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X-ray diffraction evidence for the presence of discrete water layers on the surface of membranes

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X-ray diffraction spacings in multilayered membranes obtained from frog sciatic nerves were found to increase in discrete steps of approx. 5 Å during swelling. These observed jumps in the repeat period suggest that the lipid bilayers exist in distinct states of hydration, and perhaps the swelling occurs by step-wise addition of water layers between the polar head groups. Our analysis and statistical tests of this hypothesis are presented.

Introduction

The possibility that water in biological systems might exist in a highly ordered state quite unlike that of liquid water has been considered by many investigators [1–3]. Experimental results from proteins and lipid bilayers in support of structured solvents have been discussed [4–6]. Preliminary evidence for similar structured layers of water in membranes has also been presented [7]. Membranes occur naturally as multilayers in nerve myelin, and others can be prepared as multilayers by centrifugation [8]. Low-angle X-ray diffraction patterns of multilayers of nerve myelin [9–13], and sarcoplasmic reticulum membrane [8] have been recorded in various stages of swelling. We here present X-ray analysis of such data from swollen multilayers of myelin which suggest that the widths of the fluid layers between lipid bilayers in biological membranes change in discrete steps of size w (approx. 5 Å), w being the width of the water layer.

Experimental methods and Results

A Jarrel-Ash microfocus X-ray tube was used to obtain sharp diffraction patterns (Fig. 1). The X-ray diffraction photographs were taken on an optically focussing camera [14] using slit collimation. A line focus parallel to the slits and a single mirror was used. The observed repeat periods (d_{obs}) for frog sciatic nerve (from unfed *Rana pipiens*) swollen in water are listed in Table I. In order to show that the repeat periods from swollen membrane multilayers might change in steps of w, the error in spacing measurement has to be significantly smaller than w. Following precautions were taken to increase precision of our measurements. The sample-cell holder and the film-cassette holder were rigidly attached to the base of the camera, and a sample to film distance of 188 mm was used. The capillary containing the swollen nerve was set in a groove in the sample holder. Glass capillaries of about the same diameter (1 mm) were used for all specimens, and all the exposed films (Ilford Industrial G) were processed in exactly the same manner. These steps ensure that the errors due to variations in sample to film distance, and the shrinkage of the film are kept to minimum. Distance between the diffraction lines were measured using a comparator with 10 × magnification.

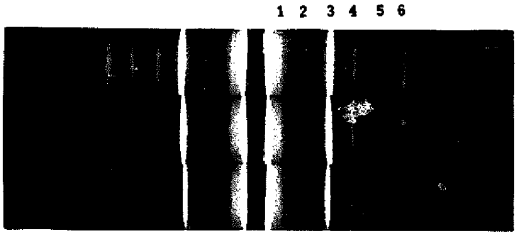

Fig. 1. Low-angle X-ray diffraction patterns from sciatic nerve myelin swollen in water. The first six-orders of reflections are numbered. Repeat periods from top to bottom are: 229.3, 233.2 and 238.0 Å; the full width of the most intense third-order reflections are 170, 150 and 130 μm, respectively (distance from the trace of the primary beam is approx. 4 mm).

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TABLE I

X-ray repeat periods for frog sciatic nerve swollen in water

d_{obs} are the observed repeat periods. d_{calc} are the repeat periods calculated at various values of n using the least-squares fit derived values of d_0 (229.1 Å) and w (4.3 Å). $d_{calc} = d_0 + nw$, and n is an integer which increases with the degree of swelling. d_0 is close to the smallest observed repeat period. w is the increment by which the repeat periods appear to change. The average value of w is 4.4 ± 0.4 Å as calculated from d_{obs} with d_0 fixed at 229.1 Å. Different experiments represent different specimens. Each nerve was swollen just once by soaking in water for 24 h. All the data obtained during this run were used in our analysis, with the following exceptions. The nerves used in experiments 5 and 12 did not swell. The d-spacing in experiments 3, 14, 15 and 16 changed abruptly on the same film along the length of the exposed nerve; the diffraction lines were not sharp enough for reliable measurement of individual d-spacings and calculating just one d-spacing would have introduced an error. The diffraction lines in experiment 22 were unusually broad and non-Gaussian due to the presence of two repeat periods giving rise to two sets of lines close to each other; measuring just one d-value would have introduced considerable error. The d-spacings in experiments 9, 20 and 26 changed along the axis of the nerve similar to that of 3, 14–16, except that the jump was not easily noticeable.

Expt. No.	d_{obs} (Å)	d_{calc} (Å)	n
1	228.7	229.1	0
24	229.3	229.1	0
4	233.4	233.4	1
6	233.9	233.4	1
7	233.1	233.4	1
8	233.2	233.4	1
23	233.2	233.4	1
2	238.8	237.7	2
17	238.0	237.7	2
18	236.8	237.7	2
21	237.2	237.7	2
25	238.4	237.7	2
19	246.8	246.3	4
27	246.5	246.3	4
28	245.9	246.3	4
13	250.3	250.7	5
11	255.1	255.0	6
10	263.5	263.6	8

In many of the photographs (Fig. 1) the reflections were fairly sharp (e.g., about 150 μm for the full width of the 3rd-order reflection which is approx. 4 mm from the trace of the primary beam). Hence, it was possible to estimate the peak positions to an accuracy of approx. 30 μm in measuring distances in the range of 6.5 to 8.5 mm, thus introducing an error of approx. 0.5%. Three sets of measurements were made and the average repeat period used for further analysis. In view of these precautions in obtaining and analyzing the X-ray photographs, we believe that our measurements have an accuracy better than 0.5%. This number is substantiated from the analysis of several photographs which had the same d-spacings (e.g., 233 and 238 Å). A typical calculation (Table II) using the data from five different samples with $d = 238$ Å show that our measurements have a standard deviation of 0.4%.

TABLE II

Data for calculation of standard deviations using the same repeat period (Å) obtained in different experiments

For $d = 237.85$ Å, $\sigma = 0.36\%$; similar calculation for $d = 233$ Å yields $\sigma = 0.26\%$.

Expt. No.	d (Å)		
Trial No:	1	2	3
2	239.0	238.7	238.6
17	238.0	238.5	237.6
18	237.6	236.9	236.0
21	237.4	237.0	237.2
25	238.0	238.5	238.7

Fig. 2 shows the continuous Fourier transform of frog sciatic nerve in water [10]. The shape of the observed reflection will be a product of the lattice interference function (containing the effects of either or both finiteness and disorder) and the square of the modulus of the structure factor of the unit cell, which is convoluted with the beam-profile. The transform is highly nonlinear near the zeros (at $x = 0.0074$, 0.0199 and 0.033–0.037 Å^{-1}; $x = 1/d$; d is the repeat period). Therefore, the shape of the reflections which lie near these zeros is considerably distorted, and some times only a part of the reflection is visible. Hence, the repeat periods calculated using these reflections will be grossly in error. Therefore, two intense and sharp reflections ($h = 2$ and 3 for $d \simeq 230$ Å, $h = 3$ and 4 for $d \simeq 260$ Å, and occasionally only $h = 3$; h is the order of the reflection; $h\lambda = 2d \sin \theta$) which are symmetric and thus had minimal distortions were used in our calculations (Table I). As will be discussed below, the decrease in the width of the 3rd-order reflection with increase in the repeat period (Fig. 1) is not due to distortion caused by the shape of the continuous transform of the membrane; the samples used for the 238- and 233-Å photographs happen to be more ordered than that used for the 229-Å photograph. The average d-spacing determined using up to six orders reflections (which could be clearly resolved in experiments 6, 17, 18, 23 and 24) were not very different from those listed in Table I.

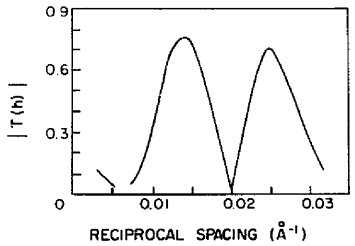

Fig. 2. Continuous Fourier transform of frog sciatic nerve obtained experimentally from low-angle diffraction data of nerve myelin swollen in water [10].

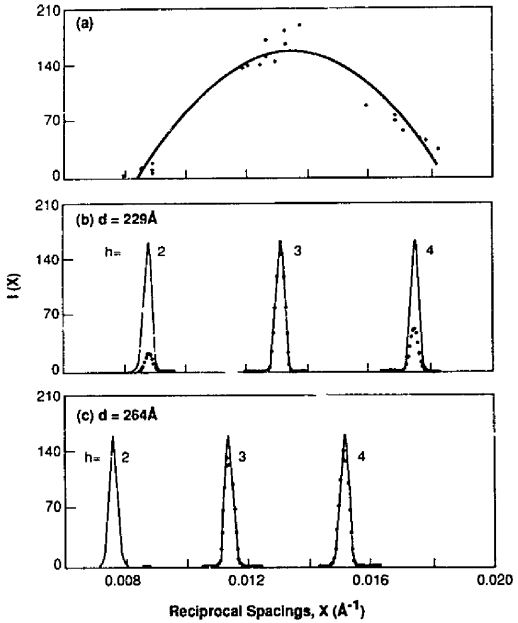

Fig. 3. (a) A plot of the structure factor modulus squared obtained by least squares fitting the observed intensity data (shown by circles) [10]. (b) and (c) Continuous curves are the interference functions broadened so as to produce a width of $0.3 \cdot 10^{-3}$ Å^{-1} in the simulated X-ray diffraction peaks which are shown by circles.

It is apparent from Fig. 2 that the square of the modulus of the structure factor of the unit cell, $|T(h)|^2$, varies over the range of lattice periodicities measured in our experiments. These variations could cause the d_{obs} to deviate significantly from d_{true}. To access these deviations, we plot in Fig. 3a the continuous intensity transform, $|T(h)|^2/h$, using published data [10]; h being the correction factor used in the calculation of $|T(h)|^2$ from the observed intensities. These data were fitted to a second-degree polynomial, $-973 + 168x - 6.3x^2$. The observed patterns were generated by multiplying this function with a Gaussian profile, $\exp(-4 \cdot \ln 2(x - x_0)^2/\Delta w^2)$, in which Δw is the full-width at half-maximum, and x_0 corresponds to the center of the peak. A Δw of $0.3 \cdot 10^{-3}$ Å^{-1} was used so as to obtain the maximum observed full width of 200 μm (at 4 mm from the trace of the primary beam). The results for the minimum and the maximum observed repeat periods of 229 and 264 Å are shown in Figs. 3b and 3c, respectively. These results show that the third-order reflection, which is used in all of the measurements, is least affected by the gradient in the structure factor modulus squared. The most affected reflection is the second-order reflection used in the calculation of some of the repeat periods at $d < 230$ Å. Even in this case the error is 0.3%. Since this is averaged with the third-order reflection, the actual error in the d-value is less than 0.3%. The error in the third- and the fourth-order reflections at higher d-spacings (e.g., $d = 264$ Å) is less than 0.1%. Since they have opposite signs, the error in the repeat period calculated by averaging the d-spacings of these two reflections will be even smaller. Thus, within the sensitivity of our measurements, the d-values listed in Table I represent the true repeat periods.

Data analysis and interpretation

The smallest value of the repeat period observed in the series of experiments for which the data is reported in this paper (Table I) is 229 Å. However, in other series of swelling experiments, we have observed repeat period as small as 202 Å for frog sciatic nerve swollen in water. We have also noticed that repeat periods of 202, 211 and 216 Å are sometimes accompanied by a 174 Å repeat. These results, as well as those of Inouye and Kirschner [14], show that there are no intermediate hydration states between the unswollen ($d \approx 170$ Å) and the minimally swollen ($d \approx 200$ Å) myelin. The 30 Å difference between the unswollen and minimally swollen myelin is likely due to changes in the charge-density on the surface of the bilayer, which we can speculate as arising from conformational changes in the proteins in the membrane and the polar head-groups of the lipid.

Worthington and McIntosh [11] have shown that the intensities of the normal nerve myelin in Ringer's solution lie on the continuous transform derived from nerve myelin swollen in 6.5% glycerol (6.5% glycerol has the same electron density as Ringer's solution). Therefore, changes in conformation and charge-density on the surface of the membrane are not accompanied by large structural changes. There is no evidence to suggest that the structure of the bilayer changes continuously with the degree of swelling. It is most likely that the changes in the membrane occur in the initial discontinuous phase of swelling, and the repeat periods, greater than approx. 200 Å have little, if any, contribution from the structural changes in the bilayer. Hence, the different repeat distances recorded from myelin swollen past the minimally swollen state (Table I) reflect the differences in the widths of the fluid layers between the apposing planes of polar groups. We will now show that these changes in the repeat periods are consistent with the hypothesis that the width of the fluid layer increases in steps of w Å.

In the statistical tests which follow, d_{calc} (listed in Table I) is obtained from the relation $d_{calc} = d_0 + nw$, where d_0 and w are constants and n is an integer. The values of d_0 and w are obtained by least-squares fitting d_{calc} values with n as an independent variable. σ^2 is the true variance and the estimated variance is s^2. $s^2 = (d_{obs} - d_{calc})^2/(N - 2)$, where N is the number of measurements and $(N - 2)$ is the degrees of freedom (df).

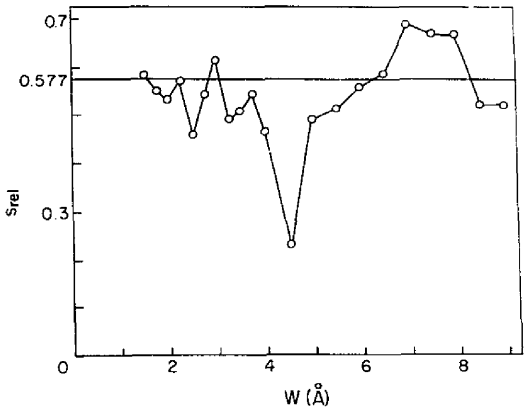

Fig. 4. The relative standard deviation (s_{rel}) is plotted as a function of w for X-ray spacings obtained from frog sciatic nerve swollen in water. An infinite set of random spacings has a s_{rel} value of 0.577 ($1/\sqrt{3}$) and is shown as a horizontal line.

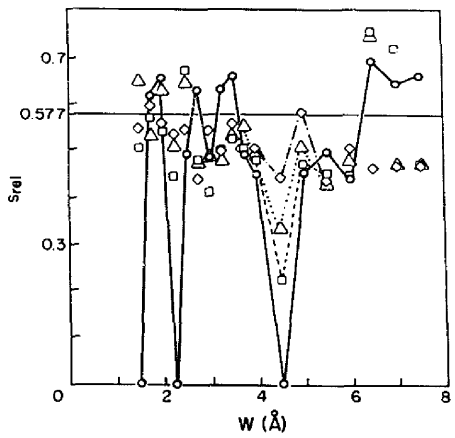

Fig. 5. The relative standard deviation (s_{rel}) is plotted as a function of w for four hypothetical sets of X-ray spacings (d). The ideal set was derived from experimental set for $d = 228.5 + n$ (4.5 Å). The s_{rel} values for the ideal set with errors of 0% (circles), ±0.2% (squares), ±0.3% (triangles), and ±0.4% (diamonds) are shown in the figure. An infinite set of random spacings has a s_{rel} of 0.577 and is shown as a horizontal line in the figure.

For convenience, we define a relative standard deviation s_{rel} as the ratio of s (or σ, for σ_{rel}) to $w/2$. A plot of s_{rel} vs. w is shown in Fig. 4. A minimum was found at $w = 4.5$ Å. F-test was used to determine whether this minimum in s_{rel} was statistically significant and different from that obtainable from a random set of X-ray spacings. The variance σ^2 of a random set of X-ray spacings can be shown to be equal to $w^2/12$ as df $\rightarrow \infty$. Thus $\sigma_{rel} = 1/\sqrt{3} = 0.577$, and is independent of w. The significant level of our minimum in s_{rel} was compared with that of an infinite set of random spacings using the ratio $(\sigma_{rel}/s_{rel})^2$. The results in Table III show that for the observed repeat periods from frog sciatic nerve swollen in water, the probability P that the minimum near 4.5 Å in s_{rel} might have occurred by chance is less than 1%. Thus, the minimum in s_{rel} Å is real, and suggests that the width of the fluid layer between the bilayers in multilayered membranes changes in increments of about 4.5 Å.

We now compare our X-ray data with an ideal set of d-spacings similar to the observed data with w hypothetically set to 4.5 Å A plot of s_{rel} vs. w (Fig. 5) shows that s_{rel} is zero at $w = 4.5$ Å and at integral fractions of 4.5 Å, since $d_{obs} = d_{calc}$ for these values of w. The effect of random errors in d_{obs} on the minimum value of s_{rel} is also shown in Fig. 5. These errors have a Gaussian distribution with a given standard deviation, and were derived from a set of random-normal numbers generated according to the algorithm of Kinderman and Ramage [15]. The results (Table III) show the probability that the minimum in Fig. 4 at $w \approx 4.5$ Å might be due to chance is less than 10%, if the accuracy of the 18 observed X-ray spacings is approx. ±0.5%. We have already shown that our measurements from frog sciatic nerve swollen in water satisfy this criterion. The probability of 10% calculated on the basis of measurement errors is higher than the probability of less than 1% obtained from the analysis of s_{rel}, and can be attributed

TABLE III

Significance levels based on F-test (16 degrees of freedom)

P = probability that minimum near 4.5 Å might have occurred by chance.

		s_{rel}	$F_{16,\infty} = (\sigma^2_{rel}/s^2_{rel})^{-1}$		P
Frog sciatic nerve in water		0.233	6.13		< 0.1%
Ideal set ±0% random normal error		0.000	∞		-
Ideal set ±0.2% random normal error		0.222	6.76		≈ 0.1%
Ideal set ±0.3% random normal error		0.331	3.04		= 1%
Ideal set ±0.4% random normal error		0.439	1.73		= 10%
Ideal set ±0.5% random normal error		0.539	1.15		> 10%
From statistical tables:	P	10%	5%	1%	0.1%
	$F_{16,\infty}$	1.72	2.01	2.75	4.06

to the exclusion of some of the data in the calculation of s_{rel} (see Table I).

The above analyses lead us to conclude that the X-ray spacings change in increments of w. The paracrystalline nature of the lamellae make it difficult to obtain a reliable value for w. Based on the results of several series of experiments similar to that in Table I, we estimate w to be about 5 Å. A more precise value of w, if it can be obtained, requires highly ordered multilayers prepared and swollen under controlled conditions. The approximate size of the water molecule is 2.5 Å, and the second nearest neighbor distance in ice is approx. 5 Å [16,17]. Thus, the 5 Å difference between successive states of hydration can be associated with one monolayer of water with each lipid bilayer. It is also possible that the water molecules are intercalated between the bilayers as layers of thickness 5 Å. The nerve membrane [18,19], like other membranes [20] is negatively charged. In general, we would expect that whenever multilayers of charged membranes swell, there will be discrete water layers between the lipid bilayers. Such ordered structures can also be expected in water layers adjacent to any charged surface such as those of proteins.

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