose.[2]

Phosphorus-31 n.m.r. was used since it is an excellent non-perturbing method for measuring the pH of inorganic phosphate solutions localised in small compartments.[3,4] The ^{31}P chemical shift of an inorganic phosphate ion is dependent upon the degree of ionisation and thus upon the pH of the phosphate solution. For orthophosphate, the acid–base equilibria shown below are rapidly established.

$$H_3PO_4 \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} H_2PO_4^- \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} HPO_4^{2-} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} PO_4^{3-}$$

$$pK_1 = 2.15 \qquad pK_2 = 7.1 \qquad pK_3 \approx 12.4$$

Increasing the alkalinity decreases the shielding of the P nucleus due to the distribution of the same number of electrons over a larger volume.[5] A shift of ^{31}P resonances downfield is thus observed at high pH and hence a calibration curve of pH against chemical shift can be drawn.

Experimental

Hydrogen-1 N.M.R. Spectroscopy.—Phosphatidylcholine vesicles (0.034 mol dm^{-3} lipid) containing Co^{II} ions (0.25 mol dm^{-3}) were prepared from egg yolk phosphatidylcholine (Grade 1, Lipid Products) and $CoCl_2$ (AnalaR grade, BDH Chemicals) by a similar method to that described elsewhere for the encapsulation of Ag^I ions.[1] Extravesicular Co^{II} ions were then removed by passing the vesicle suspension down an ion-exchange column (sodium form, Permutit) at pH 5.0. D_2O solutions were used throughout to provide a suitable resonance for the field-frequency lock of the spectrometer and to avoid the large resonance which would arise from H_2O. Hydrogen-1 spectra were recorded using a Bruker WH300 spectrometer operating at 300 MHz at a sweep width of 4 000 Hz for 100 scans. The pH of the vesicle suspension (pH_{out}) was adjusted using NaOD and the pH values quoted are the uncorrected pH-meter readings obtained with a combined calomel electrode.

Phosphorus-31 N.M.R. Spectroscopy.—Phosphatidylcholine vesicle preparations were used with the following internal and external aqueous solution compositions: (*i*) NaH_2PO_4 (1 mol dm^{-3}; AnalaR grade, BDH Chemicals), internal and external; (*ii*) NaH_2PO_4 (1 mol dm^{-3}) internal only; extravesicular inorganic phosphate was removed by passing the vesicle solution down a Sephadex G-25 column saturated in NaCl (0.20 mol dm^{-3}; AnalaR grade, BDH Chemicals) solution; (*iii*) NaH_2PO_4 (0.80 mol dm^{-3})–$NaNO_3$ (0.20 mol dm^{-3}) (AnalaR grade, BDH Chemicals), internal only; extravesicular inorganic phosphate ions were removed on a Sephadex G-25 column saturated in $NaNO_3$ solution (0.20 mol dm^{-3}).

Phosphorus-31 n.m.r. spectra were recorded at 121.49 MHz using a Bruker WH300 spectrometer. Vesicle suspensions were placed in 8-mm diameter n.m.r. tubes arranged concentrically in a 10-mm tube containing a D_2O solution of methylenediphosphonic acid (1 mmol dm^{-3}; Sigma)–2-amino-2-(hydroxymethyl)propane-1,3-diol (5 mmol dm^{-3}; Sigma) at pH 8.9. This external solution provided a chemical shift resonance of 0.00 p.p.m. and a deuterium resonance for the field-frequency lock. Spectra with adequate signal-to-noise ratio were obtained at 298 ± 2 K from 2 000 scans with broad-band proton decoupling and a 45° pulse angle giving a total accumulation time of 8.5 min.

Spectra were recorded at different pH_{out} values. The pH was adjusted by addition of aliquots of NaOH solution and the external pH measured using a glass electrode. Some precipitation of Na_2HPO_4 occurred at high pH for preparation (*i*) due to a 'salting out' effect on addition of high concentrations of Na^+ ions. No precipitation was observed at similar pH values for preparations (*ii*) and (*iii*), indicating that the vesicles were not precipitating under these conditions.

Results and Discussion

Figure 1(*a*) shows the ^1H n.m.r. spectrum for a vesicle preparation with encapsulated Co^{II} ions. These ions are initially partly bound close to the inner surface of the vesicle membrane

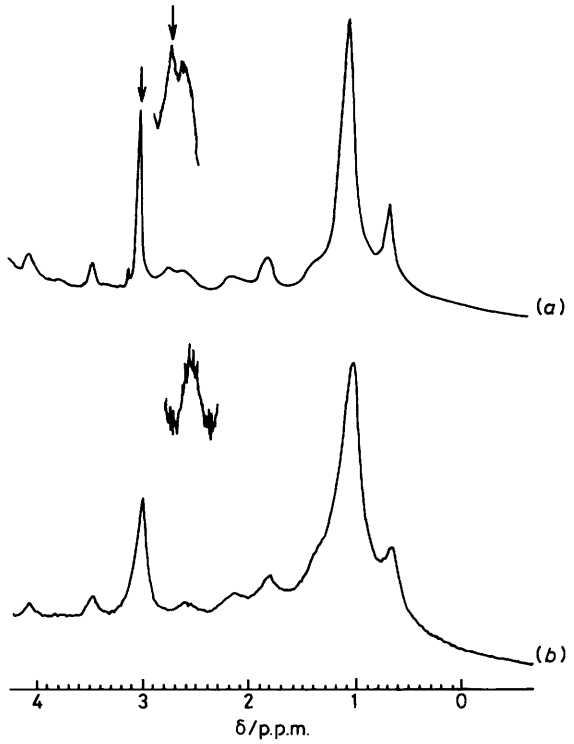

Figure 1. Hydrogen-1 n.m.r. spectra (300 MHz) of pc vesicles containing Co^{II} ions at pH_{out} values of (*a*) *ca.* 5.0 and (*b*) 12.4. The internal and external NMe_3 resonances are labelled with arrows in (*a*); the internal resonance disappears in (*b*). Intravesicular solution $CoCl_2$ (0.25 mol dm^{-3}) in D_2O; extravesicular solution NaCl (0.25 mol dm^{-3}) in D_2O

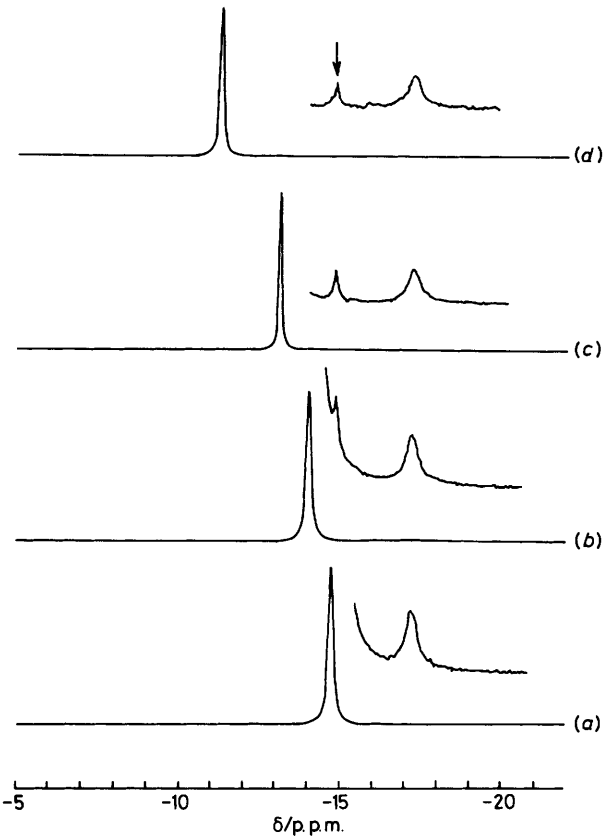

Figure 2. Phosphorus-31 n.m.r. spectra (121.49 MHz) of vesicles containing phosphate ions at pH_{out} values of (*a*) 6.67, (*b*) 7.4, (*c*) 10.95, and (*d*) 12.5. Intravesicular and extravesicular solutions, NaH_2PO_4 (1 mol dm^{-3}). The arrow indicates the intravesicular phosphate peak corresponding to pH_{in} 6.7

resulting in a splitting of the chemical shift of the $^+NMe_3$ headgroup resonance with the internal resonance at a chemical shift value of 2.82 p.p.m. and the external resonance at a value of 3.11 p.p.m. Addition of NaOD to this solution (final pH_{out} 12.4) resulted in the disappearance of the internal $^+NMe_3$ resonance leaving one $^+NMe_3$ resonance (at 3.11 p.p.m.) corresponding to both internal and external headgroups [Figure 1(*b*)]. These observations can be explained by the removal of Co^{II} ions from the headgroup binding sites and the formation of a solid phase, $Co(OH)_2$, inside the vesicles. As the concentration of Co^{II} ions falls the internal headgroup resonance shifts back to the unperturbed position of the external headgroup. The formation of paramagnetic $Co(OH)_2$ particulates within the vesicles resulted in the broadening of the $^+NMe_3$ resonance due to large bulk magnetic susceptibility effects at the membrane surface. No such extensive broadening was seen on the formation of diamagnetic intravesicular precipitates such as Ag_2O.[6]

Figure 1(*b*) was obtained immediately after the change in the external pH and it can be seen that the membrane was not able to prevent the change in the internal pH and the precipitation of $Co(OH)_2$. Under these experimental conditions $Co(OH)_2$ precipitates at a pH of *ca.* 6.0 and this value must have been reached within the vesicle compartment.

The change of intravesicular pH under these reaction conditions was studied by ^{31}P n.m.r. spectroscopy. Figure 2 shows ^{31}P n.m.r. spectra recorded for a vesicle suspension containing NaH_2PO_4 (1 mol dm^{-3}) in both the internal and external compartments. Figure 2(*a*)—(*d*) represents spectra of this sample recorded at pH_{out} of 6.67, 7.4, 10.95, and 12.5 respectively. Chemical shift values are quoted upfield from the methylenediphosphonic acid standard of 0.00 p.p.m.

The upfield resonance at −17.15 p.p.m. was unaffected by the change in pH_{out} and was assigned to the vesicle phosphodiester headgroup from a ^{31}P n.m.r. spectrum of a vesicle solution in distilled water (no inorganic phosphate). The large sharp resonance in each spectrum is the external inorganic phosphate peak (P_{out}) which as expected shifts downfield, with increasing pH_{out}. The small peak at −14.78 p.p.m., seen as a shoulder in spectrum 2(*b*) but clearly resolved in spectra 2(*c*) and 2(*d*) (see arrow), corresponds to the internal phosphate resonance (P_{in}). Increasing pH_{out} had no significant effect on the chemical shift of this resonance. Spectrum 2(*d*) was rerun after one hour and no change in chemical shifts was observed.

Similar results were observed with vesicle preparations containing only internal phosphate solutions. Spectra from these solutions (data not shown) lacked the large resonance from the external phosphate, making it easier to observe the internal phosphate resonance. As in Figure 2, increasing pH_{out} had no effect on the chemical shift of the internal phosphate. It was concluded that at an external pH of 12.5, a pH gradient of *ca.* 6 units could be maintained for at least one hour at room temperature.

It is very likely that the reason for this apparent impermeability of the lipid membrane towards hydroxide ions is due to the difficulty of the complementary process of phosphate diffusion out of the vesicle. Over the pH range considered (6—12.6) the main phosphate species present will be HPO_4^{2-} and PO_4^{3-}. The high charges on these anions will prevent

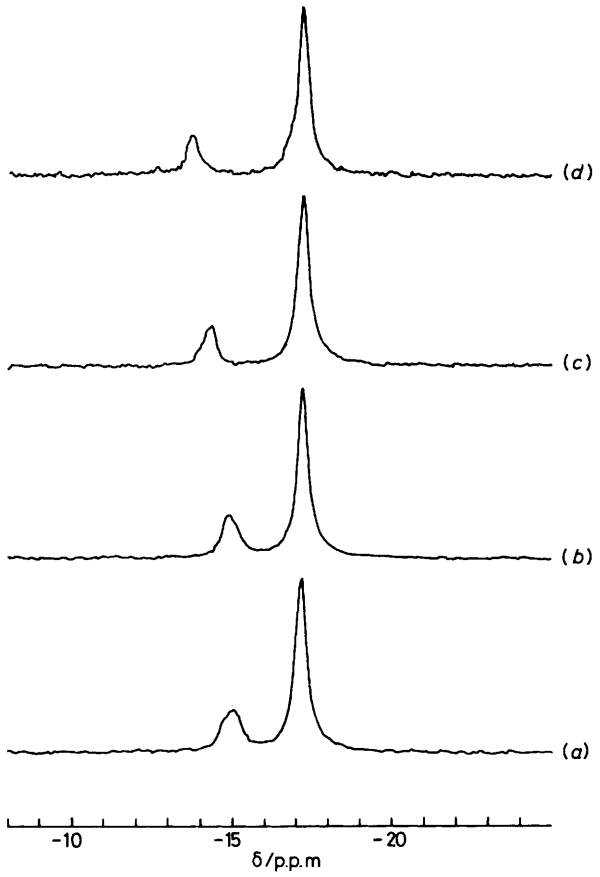

Figure 3. Steady-state ^{31}P n.m.r. spectra (121.49 MHz) of vesicles containing phosphate and nitrate ions at pH_{out} values of (*a*) 8.4, (*b*) 10.4, (*c*) 11.5, and (*d*) 12.46. Intravesicular solution NaH_2PO_4 (0.80 mol dm^{-3})–$NaNO_3$ (0.20 mol dm^{-3}); extravesicular solution, $NaNO_3$ (0.20 mol dm^{-3})

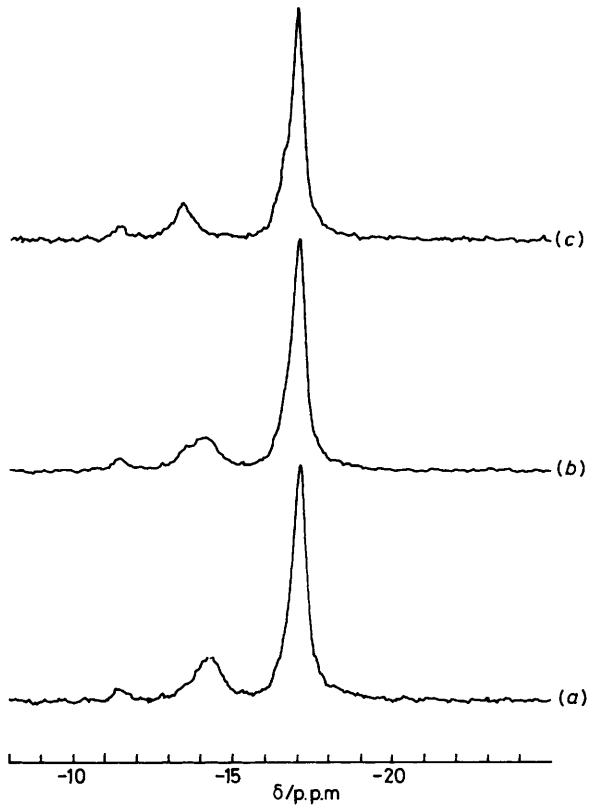

Figure 4. Phosphorus-31 n.m.r. spectra of vesicles containing phosphate and nitrate ions showing the time dependence of the spectra. The spectra were recorded (*a*) 45, (*b*) 80, and (*c*) 100 min after changing pH_{out} to 13.05 from 6.9. Intravesicular solution, NaH_2PO_4 (0.80 mol dm^{-3})–$NaNO_3$ (0.20 mol dm^{-3}); extravesicular solution $NaNO_3$ (0.20 mol dm^{-3})

diffusion through the highly hydrophobic regions of the lipid membrane. Thus a high charge potential is maintained between the two sides of the membrane preventing all but trivial ion influx.

Vesicles containing a mixture of NaH_2PO_4 (0.80 mol dm^{-3}) and $NaNO_3$ (0.20 mol dm^{-3}) and suspended in $NaNO_3$ solution (0.20 mol dm^{-3}) were prepared in order to test if the presence of the diffusable NO_3^- ion would permit the influx of hydroxide as observed for intravesicular Ag_2O formation from encapsulated $AgNO_3$ solution.[1] Figure 3 shows spectra recorded under steady-state conditions at external pH values of (*a*) 8.4, (*b*) 10.4, (*c*) 11.5, and (*d*) 12.46. The intravesicular phosphate resonance, initially at −14.98 p.p.m., did not shift significantly until above pH_{out} 11.0, after which it shifted steadily downfield. The steady state was not observed immediately after the change in pH_{out}. Phosphorus-31 spectra were recorded at intervals after a change in pH_{out} and the steady-state spectrum recorded when the individual spectra had become time-independent. For example, Figure 4 shows spectra recorded (*a*) 45, (*b*) 80, and (*c*) 100 min after changing pH_{out} to 13.05 from 6.9. The broadening of the internal phosphate resonance in Figure 4(*b*) appears to arise from an overlap between two internal phosphate resonances corresponding to the initial and final values of pH_{in}.

Figure 5 summarises the data obtained for phosphate- and phosphate–nitrate-containing vesicles at different external pH values under steady-state conditions. The ^{31}P chemical shift of the extravesicular inorganic phosphate with pH

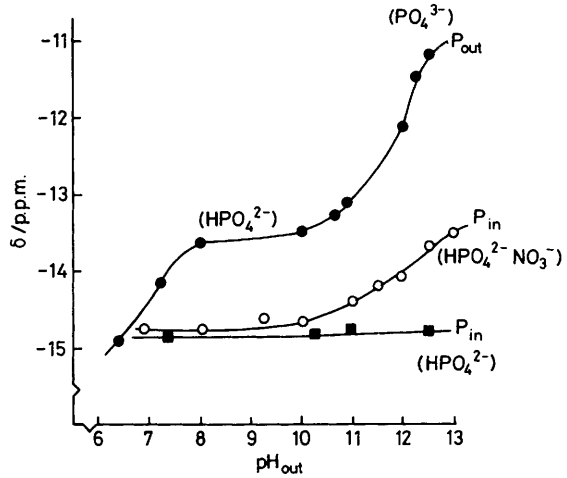

Figure 5. Summary of ^{31}P n.m.r. chemical shift data for vesicles containing phosphate, with and without intravesicular nitrate under steady-state conditions

serves as a calibration curve from which the intravesicular pH can be calculated. The corresponding plot of pH_{out} against pH_{in} under steady-state conditions is shown in Figure 6. Intravesicular pH does not appreciably rise until a pH_{out} of *ca.* 11.0 is attained. Above this value the increase in pH_{in} appears to be linear with pH_{out}, although it should be noted

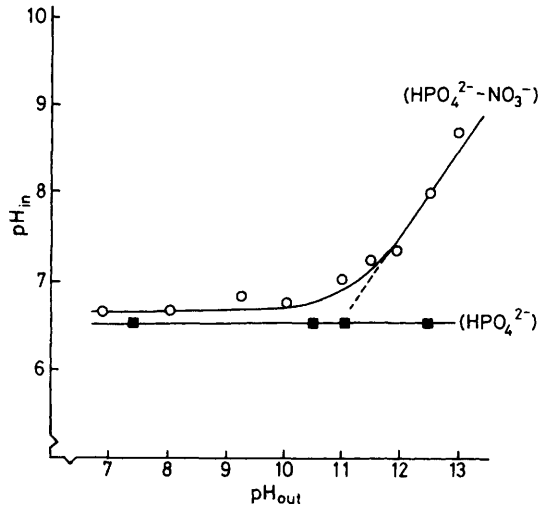

Figure 6. Plot of intravesicular pH against extravesicular pH for vesicles containing phosphate, with and without intravesicular nitrate

that the inorganic phosphate chemical shift is relatively insensitive to pH between 8 and 10 so that the error in the pH_{in} value corresponding to a pH_{out} of 13.0 is large.

The reduction of the pH gradients across the vesicle membranes in these experiments requires the net transport of OH^- ions into the vesicles or the net transport of H^+ ions out of the vesicles. In order to preserve electroneutrality this can only occur if there is an equivalent net migration of either negative ions out of the vesicles or positive ions into the vesicles. In the experiments with vesicles containing Cl^- (Figure 1) and NO_3^- (Figures 3 and 4) the internal pH responded to a change in pH_{out} above 11.0 whereas the vesicles containing only the more highly charged HPO_4^{2-} and PO_4^{3-} ions showed no change in internal pH. It is concluded that the presence of encapsulated diffusible anions is important in determining the role played by the membrane in controlling intravesicular pH.

The net rate of migration of ions through a lipid membrane is affected by two forces: (*a*) thermal agitation giving rise to a net diffusive movement due to differences in chemical concentration between the two phases, and (*b*) net movement due to electrical potential gradients established across the membrane due to diffusion of the penetrating ions creating a charge separation between the different compartments.

If a membrane separating two solutions of different composition and pH is only permeable to the OH^- ion then there can be no equilibration of the pH values of the two solutions. In this situation the membrane potential ($\Delta\psi$) cancels the difference in chemical potential of the permeable species so that the difference in electrochemical potential across the membrane is zero. As a result there is no net driving force and thus no net flow of OH^- ions. The membrane potential is given by equation (1), where ψ_{in} and ψ_{out} are the corresponding in and

$$\Delta\psi = (\psi_{in} - \psi_{out}) = \frac{RT}{zF} \cdot \ln \left[\frac{a(OH^-)_{out}}{a(OH^-)_{in}} \right] \quad (1)$$

out potentials at the two sides of the membrane, $a(OH^-)_{out}$ and $a(OH^-)_{in}$ are the external and internal activities of the OH^- ions and z, R, T, and F have their usual meaning. Under these conditions $\Delta\psi$ is the Nernst equilibrium potential for OH^- ions. This is the case for vesicles containing only inorganic phosphate solutions.

The situation is different for vesicles containing permeable NO_3^- ions since counterflow of these ions out of the vesicle will permit a change in the membran epotential across the membrane. In this case the membrane potential is given by the Goldman–Katz–Hodgkin equation obtained by integrating the Nernst–Planck equation subject to various restrictions.[7] Assuming the permeabilities of cations across lipid membranes to be some 10^5 times lower than for anions [8] the Goldman–Katz–Hodgkin equation reduces to equation (2), where P_{OH}

$$\Delta\psi = \frac{RT}{zF} \cdot \ln \left[\frac{P_{OH}a(OH^-)_{out} + P_{NO_3}a(NO_3^-)_{out}}{P_{OH}a(OH^-)_{in} + P_{NO_3}a(NO_3^-)_{in}} \right] \quad (2)$$

and P_{NO_3} are the permeability coefficients of OH^- and NO_3^- ions respectively. In reality, $\Delta\psi$ will be affected also by ion binding at the membrane surface and by vesicle swelling (osmosis).

In this system equilibrium is reached when equation (3) applies for each permeable ion j, where $\Delta\tilde{\mu}(j)$ is the electro-

$$\Delta\tilde{\mu}(j) = RT \ln \left[\frac{a(j)_{in}}{a(j)_{out}} \right] + z_j F\Delta\psi = 0 \quad (3)$$

chemical potential difference for ion j of charge z_j. The solution of this problem is unwieldy but in general it is clear that it is not possible to eliminate the pH gradient across the membrane, merely to reduce it. This condition can only be satisfied when the immediate OH^- influx becomes commensurate with the NO_3^- fluxes. A steady state is reached when the net flux of OH^- becomes zero.

These observations explain why the intravesicular precipitation of Ag_2O [1] only occurs above a pH_{out} of 11.0. Below this value trivial OH^- influx takes place and supersaturation within the vesicle is not attained. Above pH_{out} 11.0, OH^- influx is significant, raising pH_{in}, and overcoming the solubility product of Ag_2O.

Finally we draw attention to the biological significance of our observations. Mitchell [9] and Williams [10] have drawn attention to the value of pH gradients in the bioenergetics of molecular reactions. The control of proton diffusion across and within membrane spaces gives biological systems control over transport of many species both locally and over long distances. This paper shows that pH control using membranes can be exercised over precipitation reactions so that the formation and dissolution of calcium carbonates or calcium phosphates, in shells and bones, for example, may well be localised by the prevailing metabolic controls over pH. Similarly, the formation of calcium oxalate in some plants may be determined by the acidic nature of the vacuole. Oxalic acid is a stronger acid than carbonic or phosphoric acids and the vacuole pH is typically *ca.* 5.5.[11]

References

1 Part 1, S. Mann and R. J. P. Williams, *J. Chem. Soc., Dalton Trans.*, 1983, 311.
2 P. J. Strong, A. J. Skarnulis, and R. J. P. Williams, *J. Chem. Soc., Chem. Commun.*, 1978, 1030.
3 R. B. Moon and J. H. Richards, *J. Biol. Chem.*, 1973, **248**, 7276.
4 R. Iles, *Bio Science Rep.*, 1981, **1**, 687.
5 R. A. Y. Jones and A. R. Katritzky, *J. Inorg. Nucl. Chem.*, 1960, **15**, 193.
6 S. Mann, Ph.D. Thesis, Oxford, 1982.
7 S. G. Schultz, 'Basic principles of membrane transport,' IUPAB Biophysics series, Cambridge University Press, 1980, vol. 2.
8 A. D. Bangham, *Protoplasma*, 1967, **63**, 183.
9 P. Mitchell, *Nature (London)*, 1961, **191**, 144.
10 R. J. P. Williams, *J. Theor. Biol.*, 1961, **1**, 1.
11 M. J. Kime, B. C. Loughman, R. G. Ratcliffe, and R. J. P. Williams, *J. Exp. Bot.*, 1982, **135**, 656.

Received 26th May 1982; *Paper* 2/878