Biochimica et Biophysica Acta, 345 (1974) 439–447

BBA 76624

PROTON RELAXATION STUDIES OF WATER IN CONCENTRATED DIMYRISTOYLLECITHIN–WATER SYSTEMS

J. H. PRESTEGARD, and A. WILKINSON

Department of Chemistry and Department of Molecular Biochemistry and Biophysics, Yale University, New Haven, Conn. 06520 (U.S.A.)

(Received October 18th, 1973)
(Revised manuscript received January 31st, 1974)

SUMMARY

Proton relaxation studies have been performed on concentrated dimyristoyllecithin–water samples as a function of temperature. Both T_1 and T_2 data indicate a sharp increase in the mobility of the water at the hydrocarbon transition. In addition, there is the onset of an exchange process with a lifetime of a few milliseconds at this same temperature. It is suggested that this exchange encompasses diffusion over large distances and perhaps through the lipid bilayer.

INTRODUCTION

Phospholipid bilayers have, in recent years, gained acceptance as models for at least certain regions of biological membranes. Studies of the properties of these bilayers, which form spontaneously when most phospholipids are dispersed in water, have been directed mainly at the hydrocarbon layer. Accurate measurements of the dimensions of this region as well as determinations of internal fluidity have been made, and several phases which differ in dimension and fluidity have been identified. Among them are a low temperature gel phase with hexagonally packed hydrocarbon chains and a high temperature liquid crystalline phase with more disordered chains. Properties dependent on fluidity or dimensions of this layer often show abrupt changes at the transition temperature, T_m, which characterizes the gel to liquid crystal conversion.

One might expect the influence of the hydrocarbon layer and its transition to extend even to a region of interfacial water. Chapman et al. [1] have, in fact, observed a number of water molecules in lipid dispersions which do not freeze until well below 0 °C, and thus are believed tightly bound to the bilayer. Whether the behaviour of this water is directly linked to hydrocarbon properties or is mediated by the behaviour of lipid polar groups at the interface is open to question. However, the fact that polar groups seem to undergo a pretransition, T_p, independent of the hydrocarbon transition, T_m, opens the way for experiments designed to test the degree to which structural properties of interfacial water and lipid bilayer are interrelated.

Nuclear magnetic resonance studies can contribute significantly to the understanding of motional and structural properties at a molecular level. In particular, the spin–lattice relaxation time, T_1, and the spin–spin relaxation time, T_2, provide measures of the correlation time for molecular motion and hence give information on structural order. In addition, T_2 as measured by a variable pulse spacing Carr–Purcell experiment contains information on diffusion and chemical exchange of a species between different environments.

Observation of T_1 and T_2 for interfacial water would seem useful in describing the structure and properties of the interface as a whole. These relaxation times can provide information on the mobility of interfacial water, and, depending on how strongly this water is linked to head group or hydrocarbon structure, on changes in head group organization or hydrocarbon dimensions that occur through the transitions mentioned above. We shall present the results of such T_1 and T_2 observations on a dimyristoyl-lecithin–water system. Sensitivity to the characteristics of interfacial water in such experiments can be ensured by observing samples with sufficiently low water content. Sorption studies [2] have shown that a mole of lecithin can bind up to 30 moles of water of which 5–6 moles are tightly bound in the first and second hydration layers. Estimates based on other techniques [1] have placed the number of tightly bound water molecules per lecithin as high as ten. In our experiments, the mole ratio of dimyristoyllecithin to water was varied from 1 : 19 to 1 : 39. Thus, interfacial water should respresent a large fraction of all water in the sample at either of these concentrations.

Salsbury et al. [3] have performed NMR experiments on similar systems, concentrated dipalmitoyllecithin–^2H_2O, and have been able to detect abrupt changes in the properties of water near T_m. However, for dipalmitoyllecithin T_m and T_p are too close to allow a clear association of changes in T_1 or T_2 with either transition. Also, in deuterium magnetic resonance, electric quadrupole relaxation may obscure the effects of chemical exchanges which take place on a time scale of $1–10^{-3}$ s. Proton relaxation time measurements on a dimyristoyllecithin–water system offer improvement in both respects. There is a temperature separation of some 10 °C between the pre- and maintransition [4] so that a clear association of a change in relaxation with one or the other is possible. Also, water in such systems has a relaxation time long enough to allow domination by a chemical exchange mechanism [5]. Studies on the system presented here should therefore provide a clear measure of the degree of association of bound water with polar group and hydrocabon layers, and they should also provide a measure of mobility on two different time scales.

EXPERIMENTAL

β,γ-Dimyristoyllecithin was obtained from Calbiochem and used without further purification. Weighed amounts of lecithin were added to 5-mm NMR tubes each of which had a 2-mm constriction midway up the tube. Measured volumes of distilled water were added. Next, the samples were degassed, the tubes filled with N_2 at a pressure of approx. 560 mm Hg, and then sealed. Homogenization of the samples was accomplished by centrifuging the lecithin–water mixture back and forth through the constriction at a temperature greater than T_m (after Chapman).

NMR measurements were made using a modified Varian HR-60 spectrom-

eter operating at 56 MHz. This was interfaced with a PDP-8E computer which provided operating pulses and accumulated the data through a signal-averaging program. The width of the $\pi/2$ pulse produced was 150 ms. The T_1 measurements were made using the null method of Carr–Purcell. The T_2 measurements were made using the Gill–Meiboom modification of the Carr–Purcell method followed by a least squares fit of the data to a single exponential to determine T_2.

Although the accuracy of the null method for determining T_1 has been criticized as inaccurate, in the present work, comparison with T_1 values obtained from a fit of an entire set of pulse responses on a representative sample showed there to be no significant difference between the two methods for determining T_1. Measurements of T_1 and T_2 on standard samples of $CuSO_4 \cdot 5H_2O$ in water having T_1 values in the range of our lecithin samples in fact gave results within 5% of literature values and were reproducible to $\pm 5\%$ when determined by the null and exponential fit methods, respectively. Errors in T_1 and T_2 measurements for the lecithin samples are similar except for the long pulse spacing measurements on the 67% lecithin sample where the pulse spacing is greater than $\frac{1}{2}T_2$ resulting in somewhat greater errors. Temperatures were measured using a copper constantan thermocouple located next to the sample tube. Temperature regulation was to ± 1 °C.

After the completion of experiments, samples were analyzed to check for possible breakdown or variation in composition. Water content was determined by a dry weight analysis, and sample purity was checked on thin-layer chromatograms in the solvent system, chloroform–methanol–water (65 : 25 : 4, by vol.). Only trace amounts of lysolecithin were detected.

RESULTS

T_1 measurements

The temperature dependence of proton T_1 values from 10 to 45 °C for 50% (39 moles of water per mole lecithin) and 67% (19 moles of water per mole lecithin) dimyristoyllecithin in water is shown in Fig. 1. It can be demonstrated that the only significant contributions to these relaxation times come from the water protons and not from protons associated with the lecithin molecule. A sample of 50% lecithin for example gave little detectable signal on our apparatus when ^2H_2O was substituted for H_2O, probably because the response time of the spectrometer is long compared to T_2 for lecithin protons. The data from the π–$\pi/2$ pulse sequence on lecithin–water systems fit a single exponential reasonably well, implying that the bulk of the water present displays a single relaxation time characteristic of rapid exchange among several species. The value of T_1 obtained at 20 °C for water protons in a 67% dimyristoyllecithin sample (300 ms) is consistent with the data of Gottlieb et al. [5] on water–egg lecithin samples. T_1 data on a pure water sample are also included for comparison in Fig. 1.

The T_1 data for the lecithin–water system are different from those for pure water in two respects. First, the T_1 values are much shorter indicating some immobilization of the water involved. Secondly, there is an abrupt increase in T_1 between 20 and 28 °C. It is significant that this increase is near the calorimetric hydrocarbon transitions and not near the calorimetric pretransition (cf. Table I).

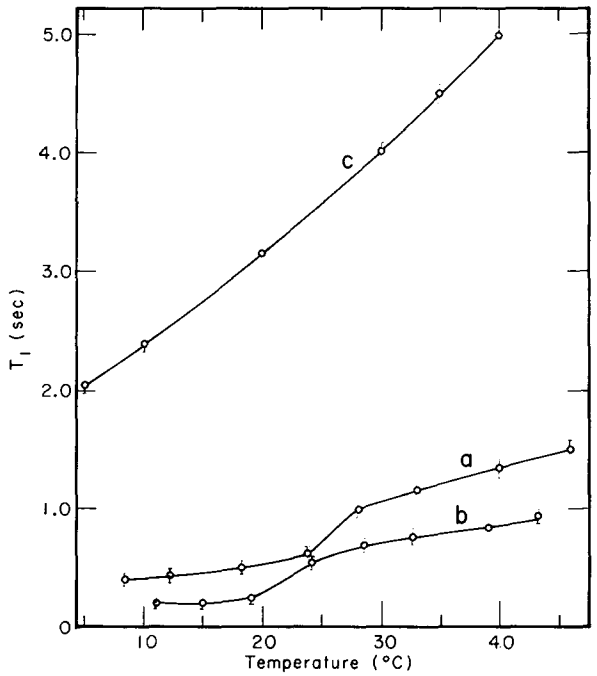

Fig. 1. Temperature dependence of T_1 for three systems: a, 50% dimyristoyllecithin; b, 67% dimyristoyllecithin; c, water. From Krynicki, K. (1966) Physica 32, 167.

TABLE I

COMPARISON OF CALORIMETRIC AND NMR RELAXATION DATA ON DIMYRISTOYL-LECITHIN–WATER SYSTEMS

The calorimetric data were obtained on a Perkin–Elmer DSC-2 calorimeter.

Concentration of DML* (% by wt)	Pretransition (°C)	Main transition (°C)	T_1 inflection (°C)	T_2 inflection (°C)
50	13	24	26	25
67	13	24	23	23

* DML, dimyristoyllecithin.

T_2 measurements

In Fig. 2, the temperature dependence of T_2 for a 50 and a 67% lecithin sample is plotted for various values of t_{cp}, the spacing between successive pulses in the Carr–Purcell experiment. The range of t_{cp} values was determined at the lower end by the limits of the spectrometer and at the higher end by the length of T_2. Again spin-echo data could be fitted reasonably well with a single exponential.

For any given value of t_{cp}, there are again two features of the plots which are immediately apparent. First, most of them show an abrupt increase in T_2 near the lipid hydrocarbon phase transition (Table I). This is consistent with the increase

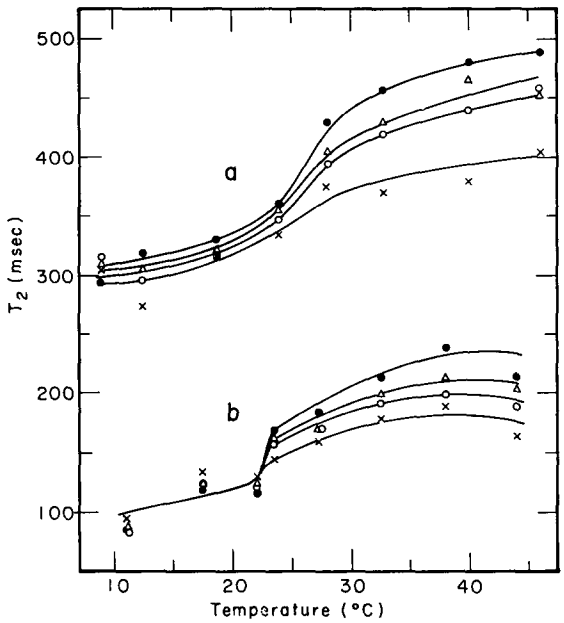

Fig. 2. Temperature dependence of T_2 for (a) 50 % dimyristoyllecithin and (b) 67 % dimyristoyllecithin with different values of t_{cp}: ×, 117 ms; ○, 64 ms; △, 45 ms; ●, 37 ms.

in mobility detected in T_1 measurements. Secondly, T_2 values are very short and in fact, much shorter than the corresponding T_1 values. This difference between T_1 and T_2 could arise from any one of several sources; contributions from species with single correlation times $\leqslant \omega_0$, contributions from species with rapid but highly anisotropic motion, or contributions from diffusion or chemical exchange mechansims of time scale $1–10^{-3}$ s.

The exchange and diffusion mechanisms can be distinguished from the others by the dependence of T_2 on the pulse spacing, t_{cp}, when this spacing is on the order of the diffusion or exchange time. Fig. 2 shows clearly that such a dependence exists least for temperatures above 22 °C. A simple calculation shows that the origin of this dependence cannot be diffusion in an externally applied field gradient. If it were, T_2 would depend on a diffusion coefficient, D, a T_2 in the absence of diffusion, T_2^0, a pulse spacing, t_{cp}, and a field gradient, γG in the following manner [6]:

$$T_2^{-1} = T_2^{0-1} + \frac{1}{12}\gamma^2 G^2 D\, t_{cp}^2 \tag{1}$$

Using a measured magnetic field gradient of $2.5 \cdot 10^3$ rad $\cdot$ s$^{-1}\cdot$cm^{-1}, and a value of D for a 67% lecithin sample at 30 °C equal to 10^{-5} cm$^2\cdot$ s^{-1} (ref. 12) the largest possible exchange contribution to $1/T_2$ would be 0.29 s^{-1}. Measured variations in $1/T_2$ are much greater. Hence, the observed dependence of T_2 on t_{cp} must arise from an exchange process.

Examination of the data makes possible some qualitative conclusions regarding the exchange processes. First, in both samples it is only at temperatures above T_m that there is a significant effect of varying t_{cp}. This implies that a drastic change in

the rate of or the number of molecules involved in the exchange process occurs at the hydrocargon transition temperature. Secondly, changing t_{cp} has a similar effect in both the 50 and 67% samples. This implies that although exchange makes a large contribution to T_2, it alone cannot account for the total change in T_2 on going from 50 to 67% lecithin.

Methodologies for obtaining a more quantitative description of the exchange process do exist [7–9]. In the present case, neither the range of t_{cp} values nor the number of points determined justified the use of the recent treatment of Carver and Richards [8]. Instead, the less complex approach of Allerhand and Gutowsky [7] was adopted. Their equation, Eqn 2, a modification of the Luz Meiboom equation for two site exchange, relates T_2 to the fractional populations of the exchanging sites, P_A and P_B, the chemical shift difference of the nucleus between the sites, $\delta\omega$, the average lifetime between exchange, τ, the Carr–Purcell pulse spacing, t_{cp}, and the relaxation time in the absence of exchange T_2^0.

$$T_2^{-1} = T_2^{0-1} + P_A P_B (\delta\omega)^2 \tau \left(1 - \frac{2\tau}{t_{cp}} \tanh \frac{t_{cp}}{2\tau}\right) \tag{2}$$

This equation was derived assuming that P_A and P_B are equal, that exchange is fast (i.e., $1/\tau \gg \delta\omega$), and that T_2^0 for each site is the same. Allerhand and Gutowsky [9] showed, however, that the Luz–Meiboom equation has a much greater range of applicability than was first indicated in that it closely approximates the exact equation even when P_A and P_B are quite different and when exchange is of an intermediate rate ($1/\tau = \delta\omega$). In the present work, if 9 or 10 water molecules were bound per lecithin in the 67% sample, they would exchange with an equal number of interstitial waters and hence P_A would very nearly equal P_B. Also, the fact that the observed relaxation was exponential suggests that the exchange rate cannot be too far from the fast exchange limit. The third assumption is not a crucial one, as it can be shown from the equations of Carver and Richards [8] that when $P_A \approx P_B$ and T_{2A}^0 and T_{2B}^0 are unequal, T_2^{0-1} can, to a good approximation, be replaced by $T_{2A}^{0-1} + T_{2B}^{0-1}$. We thus feel confident in applying Eqn 2 to our data.

Given T_2 versus t_{cp} data at two different lecithin–water ratios, it is theoretically possible to determine from Eqn 2 values for T_2^0, P_A, P_B, $\delta\omega$ and τ. Because our data were limited in quantity, we chose to estimate T_2^0 independently and to determine only τ and the product $P_A P_B (\delta\omega)^2$ at each concentration and temperature. In the limit of rapid, isotropic motion, T_2^0 can be taken equal to T_1. In the limit of restricted motion, one can say that the ratio of T_1 to T_2^0 should not exceed the value observed in the absence of exchange at low temperatures. Values of τ calculated by linear regression to Eqn 2 using these two limits on T_2^0 are presented in Table II.

Assuming $\delta\omega$ to be independent of composition and temperature, one can use values of $P_A P_B (\delta\omega)^2$ at two different lecithin concentrations to determine $P_A P_B$. If the number of sites available to one of the exchanging species is fixed by the number of lecithin molecules the fraction in that site, P_B, can be calculated. For $T_2^0 < T_1$, P_B was found to be 0.36 for the 67% sample and 0.18 for the 50% sample. This corresponds to about 7 moles water per mole lecithin. For $T_2^0 = T_1$, only meaningless values of P_B were obtained. This stems from the fact that if $T_2^0 = T_1$, the exchange term must account for approx. 75% of the observed T_2 and thus must more

TABLE II

Exchange lifetimes (τ) calculated for the two limiting values of T_2^0:(a) $T_2^0 = T_1$. (b) $T_2^0 < T_1$ (see text for details) at different temperatures.

Concentration of DML* (% by wt)	τ (ms) 28 °C	33 °C	39 °C	45 °C
50 Condition a	5	7	7	5
Condition b	7	8	9	6
67 Condition a	5	5	6	7
Condition b	11	10	9	11

* DML, dimyristoyllecithin.

than double in going from 67 to 50% lecithin. Because there is virtually no difference in values of τ between the two samples, there would then have to be a change in $P_A P_B$ by more than a factor of 2.5. This change cannot be accounted for in a model in which the number of bound sites is fixed and where the total amount of water present does not double from one concentration to the other.

The most significant thing to notice about the exchange time data in Table II is the lack of temperature dependence. When τ is related to a pseudo first-order rate constant, an activation energy much less than 8 kcal/mole is indicated for both 50 and 67% data. This fact has important implications on the nature of the exchanging species.

DISCUSSION

The preceding data show that the phase transition of the hydrocarbon region of a lipid bilayer from an ordered to a disordered state has a profound effect on the properties of water in the interfacial layer. There is an abrupt change in the mobility of the water seen in both the transverse and longitudinal relaxation times and there is evidence for the onset of an exchange process on the NMR time scale seen in the dependence of T_2 on pulse spacing.

Changes in mobility

If rapid exchange conditions exist, the increase in T_1 and T_2 on hydrocarbon melting could be due to an increase in mobility of one or more of several water species. There is evidence for several tightly bound species with numbers ranging from 2.5 to 10 molecules per lecithin molecule depending on the definition of "tightly bound" imposed by the method of observation [2, 10]. There is evidence for an additional 10–20 loosely bound molecules which exist in the interstitial zone between bilayers [1]. Any additional water added to the sample will exist as a free water phase. However, the 67% sample (19 water/lecithin) should have no free water so that the increase must occur in one or both of the interstitial or bound phases. The increase in T_1 occurs in the 22–26 °C region, one which is well removed from the pretransition temperature in which polar headgroups are thought to undergo some structural rearrangement. This suggests that the water species involved is not intimately asso-

ciated with headgroup structure, but is more sensitive to structural changes which take place on hydrocarbon melting. It has been shown that there is a substantial increase in surface area on hydrocarbon melting [11]. Hence, the increase in mobility may be associated with the increase in spacing between headgroups. These data do not necessarily contradict the deuterium NMR data of Salsbury et al. [3] which suggested a link of water structure to headgroup structure, since the relative sensitivities of our proton experiments and their deuterium experiments to a small number of tightly bound molecules differ widely. But, the data do at least suggest that the dependence of water structure on the state of the hydrocarbon region is more pronounced than the earlier work implied.

Onset of an exchange process

The T_2 data indicating the onset of an exchange process at T_m confirm the close link of the properties of at least some of the water in the sample to bilayer properties. The NMR data alone, however, place no stringent requirements on the nature of the exchanging species. In fact, they require only that there be two or more classes having chemical shift differences on the order of 0.1 ppm. Such differences may arise from association-induced shifts or simply from magnetic and chemical inhomogeneities in the sample. If one follows the assumptions of the results section the data indicate that one of the exchanging species represents about seven water molecules per molecule of lecithin. A class of this size is consistent with estimates of tightly bound species determined in sorption–desorption isotherm and NMR studies. NMR experiments of Gottlieb et al. [5] however have indicated exchange of tightly bound water to occur on the order of 10^{-4}–10^{-5} s, a time scale well out of range of our experiments. Hence the process we are sensitive to is likely to involve a more loosely bound water.

Some further insight into the nature of the exchanging species is given by the fact that the temperature dependence of their lifetimes as indicated in Table II is slight, implying a low activation energy for exchange (< 8 kcal/mole). This suggests that the particular species under observation are not tightly bound in the first hydration sphere of the polar headgroup (waters of hydration of a phosphate should have an activation energy for exchange in excess of the enthalpy of hydration which is about 8 kcal/mole [12]). Yet the fact that lifetimes are long, 5–10 ms, suggests an intimate association with the bilayer perhaps in the form of having to follow a circuitous path to reach an alternate site. Such a process has been suggested for water diffusion in concentrated lecithin samples.

This explanation is consistent with the fact that the lifetimes of exchanging species change abruptly at the bilayer hydrocarbon transition. We believe the change to be from a slow to a more rapid process. It is clear from Fig. 2 that exchange effects are not observed at low temperatures. However, inspection of Eqn 2 shows that this phenomenon could arise from one of three possibilities: from $P_A P_B \approx 0$, from a very short τ, or from a very long τ. It seems unlikely that $P_A P_B$ could change by an order of magnitude through the transition when most changes on bilayer melting, bilayer volume for example, are on the order of 5–10%. It is also difficult to envision how a transition characterized by increased fluidity of the dispersion and expansion of surface area could lead to a longer lifetime for an associated molecule. Hence, it seems likely that the lack of a demonstrable exchange below T_m is due to a long τ at those

temperatures. We have pointed to the fact that bilayer surface area and hence spacing of headgroups increases on going through the transition. This could lead to a less circuitous path for water exchange and hence shorter lifetimes above the transition. Sensitivity to hydrocarbon phase transitions could also be justified if transmembrane diffusion were important in the exchange process. The time scale for transmembrane diffusion is appropriate for the 5-ms lifetimes [14] and the dependence of diffusion rate on hydrocarbon fluidity is well established [13]. An exhaustive study of lecithins of differing internal fluidity would presumably distinguish these possibilities.

The dependence of lifetimes on the hydrocarbon transition and the low activation energy for exchange therefore suggest that the exchange involves more loosely bound, interstitial water and encompasses processes characterized by diffusion through well separated regions of chemical or magnetic inhomogeneity. Regardless of the nature of the exchange process, it is clear from both T_1 and T_2 data that a substantial fraction of water in a concentrated lecithin dispersion changes its properties (i.e. becomes more mobile) in concert with changes in the properties of the lipid bilayer. It is significant that the point of closest correlation is at the hydrocarbon transition and not at the pretransition of the system.

ACKNOWLEDGEMENT

Acknowledgement is made to the National Science Foundation (NSF-GB-32063) and to the donors of The Petroleum Research Fund, administered by the American Chemical Society for the partial support of this research. Thanks is also given to H. J. Morowitz for his encouragement and support, and to J. M. Steim for providing calorimetric data.

REFERENCES

1 Chapman, D., Williams, R. M. and Ladbrooke, B. D. (1967) Chem. Phys. Lipids 1, 445–475
2 Elworthy, P. H. (1961) J. Chem. Soc. 1961, 5385–5389
3 Salsbury, N. J., Darke, A. and Chapman, D. (1972) Chem. Phys. Lipids 8, 142–151
4 Hinz, H. and Sturtevant, J. (1972) J. Biol. Chem. 247, 6071–6075
5 Gottlieb, A., Inglefield, P. and Lange, Y. (1973) Biochim. Biophys. Acta 307, 444–451
6 Carr, H. Y. and Purcell, E. M. (1954) Phys. Rev. 94, 630–638
7 Allerhand, A. and Gutowsky, H. S. (1964) J. Chem. Phys. 41, 2115–2126
8 Carver, J. P. and Richards, R. E. (1972) J. Magn. Res. 6, 89–105
9 Allerhand, A. and Gutowsky, H. S. (1965) J. Chem. Phys. 42, 1587
10 Veksli, Z., Salsbury, N. J. and Chapman, D. (1969) Biochim. Biophys. Acta 183, 434–446
11 Melchior, D. L. and Morowitz, H. J. (1972) Biochemistry 11, 4558–4562
12 Karyakin, A. V. and Muradova, G. A. (1968) Zh. Fiz. Khim. 42, 2735–2740
13 Rigaud, J. L., Gary-Bobo, C. M. and Lange, Y. (1972) Biochim. Biophys. Acta 266, 72–84
14 Cass, A. and Finkelstein, A. (1967) J. Gen. Physiol. 50, 1765–1784