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LOCAL MEASUREMENT OF LATERAL MOTION IN ERYTHROCYTE MEMBRANES BY PHOTOBLEACHING TECHNIQUE

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Summary

The lateral diffusion coefficients (D) of the molecular fluorescence probe 3,3'-dioctadecylindocarbocyanine iodide (DII) in the membrane of discoid erythrocyte ghosts has been measured with the photobleaching technique between 7°C and 40°C. A fluorescence microscope which allows bleaching experiments within small local fields (approx. 1 μm^2) at high magnification (×1600) has been used for these measurements. The diffusion coefficient increases from $D = 9 \cdot 10^{-10}$ cm^2/s to $D = 7.5 \cdot 10^{-9}$ cm^2/s from 7 to 40°C. An increase in membrane fluidity between 12°C and 17°C indicates a conformational change of the lipid bilayer moiety in this temperature region. The diffusion coefficient measured in the regions between the spicules of echinocytes is appreciably smaller than in the untransformed discoid ghosts. In the myelin tubes originating from cells, the lateral diffusion is somewhat larger (about a factor of 2) than in the non-transformed ghosts. With the fluorescence probe technique the rate of growth of myelin tubes of 0.3 μm diameter has been estimated.

Introduction

The fluorescence recovery after photobleaching technique has become a very successful method for measuring long-distance particle diffusion in cell membranes [1—8]. Compared to the technique of cell fusion [9—11], the main advantage of the fluorescence photobleaching recovery technique is that lateral

Abbreviation: DII, 3,3'-dioctadecylindocarbocyanine iodide.

mobilities may be measured within small local areas (approx. 1 μm diameter) of the cell membrane. In contrast to the fluorescence photobleaching recovery technique, the diffusion measurement with excimer or spin label probes (cf. Ref. 12) yields rather values of the lateral mobility on a local scale (approx. 100-Å regions). In the present paper we report first measurements of lateral diffusion in the lipid moiety of membranes of erythrocyte ghosts. The main aim was the application of local diffusion measurements as a tool to study the local composition and structure of the lipid bilayer regions. It is hoped that such experiments will show whether the lateral lipid organisation may influence the cell shape or whether it is determined solely by the cytoplasmic network of spectrin and microfilaments [13].

Materials and Methods

Incubation of cells. The fluorescence probe was 3,3'-dioctadecylindocarbocyanine iodide (DII). Since it is not incorporated spontaneously into the plasma membrane the following incubation procedures were applied: (1) 250 μg DII were dissolved in 0.25 ml ethanol. The solution was added to 5 ml freshly withdrawn blood. (2) A more gentle method. The blood cells were first sedimented (in the gravitational field) for several hours. Then the dye solution was added at the top of the suspension. After 30 min, the supernatant was removed. The uptake of dye by the cell membrane was accompanied by hemolysis of the cell. This is supposed to be caused mainly by the alcoholic solvent which evocates also hemolysis in the absence of dye. Since the incorporation of dye is dependent on the hemolysis it was an all or nothing process. 10% of the erythrocytes had taken up dye and showed strong fluorescence while the other cells did not exhibit an observable emission. Intensity measurements indicated that the dye concentration in more than 90% of the labelled cells varied at most by a factor of 4. Unfortunately, it was not possible to measure the dye distribution between the incubation medium and the cell membrane. We can therefore only give an upper limit of the final dye concentration in the membrane, which is 3 mol%. At this concentration, the perturbation of the membrane structure by the dye is supposed to be negligibly small. This assumption is confirmed by the finding that for several hours after incubation, the shape of the labelled ghosts remained biconcave. This would easily be checked by measuring the distances between the two opposing membranes in the middle of the discoids. As shown below this distance can be measured with a high accuracy of ± 0.1 μm by focusing the irradiation light on the membrane. The formation of myelin threads starts after about 1 h above 30°C and after some days at room temperature. The latter stability was achieved by keeping the cells in their own plasma. The best ghost cells were selected for the measurements.

The microfluorometer. For the photobleaching experiment an inverted Zeiss Axiomat microscope is used. It is distinguished by an outstanding high mechanical stability allowing longtime measurements of light originating from very small areas. The photobleaching is performed with an argon laser (Spectra Physics, Mod. 162) at 488 nm while the fluorescence is irradiated with a 100 W Hg lamp (Osram HBO 100/W). Both the laser light and the measuring light are focused simultaneously on the membrane (plane A') via the same pathway (cf.

Fig. 1). For that purpose, the diaphragm A, is imaged on the membrane by the objective lens. The incident laser beam is focused on the diaphragm A, in such a way that only its central region (less than 50% of the whole beam width) is entering the opening of A. The wings of the Gaussian intensity profile of the laser beam are cut off. The spatial variation in intensity along the profile of the light beam (entering A) is thus smaller than 20% of the maximum intensity.

The edges of the beam profile behind A are only slightly distorted by Fraunhofer diffraction at the diaphragm. More serious is the distortion of the profile profile edges by the objective lens. The width of the edges at A′ is determined by the resolution power of the onjective lens. Due to the high numerical aperture (N.A.) of the objective used in our experiments (N.A. = 1.30) the width of the edges is expected to be not more than 0.25 μm (at the excitation wavelength of 488 nm). On the other side the Fraunhofer diffraction favours the formation of a rectangular profile in the inner region of the light beam. The above estimation of the width of the edges is confirmed by the following finding: by optimizing the sharpness of the image of A observed on the membrane, the diaphragm may be focused on the membrane plane to an accuracy of ± 0.1 μm. The position of the membrane may thus be measured to this accuracy. The diameter of the bleaching light spots as well as of the measuring light may be varied between 1 μm and 30 μm. Since the bleaching and the measuring light beams enter the microscope along the same pathway, they irradiate exactly the same membrane region.

Measurements were performed with so-called Torocytes [17] were the two bilayers in the centre of the cells are nearly in contact and with discoid ghosts with well-separated bilayers. In the first case the bleached spot sizes in the upper and lower membranes are nearly identical. In the latter case only the upper membrane is bleached substantially since the high numerical aperture of

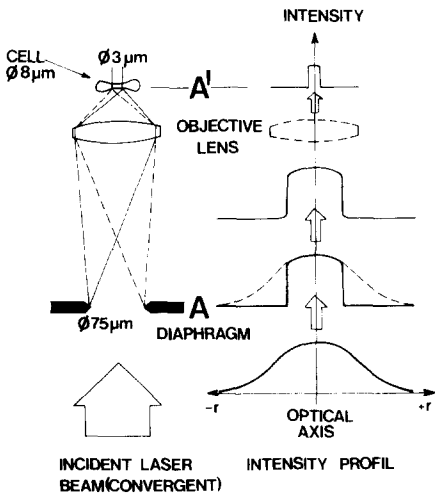

Fig. 1. Simplified diagram of the irradiation technique. The diaphragm A, is imaged on the cell surface by the objective lens. Only the central region of the laser beam is entering the opening of A, while the wings are cut off by a further diaphragm in front of A. Sharp focusing is allowed for a nearly rectangular intensity profile on the cell surface.

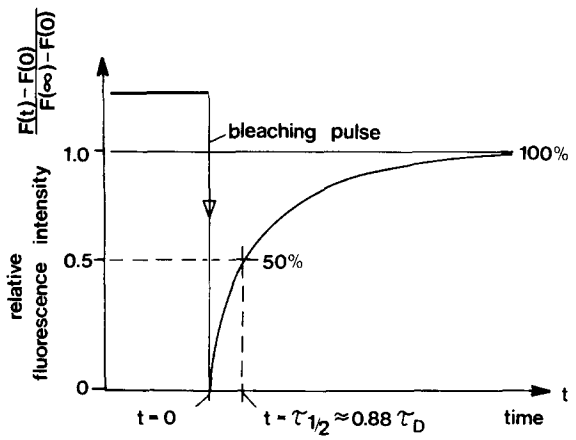

Fig. 2. Typical fluorescence recovery curve. The ordinate gives the normalized difference, $F(t) - F(o)$, of the fluorescence intensities measured at time t and at time $t = 0$, that is immediately after termination of the bleaching pulse. The recovery curve for an erythrocyte as obtained for a diameter of the bleaching spot of approx. 3 μm is shown.

the microscope allows to focus the bleaching spot very sharply (to ± 0.1 μm). Therefore the diffusion coefficients as measured for the two types of ghost agreed well.

The fluorescence emission was filtered out and imaged on the photomultiplier through the camera exit of the microscope. The measurements were made using the photon-counting technique (PCM 03 A, Photonic, Munich, F.R.G.). The signal was displayed on the screen of a storage oscilloscope (Tektronix 7623A) and registered by a chart-recorder. The cells can be observed simultaneously by the phase-contrast method. For that purpose normal transmitted light illumination is used. In this way the shape of the cell and the region of interest can be observed and selected before each measurement.

In the fluorescence recovery after photobleaching technique, the diffusion coefficient is obtained by first bleaching the fluorescence probes irreversibly and by recording the influx of fresh probe molecules into the irradiated region after bleaching. The bleaching time with the argon laser was about 0.25—1 s (488 nm, approx. 10 mW). Diffusion-coefficients between 10^{-12} cm^2/s and 10^{-7} cm^2/s could be measured. The evaluation procedure has been worked out in full detail by Axelrod et al. [2]. It becomes rather simple if bleaching is effected with light beams with rectangular intensity profiles. A typical fluorescence recovery curve is shown in Fig. 2. The normalized difference between the fluorescence intensities measured at time t ($F(t)$) and immediately after bleaching is accomplished ($F(0)$) is plotted as a function of the time t (Fig. 2). The photobleaching is a first-order reaction *. Therefore, the concentration of

* The first-order kinetics of the bleaching process was tested for each measurement. For that purpose, a single spot on the cell membrane was bleached in a repeated manner by a train of light pulses of well-defined intensity and duration. The emission intensity after each pulse was measured after equilibration by diffusion. The logarithm of this intensity was plotted as a function of the pulse number and was analysed by linear regression. In all cases the correlation coefficient was better than $c = 0.995$, indicating a well-defined exponential function.

dye immediately after bleaching ($t = 0$) is $c(0) = c_0 \exp[-\alpha TI]$. c_0 is the initial concentration, I is the bleaching light intensity, α is the first-order reaction constant of bleaching and T is the duration of the bleaching pulse.

The main advantage of a rectangular intensity profile of the bleaching light is, that the intensity profile of fluorescence originating from the bleached area is rectangular at time $t = 0$. Axelrod et al. [2] have calculated fluorescence recovery curves for this case. They found that the time $t = \tau_{1/2}$ after which half of the final fluorescence intensity, $F(\infty)$, is reached is given by

$$\tau_{1/2} = 0.88\ \tau_D \tag{1}$$

For two-dimensional diffusion, the time τ_D is related to the lateral diffusion coefficient by

$$\tau_D = \frac{w^2}{4D} \tag{2}$$

Results

The apparatus was tested with model membranes of dimyristoyl phosphatidylcholine. Large vesicles of the order of 10 μm diameter were incubated with DII. The diffusion coefficients measured below the phase transition are well within the dynamic range of the apparatus. For 15°C a value of $D = (2.6 \pm 1.4) \cdot 10^{-11}$ cm^2/s was observed in good agreement with the corresponding value of $4 \cdot 10^{-11}$ cm^2/s reported recently by Smith and McConnell for multilayers [7] and of $D \approx 5 \cdot 10^{-11}$ cm^2/s reported for thin-walled vesicles of this lipid [8]. At 25°C a value of $D = (7.5 \pm 2.5) \cdot 10^{-8}$ cm^2/s was measured. The corresponding value obtained with the excimer technique is $D = 7 \cdot 10^{-8}$ cm^2/s [12]. The value agrees also well with the result of Wu et al. [6] ($D = 5.5 \cdot 10^{-8}$ cm^2/s at 30°C). It is by a factor of 5 larger than the value reported by Fahey and Webb [8].

The results measured for the red cell ghosts are summarized in Fig. 3. For discoid-shaped cells the diffusion coefficient was measured in the centre of the discoid as a function of temperature. The curve X was obtained for ghosts incubated with the fluorescence dye by the first method. The curve ⊗ was measured for cells incubated by the more soft second method. The diffusion coefficient versus temperature plots show an inflection between 12°C and 17°C. This is more pronounced in the central trace plotted in Fig. 3. Since the experimental points and the error bars were obtained by many measurements (between 18 and 44) at different cells the inflection in the slope is well established. The diffusion coefficients of curve X are by about 30% smaller than those of curve ⊗ . Above 45°C no measurements were possible since denaturation of the ghosts started.

At low temperature, the diffusion coefficient in the membranes of myelin threads originating from the ghosts can be measured since the threads move only slowly. The corresponding value of D at 7°C is included in Fig. 3 (point ⊜). Note that a freshly formed myelin thread has an observed thickness of 0.3—0.4 μm.

Measurements were also performed with echinocyte-shaped ghosts. The

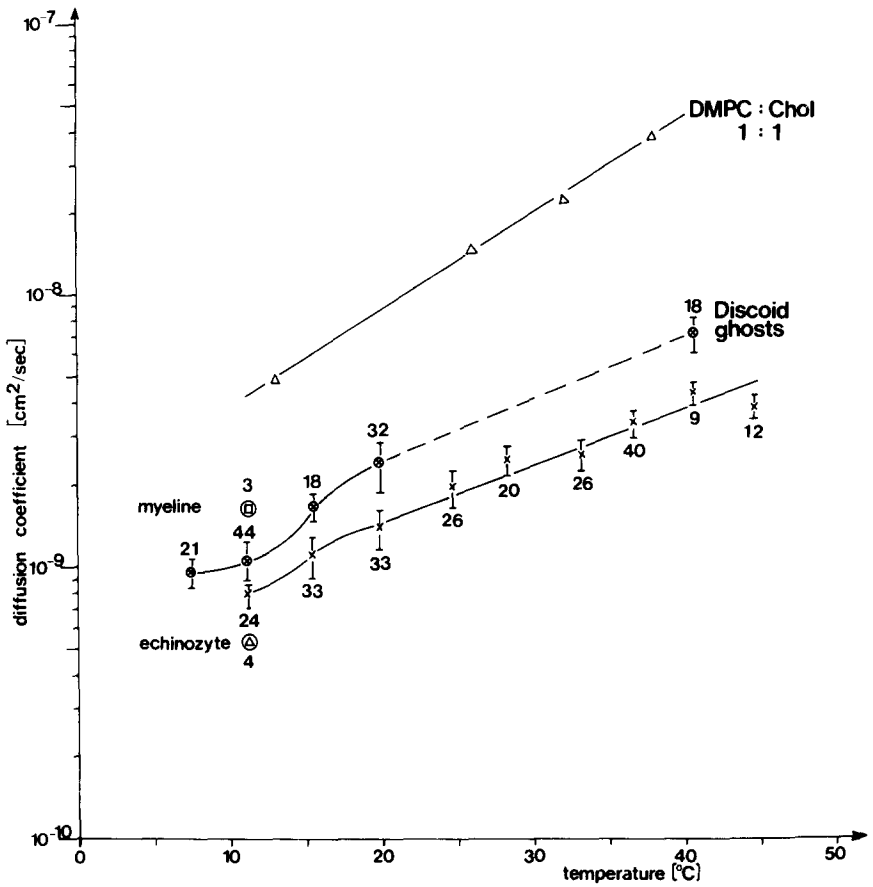

Fig. 3. Summary of diffusion coefficients D of DII in erythrocyte plasma membranes and comparison with D value of *N*-4-nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) probe in 1 : 1 mixture of cholesterol and dimyristoyl phosphatidylcholine (△———△) [6]. Diameter of bleaching spot approx. 3 μm. X; values of D in biconcave ghosts incubated by the first method. ⊗, values of D in same ghosts prepared by the second method. ⓞ, value of D in membrane of freshly formed myelin tube of about 0.3 μm diameter. ◬, value of D in ghost with echinocyte shape as obtained in regions between spicules. The figures at the error bars give the numbers of measurements performed for this temperature. For each value given, between three and ten cells were investigated. Error bars give the range of uncertainities of the $\tau_{1/2}$ measurements. An addditional error of ±20% results from the measurement of the irradiation spot size.

diffusion coefficient could be measured within the relatively flat regions between the spicules. The point ◬ gives a typical result. A considerable reduction in D (by about a factor of 2) as compared with the undeformed ghost is observed. For comparison the diffusion coefficient for bilayers of a 1 : 1 mixture of dimyristoyl phosphatidylcholine and cholesterol as measured by Wu et al. [6] are included.

Discussion

In the present work we report photobleaching measurements in erythrocyte ghost membranes at high optical magnification (×1600). The diffusion coeffi-

cient of DII in the lipid moiety of freshly prepared discoid ghost cells (as measured in the central region of the discs) varies between $D = 9 \cdot 10^{-10}$ cm^2/s at 10°C and $D = 7.5 \cdot 10^{-9}$ cm^2/s at 40°C. It has been shown by several workers that in discoid ghosts all membrane function are well preserved (cf. Ref. 17). Thus ghosts membrane closely resembles that of intact cells. The diffusion coefficients of undeformed (discoid) ghosts and non-hemolyzed cells are then expected to be rather similar.

Diffusion measurements in model membranes performed with excimer and spin label probes have shown that diffusion coefficients of lipophilic probes of equal molecular weight differ only by a few percent [12]. The diffusion coefficient of the DII probe is thus expected to give a good approximation to the lipid self-diffusion coefficient in the ghost membrane. The diffusion coefficient obtained in the present work is somewhat lower than the values obtained for chicken embryo fibroblasts [3] or L-6 (rat) myoblasts [4].

The logarithm of the diffusion coefficient in the lipid bilayer regions increases linearly with temperature between 15°C and 40°C (Fig. 3). From the slope of the straight line one estimates an activation energy of $E_{act} = 50$ kJ/mol. This value is by about 50% higher than for pure lipid bilayer. It agrees rather well with the activation energy observed for lipid bilayer with a high content of cholesterol (greater than 30 mol%) [12]. This seems to agree with the fact, that normal erythrocyte membranes contain a nearly equimolar mixture of phospholipid and cholesterol. In contrast to this, the absolute values of the diffusion coefficients in 1 : 1 mixtures of cholesterol and dimyristoyl phosphatidylcholine are by about a factor of 5—10 larger than in erythrocytes (cf. Fig. 3). Measurements with excimer probes have shown that at a given temperature the local diffusion coefficient, D, in fluid membranes of different lipids are rather similar. Thus the D values in egg phosphatidylcholine and dipalmitoyl phosphatidylcholine differ only by about a factor of two. Addition of 30 mol% of cholesterol to dipalmitoyl phosphatidylcholine reduces the diffusion coefficient also only by about a factor of two [12]. In view of this result the discrepancy of the D value in erythrocytes and the 1 : 1 mixture of egg phosphatidylcholine and cholesterol is rather serious.

The discrepancy may be explained in terms of a mosaic-like distribution of fluid lipid regions and of rigidified lipid-protein assemblies. In the photobleaching technique the diffusion over distances large compared to the molecular dimensions is observed. If the transfer of lipids between the fluid domains in impeded by less fluid transition regions (acting as bottlenecks of transport), the long-range lateral migration is expected to be considerably slower than the random walk on a local scale. The possibility of fast local diffusion in the presence of restricted long-distance motion has been discussed recently by Cherry et al. [5]. This view is further confirmed by local diffusion measurements in erythrocyte ghosts by the excimer probe technique. Thus we found a value of $D = 10^{-7}$ cm^2/s at 40°C for pyrene decanoic acid [12]. This is in reasonable agreement with the D value of the larger DII probe in the 1 : 1 cholesterol/phosphatidylcholine mixture (cf. Fig. 3) since the diffusion coefficient of a lipid probe is about inversely proportional to the square root of its molecular weight [12].

A further indication for the non-homogeneous membrane structure is the

finding that the diffusion coefficient of the dye in the membrane of the myelin tubes is substantially larger than in the intact ghost membrane (Fig. 3). At 10°C, the diffusion coefficient in the myelin agrees within a factor of two to three with the corresponding value of 1 : 1 mixtures of dimyristoyl phosphatidylcholine and cholesterol. A mosaic-like lateral organisation of the plasma membrane is also suggested by the measurement of the diffusion coefficient in the echinocyte. According to Fig. 3 the mean value of D in the regions between the spicules is by about a factor 2 smaller than in intact discoid ghosts prepared by the sedimentation technique. The measured differences in the diffusion coefficients in echinocytes, discoid ghosts and myelin threads may seem to be of marginal importance. As pointed out already above, the diffusion coefficients in fluid lipid layers are only slightly dependent on lipid structure (e.g. on its degree of saturation) [12]. Thus the observed differences strongly suggest a substantial difference in the lipid structure at least between the echinocytes regions studied and the myelin threads.

Another interesting feature of the D versus temperature plot is the small but well-established inflection of the diffusion coefficient between 12 and 17°C. This indicates some conformational change in the membrane within this temperature region. The nature of this conformational change is not understood yet. It has been observed before by many groups and by completely different methods. Verma and Wallach found a membrane-state transition at +17°C using Raman spectroscopy [14]. Zimmer and Schirmer [15] observed a transition between 18 and 19°C from viscosity changes of suspensions of sonicated erythrocyte membranes. Using ^{31}P-NMR, Cullis [16] observed a transition at 20°C both for normal ghosts and cholesterol-depleted ghosts. Some kind of lipid phase transition at 18°C was also found in a spin label study by Tanaka and Ohnishi [19].

The diffusion coefficient decreases steady in the course of the transformation of the cells after some hours. Obviously, this decrease is caused by the loss of fluid lipid which detaches from the cells in the form of small vesicles and myelin threads. This detachment can be directly observed in the microscope. As shown by Elgsaeter et al. [18], the pure lipid vesicles may be expelled from the plasma membrane by contraction of the cytoplasmic spectrin meshwork. The loss of lipid most probably is also responsible for the lower values of D found for the ghosts as prepared according to the first method. The direct addition of organic dye solution may lead to a substantial loss of lipid.

A very interesting benefit of the microfluorescence technique is the possibility to detect very thin myelin extension of 0.3 μm diameter. In phase contrast these structures become only visible when they contain hemoglobin or when, after some time, their thickness has increased considerably due to the adsorption of small vesicles. With the fluorescence-labelling technique the dynamics of myelin formation may thus be observed in a direct way. We found that this process can be rather fast. At 20°C the myelin thread can grow with a maximum speed of 45 μm/s if the cell is exposed to a weak streaming field while the free end of the myelin thread is attached to the glass surface. A myelin growth rate of several μm/s can be explained in terms of the lateral diffusion. A freshly formed myelin has a diameter of $d \leqslant 0.3$ μm. Assume (1) that half of the membrane lipid is available for myelin formation *, and (2)

that the speed of myelin formation is diffusion controlled. The change in length, Δl, of myelin with time ($\Delta l/\Delta t$) may then be related to the rate of transport of new lipid bilayer to the location of myelin growth. If this transport is measured in terms of bilayer area (ΔA) transported to the location of growth per unit time $\Delta A/\Delta t$) one obtains

$$\frac{\Delta l}{\Delta t} = \frac{1}{\pi d} \cdot \frac{\Delta A}{\Delta t} \tag{3}$$

where d is the thickness of the myelin tube. For two-dimensional diffusion it is $\Delta A/\Delta t = 4\pi D$ and therefore

$$\frac{\Delta l}{\Delta t} = \frac{4D}{d} \tag{4}$$

For 20°C, $D = 2.5 \cdot 10^{-9}$ cm^2/s and one obtains $\Delta l/\Delta t \approx 3$ μm/s which is of the right order of magnitude.

References

1 Peters, R., Peters, J., Tews, K.H. and Bähr, W. (1974) Biochim. Biophys. Acta 367, 282—294
2 Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E. and Webb, W.W. (1976) Biophys. J. 16, 1055—1069
3 Schlessinger, J., Bark, L.S., Hammes, G.G., Yamada, K.M., Pastan, I., Webb, W.W. and Elson, E.L. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2909—2913
4 Schlessinger, J., Axelrod, D., Koppel, D.E., Webb, W.W. and Elson, E. (1977) Science 195, 307—309
5 Cherry, R.J., Bürkli, A., Busslinger, M., Scheider, G. and Parish, G.R. (1976) Nature 263, 389—393
6 Wu, E.S., Jacobson, K. and Papahadjopoulos, D. (1977) Biochemistry 16, 3936—3941
7 Smith, B.A. and McConell, H.M. (1978) Proc. Natl. Acad. Sci. U.S. 75, 2759—2763
8 Fahey, P.F. and Webb, W.W. (1978) Biochemistry 17, 3046—3053
9 Frye, L.D. and Edidin, M. (1970) J. Cell Sci. 7, 319—335
10 Edidin, M., Zagyansky, Y. and Lardner, T.J. (1976) Science 191, 466—468
11 Fowler, V. and Branton, D. (1977) Nature 268, 23—26
12 Galla, H.J., Hartmann, W., Theilen, U. and Sackmann, E. (1979) J. Membrane Biol., in the press
13 Edelmann, G.M. (1976) Science 192, 218—226
14 Verma, S.P. and Wallach, D.F.H. (1976) Biochim. Biophys. Acta 436, 307—318
15 Zimmer, G. and Schirmer, H. (1974) Biochim. Biophys. Acta 345, 314—320
16 Cullis, P.R. (1976) FEBS Lett. 68, 173—176
17 Bessis, M. (1973) Living Blood Cells and their Ultrastructure, pp. 140—164, Springer-Verlag, Berlin
18 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101—122
19 Tanaka, K. and Ohnishi, D. (1976) Biochim. Biophys. Acta 426, 218—231

* This assumption leads to the conclusion that the maximum length of myelin (of 0.3 μm diameter) that can evolve from one ghost would be 100 μm. This has indeed been confirmed.