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Surface charge effects upon membrane transport processes: the effects of surface charge on the monensin-mediated transport of lithium ions through phospholipid bilayers studied by ^{7}Li-NMR spectroscopy

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Addition of monensin to preparations of large unilamellar vesicles prepared from egg-yolk phosphatidylcholine (egg PC) or egg PC containing 5% phosphatidylserine (PS^-) or cetylpyridinium (CP^+) ions in lithium chloride solution allows the transport of Li^+ ions to be monitored by an NMR magnetisation transfer technique. The kinetics of the transport are followed as a function of the metal ion and monensin concentrations and are compatible with a model in which one monensin molecule transports one Li^+ ion. The data allow the extraction of the rate constants for the association and dissociation of the monensin-Li^+ complex in the water/membrane interfaces and the evaluation of the stability constants for complex formation in the interfaces. Placing positive charge (CP^+) on the membrane surface reduces the formation rate by a factor of about three but hardly alters the dissociation rate. Placing negative charge (PS^-) on the membrane surface hardly alters the formation rate but speeds the dissociation rate by about a factor of three. Data from relaxation times of $^7Li^+$ inside the vesicles and from the total enclosed volumes as the vesicles are formed, point to appreciable Li^+ surface interactions that increase as the charge on the surface is made more negative. The size of the vesicles formed by the dialytic detergent removal technique increases with the surface charge. The results support a view that enzyme–phospholipid or substrate–phospholipid interactions could play an important role in determining the efficacity of action of membrane bound enzymes. The relevance of the results in the role of Li^+ in the control of manic depression is also discussed.

Introduction

The limiting membranes of cells serve many functions. One of their main roles is to serve as a support for enzymes. In many instances it is known that specific phospholipids are required in the membrane for the enzyme to function correctly [1] and it has been shown that some enzymes have a motionally restricted lipid shell around them [2]. It is generally assumed that such effects arise from solvation of the enzyme in the interior of the membrane by the required phospholipids and that these interactions maintain the correct conformation of the lipophilic sections of the enzyme. Much less is known about interactions between phospholipids and enzymes in the membrane surface although evidence of such interactions does exist [3].

Such solvation effects can broadly be described as thermodynamic – the effect of changes in phospholipid composition on the solvation and stability of membrane bound enzymes. What has not been investigated to any appreciable extent is the kinetic effect of phospholipid composition – the

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effect of changes in phospholipid composition, particularly changes in head groups – on the rates of enzyme catalysed reactions.

The work that has been carried out in this area shows evidence of specific activation of enzymes by certain lipids [4]. There is evidence, for example, that β-hydroxybutyrate dehydrogenase has an allosteric requirement for two molecules of phosphatidylcholine [5] and that other membrane-bound enzymes may have specific lipid requirements for them to function correctly. This evidence is of activation: the requirement of an enzyme for specific lipids to be present. It does not address the general question of how changes in lipid composition, particularly at the surface, change the rates of enzyme-mediated reactions.

Enzymes or any other molecules involved in reactions at membrane surfaces must recognise their substrate and complex with it in the membrane surface. Interactions between enzyme and lipid headgroups or between lipid headgroups and substrate potentially alter the rates of these reactions. It is mainly to the latter problem that the current work is directed.

A universal feature of living things is their ability to transport the alkali metal ions Na^+ and K^+ across their plasma membranes. Membrane-bound enzymes are responsible for maintaining the concentration gradients of these ions and the membrane potential across cell membranes. Ionophores such as nigericin, monensin and valinomycin are also able to mediate ion exchange across membranes. We have previously demonstrated that the transport of alkali metal ions mediated by the ionophores monensin and nigericin can conveniently be studied by alkali metal NMR spectroscopy [6–9]. Further, we have demonstrated that it is possible to measure the rates of the reactions at the membrane surfaces for formation and dissociation of the metal/ionophore complex [7]. Ionophores, although not proteins, can be regarded as models for membrane-bound enzymes and thus should allow insights into the effects of changes in the membrane composition, particularly those on the surface, on the rates of enzyme-like reactions on membrane surfaces.

We have observed variations in surface reaction rates for monensin transporting Na^+ and Li^+ when both negative (phosphatidylserine (PS)) and positive (cetylpyridinium (CP^+)) charges are placed on the surfaces of egg-yolk phosphatidylcholine (egg PC) bilayers. Some of the effects observed with Li^+ are reported in this paper. Interest in Li^+ chemistry in biological systems is heightened by the physiological effects of Li^+, particularly because of its use in the control of manic depression [10]. Speculation as to the origin of this action has been widespread with one major suggestion being that it arises from a specific Li^+–phospholipid interaction [11,12].

Significantly important interactions between Li^+ ions and phospholipid membranes, particularly those with negatively charged surfaces as in for example dimyristoylphosphatidylserine (DMPS), and dipalmitoylphosphatidylglycerol (DPPG) have been reported. Hauser and Shipley [11] demonstrated that Li^+ induces an isothermal crystallisation of DMPS bilayers presumably by a strong ionic interaction with the negatively charged head group of the lipid. Cevc et al. [13] showed that thermodynamic and structural properties of PS bilayers were modified in the presence of Li^+ ions with a time-dependent alteration of the phase transitions associated with a specific ion binding to the headgroup that dehydrates the lipid and is similar to the interaction with H^+ ions. This result has been reinforced more recently by Roux and Neumann [14] who suggested that the binding constant for Li^+ to DMPS could be as much as 200 times that for Na^+. Fossel et al. [12] showed that there was a significant interaction between Li^+ and DPPG in phosphatidylcholine bilayers with a substantial formation constant for the Li^+-DPPG complex. Other workers have shown that there are measurable interactions of Li^+ and Na^+ ions with the surfaces of phosphatidylcholine bilayers but that these are weaker than those with DMPS bilayers.

The transport of Li^+ mediated by monensin is up to 100-times slower than that of Na^+ and falls conveniently into the range measurable by magnetisation transfer [8]. This technique, for a two site case, involves placing a magnetic label at one site by inverting the spin populations (inverted signal) and then following the intensity at the other site as the inverted signal relaxes back. If there is chemical exchange between the two sites at around the timescale of the relaxation process a

reduction in intensity of the monitored signal will be observed. The longer the relaxation times of the relevant signals the slower the rate of exchange that can be measured. Although ^{7}Li is a quadrupolar nucleus its quadrupole moment is so small as to give sufficiently long relaxation times in aqueous solution (up to 20 s at our magnetic field strength) to allow this technique to be used effectively in our systems for exchange rate constants as low as 0.1 s^{-1}.

A related saturation transfer technique has been used in ^{13}C studies of membrane transport of glucose and bicarbonate in erythrocytes [15,16]. This technique uses a DANTE sequence [17] to saturate one of the exchanging resonances. Our technique specifically inverts one of the exchanging resonances and so gives twice the range of magnetisation available from saturation transfer.

Experimental

Large unilamellar vesicles were prepared from egg-yolk phosphatidylcholine (egg PC) or egg PC loaded with 5% bovine spinal chord phosphatidylserine or with 5% cetylpyridinium bromide as described previously [7–9]. A typical preparation would have a total of approx. 27 μmoles of lipid. The lipids were mixed as solutions in chloroform/methanol before being evaporated under high vacuum and treated as described in our previous papers. Three dialyses at 40 °C against LiCl produced large detergent-free unilamellar vesicles with the same concentrations of Li^+ inside and out. Two final dialyses introduced a small amount (0.5–5 mM) of lithium linear tripolyphosphate ($LiPPP_i$) into the external medium. The Li^+/PPP_i^{5-} ratio in the external medium was 25 : 1 with the ionic balance being made up by chloride. Sufficient $DyCl_3$ (typically a few microlitres of an 80 mM aqueous solution) was then added to generate a shift difference of between 25 and 45 Hz (νHz).

Spectra were obtained on a Bruker WP 80 spectrometer operating at 31.14 MHz. Longitudinal relaxation times (T_1) were obtained for each site by the inversion recovery method before any addition of monensin. To measure exchange rates aliquots of monensin were added, typically a few microlitres of a standard solution of monensin in methanol (approx. $6 \cdot 10^{-3}$ M). The pulse sequence 90°-t_1-90°-τ-90°-FID-d_1 was then employed with the transmitter set ν Hz to high frequency of the Li^+(in) signal and 2 ν Hz to high frequency of the Li^+(out) signal and with $t_1 = 1/2\nu$. The first two pulses of the sequence specifically invert the Li^+(out) magnetisation. The variable delay τ then allows chemical exchange and relaxation to occur. This simple three pulse sequence can be used for two exchanging sites in preference to the DANTE sequence [17] or to two-dimensional methods.

In a typical experiment the FID was collected into 4K data points with a spectral width of 600 Hz. A relaxation delay (d_1) of at least five times T_1(in) was inserted in the sequence. The spectra were line broadened by 1 Hz and transformed in the same sized block. All samples were field/frequency locked on an external ^2H_2O sample placed in the inner compartment of a coaxial tube. Signal to noise ratios were sufficiently good to allow the amount of Li^+ enclosed in the vesicles to be determined to a accuracy of better than 1%.

Rates of exchange were extracted from the time dependence of the intensity of the Li^+(in) signal by application of the equations derived by Morris and Freeman [17] and a least-squares technique in a program written for a BBC microcomputer.

Transport rates in vesicle systems are a function of vesicle size. The total enclosed volume of the EPC vesicles was nearly constant (11.5 ± 1%). Where the charge on the membrane surface altered the total enclosed volume (Tables II and III) from that obtained with pure egg PC a volume correction was applied to the observed rate such that:

$$\text{reported rate} = \frac{\text{observed rate} \times \text{\% volume enclosed}}{\text{average \% volume for PC vesicles}}$$

This correction ensures that rates are reported under as nearly comparable conditions as possible.

At low concentrations of Li^+ (< approx. 25 mM) a time-dependent decrease of the chemical shift difference by approx. 2–3 Hz over a 48-h period was observed. This decrease, attributable to slight hydrolysis of the PPP_i was faster with PS present in the membranes than in other cases.

All lipids were purchased from Lipid Products. The monensin was supplied by Sigma as its sodium

salt and purified by dissolving in ethanol and precipitating with water. Analytical thin-layer chromatography performed on lipids extracted from used nmr samples showed the starting lipids in their expected ratios with negligible traces $\ll$ 1% of other lipids present.

Results and Discussion

Li^+ relaxation times of intra- and extravesicular Li^+ ions were measured in order to extract rates of exchange. ^7Li is a quadrupolar nucleus which has a low quadrupole moment. Quadrupolar nuclei relax by interaction of the nuclear quadrupole with the electric field gradient (EFG) at the nucleus [18]. In the case of ^7Li in symmetrical environments the low quadrupole moment gives rise to relatively long quadrupolar relaxation times and dipolar relaxation contributes 30–50% of the total nuclear relaxation [19]. When Li^+ ions are subject to a substantial EFG or are immobilised as for example on a membrane, the relaxation times of the ^7Li should decrease–the lower the relaxation time the stronger the interaction.

For the extravesicular Li^+ the relaxation time is determined by the interaction of the Li^+ with the $Dy^{3+}PPP_i$ complex [20]. The chemical shift difference between the intra- and extravesicular Li^+ is also determined by this. Relaxation times of extravesicular Li^+ with the chemical shift differences established of between 25 and 45 Hz were typically between approx. 0.8 and 0.5 s^{-1}. Such relaxation times are convenient for our rate measurements. The extravesicular relaxation times, depending only upon the chemical shift established are not instructive as to membrane–Li^+ interactions. However, the intravesicular Li^+ relaxation times are.

The measurements of spin-lattice relaxation times of intravesicular Li^+ show evidence of interaction between the Li^+ ions and the membrane surface. Measured $^7Li^+$ relaxation times in free aqueous solution (curve a), inside vesicles formed from egg PC (curve c) and inside vesicles from egg PC loaded both with 5% PS (curve d) and 5% CP^+ (curve b) are shown in Fig. 1. In all cases the relaxation times of intravesicular Li^+ are shorter than for free Li^+ in H_2O indicating a membrane–Li^+ interaction.

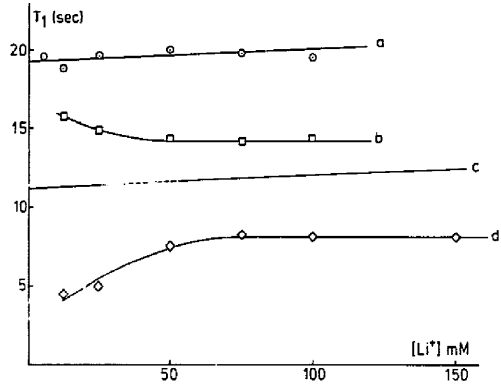

Fig. 1. Variation in $^7Li^+$ T_1 values in free aqueous solution and inside the vesicles against $[Li^+]$.

The relaxation times of LiCl in H_2O are approx. 20 s and show what may be a slight variation with concentration. The relaxation times of Li^+ inside egg PC vesicles are about half those of free aqueous Li^+ showing that there is an appreciable interaction between Li^+ and phosphatidylcholine in accord with previous suggestions.

When 5% PS is included in the membrane the relaxation time of the intravesicular $^7Li^+$ decreases further and shows a fall to lower values at concentrations below approx. 75 mM. There is a limiting value at approx. 8 s for higher values of $[Li^+]$ and a decrease to approx. 4 s at low values of $[Li^+]$. The importance of individual Li^+–PS interactions increases at low $[Li^+]$. For a vesicle of radius 400 nm containing 5% PS and 10 mM Li^+ there are approx. 6 Li^+ ions per PS on the interior face. Thus at low $[Li^+]$ the expected importance of one on one Li^+–PS^- interactions is observed. There is clearly a very strong interaction between Li^+ and membranes containing PS even at 5% loading.

By contrast with positive charge, when 5% CP^+ ions are incorporated in the membrane, the relaxation time increases slightly at low $[Li^+]$ indicating a reduced interaction between Li^+ and the membrane surface. As with the PS loaded membranes the effect increases at low ionic concentration as the individual ion–charge interactions become more important.

A similar conclusion about Li^+–PS interactions can be reached from a study of the relative sizes of

the vesicles formed by our dialytic detergent removal technique. These results are presented in Fig. 2. With egg PC the size of the vesicles, as measured by the total enclosed volume in the NMR spectrum, hardly varies with concentration and is approx. 11.5%. With 5% charged species in the membrane at low ionic strength the enclosed volume more than doubles (approx. 24% CP^+; approx. 29% PS^-). This increase arises because electrostatic interactions between charges are greater on the inside of a curved surface than on the outside. Thus, as the detergent is being removed and the bilayer being formed there is less tendency for it to curl back upon itself giving rise to larger vesicles.

With 5% CP^+ in the membrane there is no appreciable decrease in internal volume at up to 100 mM Li^+. By contrast, the internal volume and therefore the size of the vesicles containing 5% PS falls rapidly between 50 and 100 mM Li^+ to almost the value of egg PC vesicles. This can be explained if there is a substantial hard interaction between Li^+ and the PS negative charges on the membrane surface that neutralises the charges of the PS and hence the electrostatic effect at higher [Li^+]. With positive charge on the membrane surface the interaction between the pyridinium ion and the anions in solution, principally Cl^-, must be much weaker.

The evidence from the relaxation time measurements and from the internal volumes is consistent with the ideas advanced by other workers of a

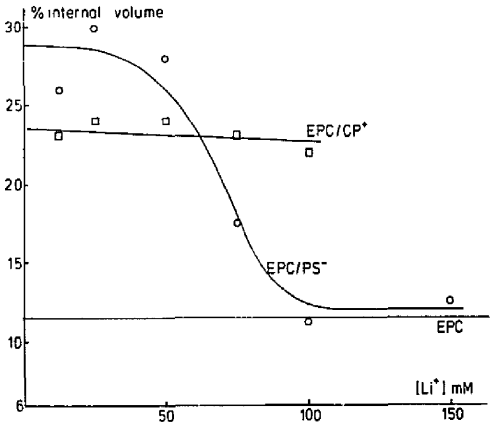

Fig. 2. Variation in internal volume for egg PC vesicles and vesicles carrying surface charge against [Li^+].

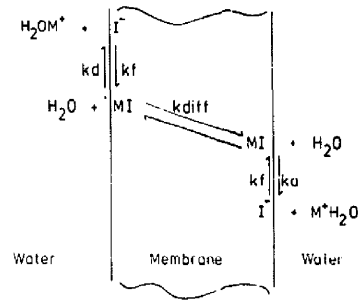

Fig. 3. Mechanism for ionophore-mediated transport of metal ions through phospholipid membranes.

hard interaction between Li^+ and the headgroups of PS molecules [11–13]. The evidence also points to a somewhat weaker interaction of Li^+ ions with the zwitterionic headgroups of the egg PC and to an even weaker interaction when positive charge is incorporated in the membrane surface. These are thermodynamic effects that will affect the concentrations of Li^+ ions near the membrane surface. They indicate a higher concentration of Li^+ near the PS-loaded surface than near the egg PC surface, and a lower concentration of Li^+ near the CP^+ loaded surface than near the egg PC surface.

We have previously shown that the mechanism of transport shown in Fig. 3 gives a satisfactory explanation of our results for Na^+ and K^+ transport mediated by nigericin [7] and monensin [9]. We have also shown that diffusion is not rate limiting in these cases. The rates of transport of Li^+ by monensin (Tables I–III) are typically 100-times slower than those of Na^+ and K^+. One would not expect diffusion of the Li^+ complex to be 100-times slower than for the other metals therefore diffusion is not rate limiting in the current experiments. From the rate equation for transport with rapid diffusion (Eqn. 1) [7] we can show that a plot of k^{-1} vs. [Li^+] should give a straight line with intercept $(k_f')^{-1}$ and slope $(k_d')^{-1}$. These results are presented graphically in Fig. 4.

$$\frac{1}{\tau_{M_{in}^+}} = \frac{A\,k_d[L]_T}{V_{in}2([M^+] + k_d/k_f)} \qquad (1)$$

where $\tau_{M_{in}^+}$ is the lifetime of a metal ion M^+ inside a vesicle of volume V_{in} and surface area A at a total ionophore concentration of $[L]_T$.

TABLE I

RATE CONSTANTS FOR Li^+ TRANSPORT IN EGG PC VESICLES

The average value of T_1(in) from these and other experiments with egg PC vesicles was 11.5 ± 1 s. From these results: $k_f' = 1031 \pm 145\ s^{-1}$, $k_d' = 55.8 \pm 5.0\ M \cdot s^{-1}$, $K = 18.5 \pm 3.4\ M^{-1}$.

$[Li^+]$(M)	k(mol lipid·mol monensin^{-1}·s^{-1})
0.0125	1081
0.025	751
0.050	471
0.075	401
0.100	339
0.125	333
0.150	294

TABLE II

RATE CONSTANTS FOR Li^+ TRANSPORT IN EGG PC/5% PS VESICLES

From these results: $k_f' = 1174 \pm 87\ s^{-1}$, $k_d' = 148 \pm 17\ M \cdot s^{-1}$, $K = 7.9 \pm 1.7\ M^{-1}$.

$[Li^+]$(M)	k(mol lipid·mol monensin^{-1}·s^{-1}) (corrected for vol.)	T_1(in) (s) ±0.5 s	Internal volume(%) ±1%
0.0125	1087	4.43	26
0.025	1060	4.89	30
0.050	851	7.52	28
0.075	710	8.28	17.5
0.100	631	8.06	11.2
0.150	578	8.13	12.5

The effect of placing positive charge (5% CP^+) on the membrane surface is to reduce the transport rate. The rate of formation of the monensin Li^+ complex is about one third of the rate for egg PC and the dissociation rate is not significantly altered. The presence of positive charge on the membrane surface should reduce the concentration of Li^+ ions near the surface as shown by the relaxation time data and this can account for the reduction in the rate of formation of the metal ionophore complex. Once the complex has reached the opposite surface and dissociates the release of Li^+ from a neutral complex into the positively charged surface will meet electrostatic opposition and this should slow the rate relative to egg PC.

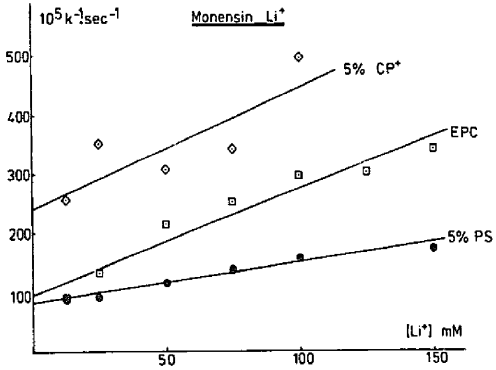

Fig. 4. Variation of reciprocal of corrected transport rate with metal ion concentration to allow extraction of rates of processes at membrane surfaces.

The effect of placing negative charge (5% PS) on the surface is recorded in Table II. The formation rate of the complex is not significantly altered. Although the presence of negative charge on the surface should result in a higher concentration of Li^+ near the surface, energy is needed to break the strong Li^+–PS interactions as the metal ion moves into a neutral complex inside the membrane. Thus, if the transition state for complex formation is 'reactant-like' we would expect a reduction in the rate of reaction. These two effects almost cancel each other and there is little change in the rate of complex formation. It should be noted that the simple explanation of a pre-equilibrium of Li^+ with the membrane surface prior to reaction does not account for the near similarity of rates. A specific Li^+–PS interaction is needed.

At the opposite membrane surface the metal ion is being released from the neutral complex inside the membrane into a negatively charged surface with strong Li^+–PS interactions. If the transition state for this transfer is 'product-like', the energy of the Li^+–PS interaction will lower the activation energy and increase the rate of reaction. There is a approx. 3-fold increase in rate compared to egg PC which we suggest arises from this effect.

The stability constants for the metal ionophore complex in the membrane surface are presented in Tables I–III. The stability constants for the

TABLE III

RATE CONSTANTS FOR Li^+ TRANSPORT IN EGG PC/5% CP^+ VESICLES

From these results: $k_f' = 415 \pm 59\ s^{-1}$, $k_d' = 48.5 \pm 15\ M \cdot s^{-1}$, $K = 8.5 \pm 2.7\ M^{-1}$.

$[Li^+](M)$	k(mol lipid·mol monensin^{-1}·s^{-1}) (corrected for vol.)	T_1(in) (s) ±0.5 s	Internal volume(%) ±1%
0.0125	390	15.6	23
0.0250	286	14.8	22
0.050	326	14.3	24
0.075	292	14.1	23.5
0.100	202	14.3	22

charged surfaces (approx. 8 M^{-1}) are lower than for the neutral surface (approx. 19 M^{-1}). In the case of PS-loaded membranes this is because of an increase in the dissociation rate with the association rate being similar. In the case of the CP^+-loaded membranes it is because of a decrease in the association rate with the dissociation rate being similar.

There has been speculation in the biochemical literature about phospholipid–enzyme interactions being important for the ability of enzymes to operate, or for the specificity of enzyme action [1,4,5]. Here we have evidence of a phospholipid headgroup effect on the rate of ion transport through a membrane. The concentrations of phosphatidylserine used in our experiments are similar to those found in physiological systems. Specific interactions between phospholipid and metal ions are almost certain explanations for the rate differences that we observe. Our results suggest that specific cation–phospholipid interactions could be important in determining rates of enzyme catalysed reactions such as those of the metal ATPases and any others which involve the removal of charged species from the membrane surface into the interior of the membrane. This of course would not preclude the existence of specific enzyme–phospholipid interactions of importance to enzyme function and may even enhance the importance of such effects.

Several suggestions have been made in the literature as to the role of Li^+ in the control of manic depressive illness [10–12]. One major hypothesis as to the mode of action speculates that it involves the interaction of Li^+ with cellular membranes by altering some functional properties of these membranes [11,12]. An alterative view has recently gained increasing acceptance, that the mode of action arises from a blockage of the enzyme that hydrolyses inositol phosphate [21]. Our results may lend some credence to the former suggestion without affecting the possible validity of the second. It is known that phosphatidylserine is a major phospholipid of excitable membranes [22]. The interactions that we have demonstrated between Li^+ ions and membranes containing physiologically normal amounts of phosphatidylserine suggest that localised high concentrations of surface Li^+ could be established in membranes containing high concentrations of phosphatidylserine. Such membranes may be those where Li^+ exerts its therapeutic effects.

Our results show that such surface concentrations of Li^+ can affect the function of molecules involved in ion transport and hence the nervous system. Alternatively, the absorption of Li^+ on the surface could result in the exclusion of Mg^{2+} or Ca^{2+} ions from membrane surface sites again affecting the ability of membrane systems to transport these metals and hence affecting the nervous system. A further hypothesis is that the Li^+–membrane interaction could react to changes in the concentrations of phosphatidylserine or other negatively charged lipids in cell membranes and thus control the action of these lipids in modulating enzyme function. We are examining the other naturally occurring negatively charged phospholipids to see if their behaviour parallels that of phosphatidylserine.

TABLE IV

^7Li T_1 MEASUREMENTS IN WATER

$[Li^+](M)$	T_1 (s) ±0.5 s
0.0050	19.5
0.0125	18.8
0.0250	19.6
0.0500	20.0
0.0750	19.8
0.1000	19.5

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