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PROTEIN-CATALYZED PHOSPHOLIPID EXCHANGE IN BILAYER VESICLES DETERMINED BY FLOW CYTOMETRY AND ELECTRON MICROSCOPY

Y.H. XÜ, D. RÜPPEL, H. ZIEGLER, W. HARTMANN and H.-J. GALLA

Department of Biophysics, University of Ulm, Oberer Eselsberg, D-7900 Ulm (F.R.G.)

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The protein-induced lipid transfer between phosphatidylcholine vesicles was investigated. Measurements of the degree of polarization at single vesicles were made by flow cytometry using diphenylhexatriene as the optical probe. Vesicles differing in phase transition temperature could be distinguished by their degree of polarization at a temperature where one population was in the fluid ($T > T_t$) and the other one in the quasi-crystalline ($T < T_t$) state. Besides vesicles containing exchanged lipids we also observed fractions of unaffected vesicles. The lipid exchange was visualized directly by freeze-fracture electron microscopy. The characteristic 'ripple' structure of phosphatidylcholine vesicles disappeared upon exchange with lipid in the fluid state.

Introduction

The exchange of phospholipids between natural membranes occurs via cytosolic proteins [1]. So-called phospholipid exchange proteins isolated from bovine heart, brain or liver catalyze the transfer of phospholipids in natural and artificial membranes [2–4]. Kinetic studies showed the formation of a strong protein-membrane complex during the exchange process [5]. We investigated this process by measurements of fluorescence polarization because the progress of the lipid exchange can be followed from the lipid phase transition curve. However, such measurements yield only the average degree of polarization of the whole vesicles preparation, so we have developed a method of flow-cytometric investigation which we report here. In this we measured the fluorescence polarization of 10000 individual vesicles and took the means of the results, which gives a more accurate representation than sampling the average degree of polarization. We also studied the events in single bilayer vesicles using electron microscopy.

Materials and Methods

Distearoyl- (DSPC), dipalmitoyl- (DPPC), dimyristoylphosphatidylcholine (DMPC) and diphenylhexatriene were obtained from Fluka (Neu-Ulm, F.R.G.). Vesicles for fluorescence polarization measurements were formed from dried lipid films containing 1% diphenylhexatriene by ultrasonication in a bath sonifier for 10 min above the corresponding lipid phase transition temperature. The lipid concentration was 1 mg/ml in phosphate buffer at pH 7.0. Phase transition curves were obtained automatically by recording the fluorescence polarization $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ as a function of temperature with a Schoeffel Fluorescence Spectrometer RRS 1000 [6]. The basic layout of the flow cytometer for fluorescence anisotropic measurements is given elsewhere (Refs. 7, 8 and references therein). A mercury lamp (100 W) was used as light source in a Köhler illumination design combined with a UG 11 filter to allow the excitation of diphenylhexatriene-stained single vesicles at 360 nm. The flow rate of the lipid

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dispersion medium was 40 μl/min which corresponds to about 100 vesicles passing the irradiation beam every second. Between 5000 and 10000 vesicles were measured in each experiment. Polarization histograms were composed with the aid of a microprocessor-based data system. Phospholipid exchange protein was isolated from bovine liver following the method of Kamp and Wirtz [9]. The protein concentration was brought to 15 μg/ml and incubation was performed at 42°C. Giant vesicles for freeze-etch electron microscopy were prepared as described by Reeves and Dowden [10]. The lipid concentration was 1 mg/ml. Before freezing the probes were incubated for 10 h at the given temperature. Fast freezing was performed by the so-called 'sandwich procedure' [11], which yields cooling rates of about 10^4 K/s. Platinum-carbon replicas were obtained after freeze-fracturing in a Balzers freeze-etching device BAF 400 D and were inspected in a Philips EM 301 electron microscope.

Results and Discussion

The phase transition curve of a mixture of equal amounts of dipalmitoyl- and distearoylphosphatidylcholine vesicles is shown in Fig. 1. A two-step phase transition was obtained where the step height can be taken as a measure of lipid in the corresponding phase. After addition of exchange protein (15 μg/ml, incubation at 42°C for 30 min) the horizontal trace between 40 and 50°C changes into a slope that is characteristic for a mixed layer containing both types of lipid. The step height of the mixed layer is then a measure for the amount of lipid exchanged. The protein concentration and incubation time were chosen to yield a saturation value for the exchange (Xü, Y.H., Galla, H.-J., Gietzen, K. and Sackmann, E., unpublished data). One result was that we found that the phase transition of both lipids are observable, together with the broad transition of the mixed layer, even in the presence of exchange protein. Thus the question arises as to whether the phase transitions of the pure lipids correspond to a non-exchanged inner layer of the vesicles or if there exist vesicles that are not affected by the

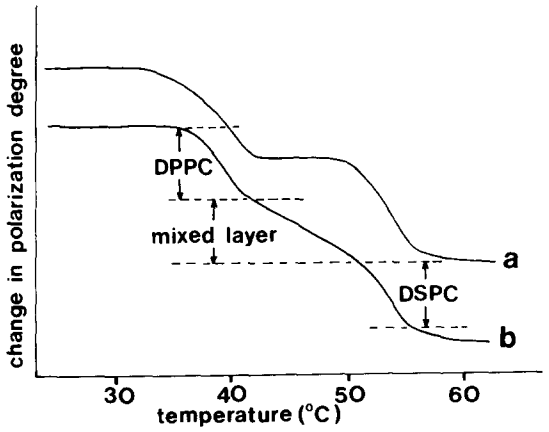

Fig. 1. Phase transition curves of mixtures of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine vesicles (a) in the absence and (b) in the presence of 15 μg/ml exchange protein. The incubation was performed at 42°C for 30 min. Diphenylhexatriene was used to determine the fluorescence polarization degree. The y axis gives the relative change in fluorescence polarization. Curves a and b are shifted along the y axis.

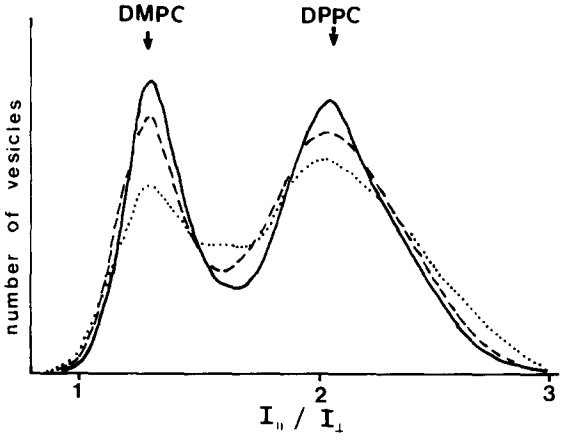

Fig. 2. Histogram of the fluorescence intensity ratio of the emission parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the incident light beam measured at individual bilayer vesicles in a flow cytometer at 33°C. Two vesicle preparations made from dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were mixed in the absence of exchange protein (solid line). Two peaks are visible; one from the fluid DMPC and the other from the crystalline DPPC. The broken line shows the result of a measurement of the mixed vesicles after 15 min incubation at 42°C in the presence of 15 μg/ml exchange protein. The dotted line represents the results of a measurement after 30 min incubation. Note that the amount of pure DPPC and DMPC decreases whereas a fraction of medium fluidity ($I_{\parallel}/I_{\perp} \sim 1.8$) increases with increasing incubation time.

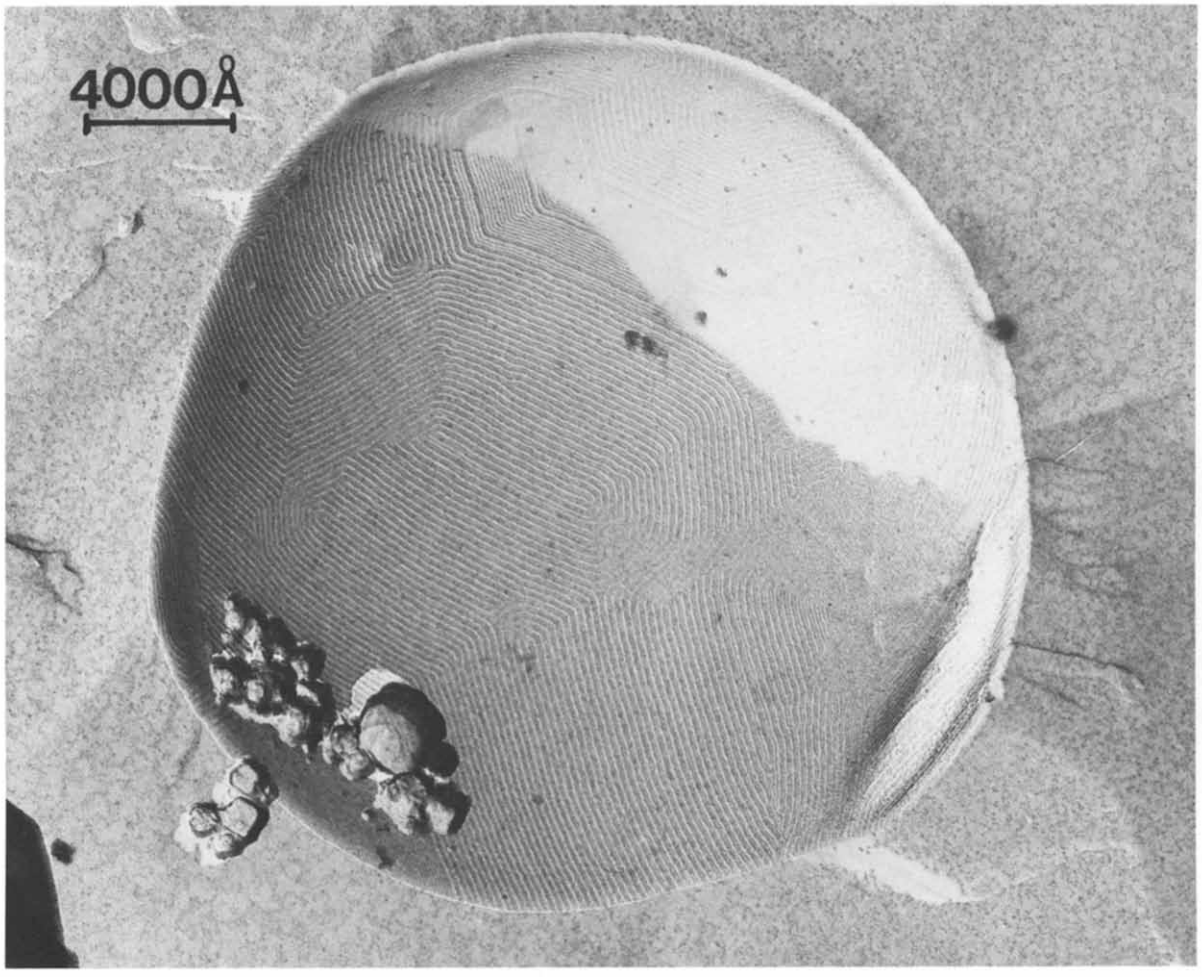

Fig. 3a. Electron micrograph of a giant dipalmitoylphosphatidylcholine vesicle snap cooled from 37°C, which is between the pre- and the main transition temperatures of the lipid. The 'ripple' structure is characteristic for this phase.

protein. This cannot be answered from the evidence of the phase transition curves, but with flow cytometry one is able to measure the polarization anisotropy in individual vesicles.

A histogram of the fluorescence intensity ratio $I_{\parallel}/I_{\perp}$ of the emission parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization of the incident light beam is given in Fig. 2 for two vesicle preparations, one made from dimyristoyl- and the other from dipalmitoylphosphatidylcholine. The phase transition temperatures of the two are 23°C and 41°C. The flow cytometric measurements were performed at 33°C, where dimyristoylphosphatidylcholine is in the fluid and dipalmitoylphosphatidylcholine in the quasi-crystalline state. Two peaks (solid line in Fig. 2) are observable in the histogram in the absence of protein, corresponding to the fluid DMPC vesicle and the quasi-crystalline DPPC vesicle. The area under each peak is equivalent to the number of vesicles in that population. Addition of phospholipid exchange protein (15 μg/ml) resulted in histograms as shown in Fig. 2 after 15 min (broken line) and 30 min (dotted line) incubation at 42°C. The amounts of pure DMPC and DPPC decrease whereas a fraction with the intermediate fluidity ($I_{\parallel}/I_{\perp} \sim 1.8$) increases with increasing incubation time. Obviously a third component corresponding

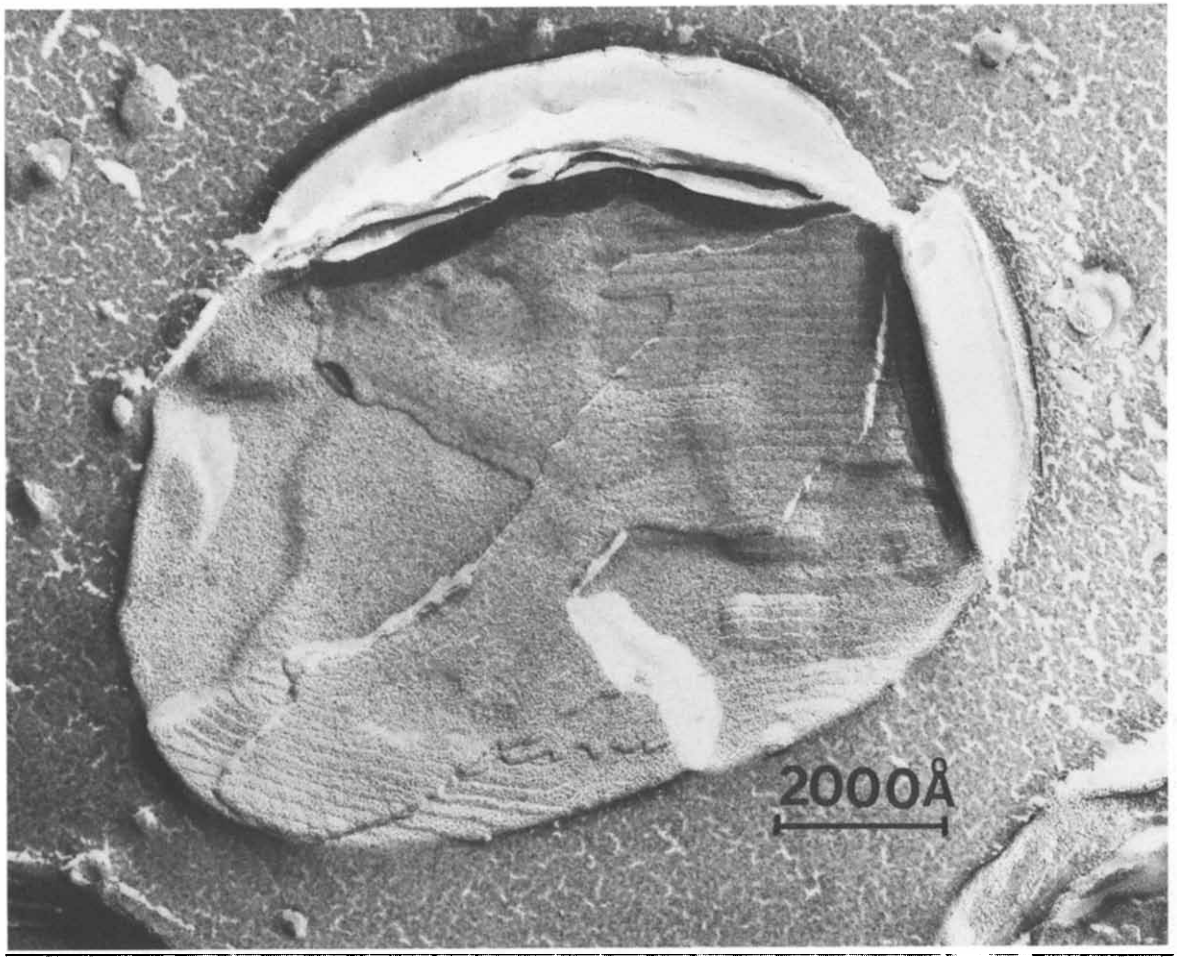

Fig. 3b. Large DPPC vesicle fast-frozen from 37°C, as shown in Fig. 3a but after an incubation with exchange protein (13 μg/ml for 0.5 h at 42°C). Note the extended areas without ripple structure. The disappearance of such structure gives evidence for the transfer of DMPC into DPPC vesicles.

to the mixed layer (e.g. Fig. 1) develops in the DMPC/DPPC vesicle mixture in the presence of exchange protein, even though pure DMPC- and DPPC vesicles are still present. One can infer that not all vesicles are affected equally by the exchange protein; infact, some remain unaffected.

These results were confirmed by electron microscopy. We used the disappearance of the so-called 'ripple' structure [11] to follow the exchange process. The ripple structure is visible only if pure phosphatidylcholine vesicles are snap frozen from a solution at a temperature between the pre- and the main transition temperatures [12]. A typical example of the ripple structure is shown in Fig. 3a for a large DPPC vesicle of about 20000 Å diameter. This preparation was frozen from 37°C which is indeed between the pre- and the main transition temperatures of DPPC. DMPC vesicles are in the fluid state at 37°C and so do not exhibit ripples. In this experiment we used small sonified DMPC vesicles of about 300 to 1000 Å diameter because then they are easy to distinguish from the giant DPPC vesicles. Fig. 3b shows such a giant DPPC vesicle in the presence of the small DMPC vesicles after incubation with 13 μg/ml of exchange protein for 30 min at 42°C. The preparation was again frozen from 37°C. Extensive areas without ripple structure are visible. Another vesicle with a

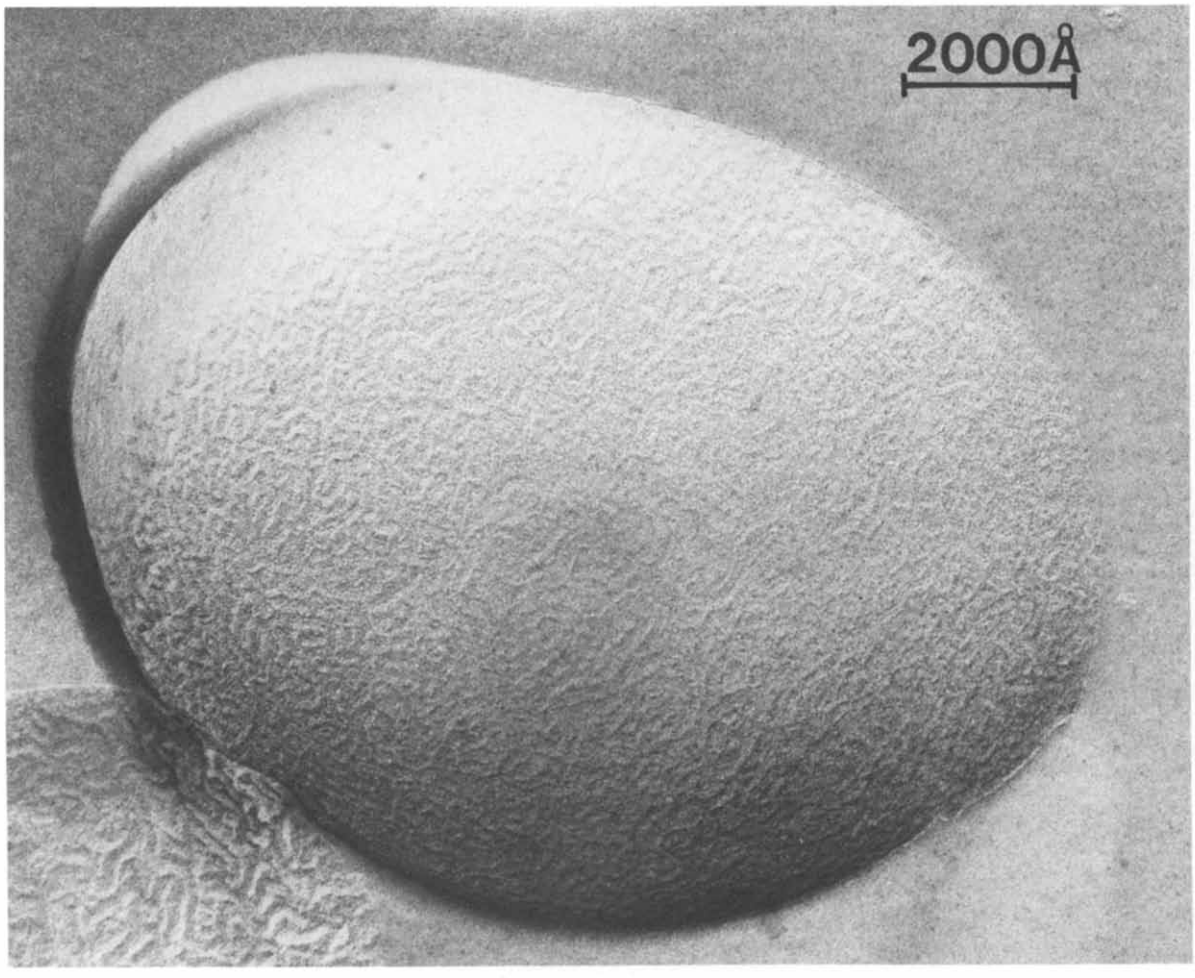

Fig. 3c. Large DPPC vesicle rapidly cooled from 37°C after 0.5 h incubation with exchange protein. An exchange process with small DMPC vesicles has occurred leading to the smooth surface of this vesicle.

smooth surface obtained under the same conditions is shown in Fig. 3c. The disappearance of the ripple structure gives evidence for the transfer of fluid DMPC- into DPPC vesicles which leads to a lowered phase transition temperature of the mixed lipid phase and so to the disappearance of the ripple structure. However, even in the presence of exchange protein we still observed fully rippled vesicles as well as smooth vesicles.

The results from the electron microscopy support our results from the study of single vesicles. A fraction of the phospholipid vesicles remain unchanged during an exchange process extending to a period of one hour. Longer incubation times cannot be applied because of the concomittant natural lipid transfer in the absence of catalytic protein [6]. This can be explained if a nucleation step in the exchange process is assumed. If a protein catalyzes the transfer of lipids at the vesicle surface it would create a fluid defect which would in turn facilitate further lipid exchange, thus leading to a completely exchanged outer bilayer of the vesicle. Therefore totally smooth vesicles would be found besides rippled vesicles. Fig. 3b shows an example of such a minority of vesicles which may have been frozen in an intermediate stage of the exchange process.

The interesting point in the exchange process is

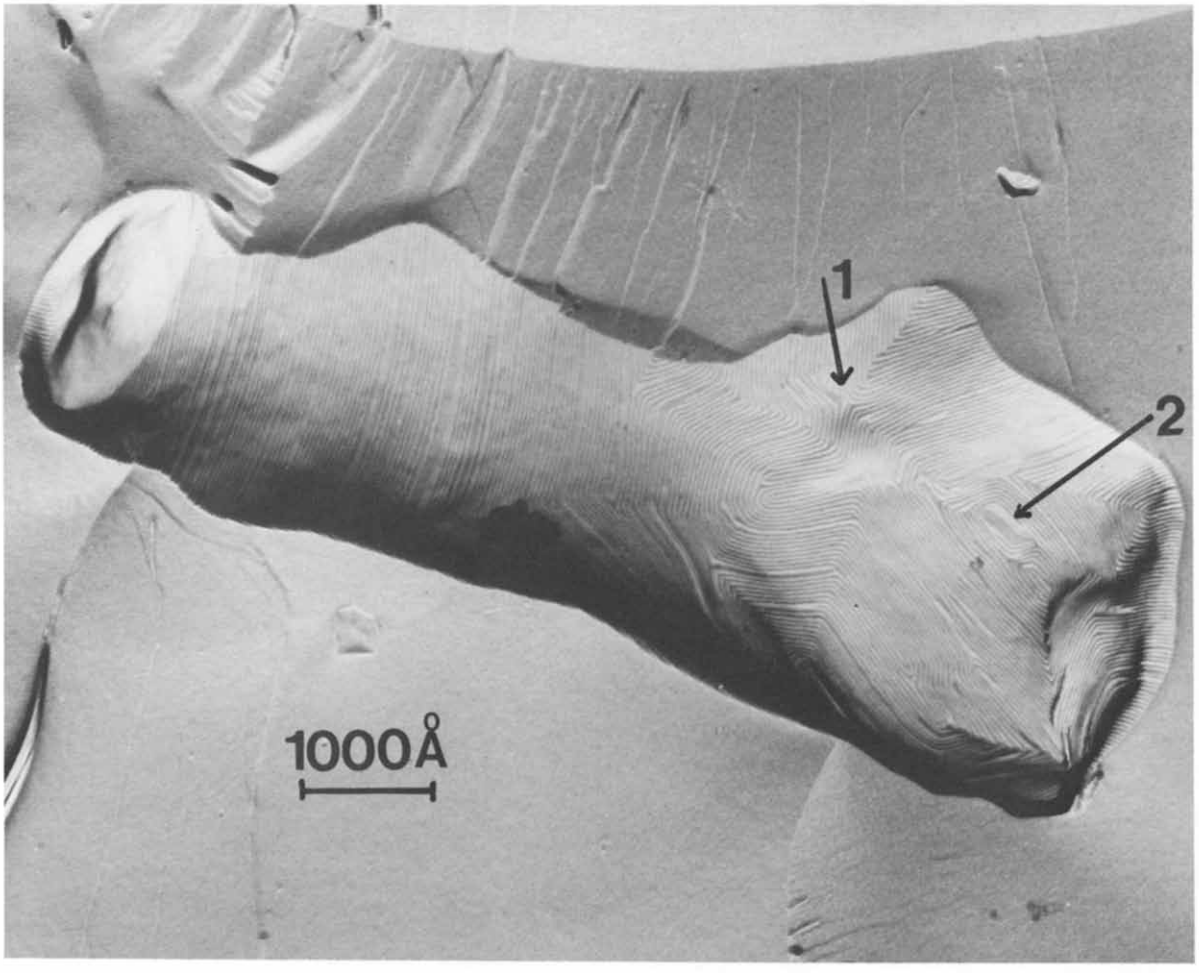

Fig. 3d. Large DPPC vesicle obtained from the same preparation as a–c, with vesicles as shown in Figs. 3b and 3c. Arrow 1 points to an $s = -1$ disclination that is unstable in pure DPPC layers. Its existence in the presence of DMPC vesicles and exchange protein gives evidence for the incorporation of DMPC. This might form the nucleus of an exchange process and lead to a small smooth area such as is marked by arrow 2, which in turn could spread over the whole vesicle.

this possible nucleation step. Fig. 3d shows a vesicle almost completely covered by ripple structures. However, there are two remarkable differences in it compared to the defect pattern of pure phosphatidylcholine vesicles. Arrow 1 points to a so-called $s = -1$ disclination which is instable in pure systems and therefore dissociates into an $s = -\frac{1}{2}$ and $s = +\frac{1}{2}$ pair [11]. Such a disclination is only stable in the presence of impurities [13] which may be, for example, exchanged DMPC in a DPPC vesicle, and may thus function as a nucleus for the exchange process. Arrow 2 points to a small smooth area surrounded by a defect pattern. This smooth area may originate from the growth of such a nucleus during the first stage of the exchange process.

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