Fusion of Isolated Sarcoplasmic Reticulum Membranes

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Fragmented sarcoplasmic reticulum (FSR) vesicles from rabbit muscle were suspended in 1.5–5% glycerol solutions and were pelleted onto aluminum foil disks in a modified centrifuge tube. Examination of these pellets in the electron microscope after drying for 2–2.5, 4–5.5, and 21 hours revealed a progression of changes. First, distances between individual, round vesicles decreases. Next, somewhat flattened vesicles establish limited areas of contact with adjacent vesicles. Finally, vesicle fusion occurs and extended areas of double bilayers are formed. A water loss-time interaction appears to be needed for the fusion process. A Hg-phenyl azoferritin compound was used as a marker to identify intra- and extra-vesicular space in the fused samples.

Quantitative measurements of birefringence during imbibition of pellet slices in a graded series of glycerol solutions indicates a steadily increasing amount of birefringence until 60–80% glycerol ($\eta = 1.41-1.43$) is reached. The plateau seen in this part of the curve is again followed by steadily increasing birefringence at higher glycerol concentrations. This interruption in the birefringence curve is presumably due to a matching of the refractive indices of the glycerol solution and a lipid component in the membranes.

Introduction

The process of fusion of biological membranes has intrigued researchers for many years. This interest has been heightened by a recent change in the model for biological membranes [1], by a theory for membrane fusion [2, 3] and by the finding that viruses [4], lysolecithin [5], DMSO, glycerol, sorbitol, manitol and sucrose [6] all apparently cause fusion of various types of membranes or cells. Certain of these agents, however, reduce or eliminate cell viability. A model system involving fusion of fatty acid enriched lecithin vesicles has also been studied [7].

Of the three published reports involving fragmented sarcoplasmic reticulum (FSR) membrane fusion, one [8] used mainly x-ray diffraction to characterize the fusion while the other two [9, 10] used electron microscopy. The study by Deamer [10] reported that lysolecithin, which solubilizes approximately 75% of the vesicular lipids and 50% of the proteins, will compex with the ATPase and, after drying, this complex will form layers. These lipid and protein modifications appear to make this system differ significantly from the native preparation. The study by Coleman *et al.* [9] involved, in part, a comparison between the structure of layers formed by native vesicles and by trypsin-treated vesicles. Intermediate steps in the "layering" process were not shown and resolution in the negative contrast section makes it difficult to accurately determine the origin of various layers. The purpose of the investigation reported here is to study the fusion of native FSR vesicles, to identify the intra- and extravesicular space and to quantitatively measure the behavior of the fused vesicles under polarized light during imbibition in glycerol solutions.

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Materials and Methods

FSR was isolated from rabbit muscle by the method of Hasselbach and Makinose [11]. Inserts with a flat upper surface were placed into polyallomer centrifuge tubes (Beckman, Palo Alto, Cal.) and a pre-weighed aluminum foil disk approximately 13 mm in diameter was placed on top of the insert. The tubes were filled nearly to the top with various concentrations of glycerol (1.5–5%) in 0.08 M KCl, 0.1 M PO_4, pH 7.0. An aliquot of the FSR vesicles was layered on top of the glycerol solution. Centrifugation typically was done at 10 000 rpm for 20 min and then at 35 000 rpm for 100 min, both at 2–5 °C, in SW-40 rotor (Beckman). To determine the effect of centrifugal force on fusion, one of the 35 000 rpm runs was extended to 14 h. After the 35 000 rpm centrifugation, the thin pellets (approx. 0.3–0.5 mm thick) which formed on the aluminum foil disks were carefully removed

from the tubes and were weighed. The pellets were then placed in a shallow petri dish lid in a 5 – 8 °C refrigerator. After 2 – 2.5 h of drying, pellets were weighed, and the first pair of pellets was fixed for electron microscopy. After an additional 2 – 3 h drying under identical conditions, the pellets were again weighed and the next pair of pellets was fixed for electron microscopy. The remaining pair was placed in a vacuum desiccator over silica gel, a vacuum was drawn with an aspirator and the desiccator was placed in a cold room overnight (usually about 16 h). The weight loss overnight was determined and fixation was initiated. A Hg-phenyl azoferritin marker [12] was used in some experiments to distinguish intra- from extravesicular space.

All pellets were fixed 2 h in Karnovsky's paraformaldehyde-glutaraldehyde mixture [13], rinsed in Millonig's PO_4 buffer and were cut into 1 mm square pieces. They were then post fixed in 1% OsO_4, dehydrated in graded acetones, infiltrated with an Epon-Araldite mixture [14] and were flat-embedded. Because the pellets were very thin, (usually 0.1 – 0.4 mm) the 1 mm squares always oriented themselves in the same way in the flat-embedding molds. Block were trimmed so that the entire thickness of the pellet could be included and examined in one section and were sectioned on an LKB Ultrotome III with either glass or diamond knives. During sectioning, the knife edge was perpendicular to the direction of the centrifugal force which formed the pellet. This precaution was taken to insure that the longest diameter of the flattened vesicles would not be enhanced during sectioning. Sections were stained with 2% uranyl acetate in methanol followed by lead citrate. Sections were examined in a Siemens EM101 equipped with an anticontamination device and operated at 80 KV.

For quantitative polarized light microscope measurements, glutaraldehyde-fixed pellets were cut with a Smith and Farquhar tissue sectioner (Ivan Sorvall, Inc., Newtown, Conn.) at a setting of 50 μm. The slices were mounted on a coverslip with nitrocellulose and were oriented so that the polarized beam passed through the slice at right angles to the direction of centrifugal force. A Leitz Orthoplan microscope equipped with pol objectives, rotating stage and a tilting Type M compensator was used for the measurements. Actual thickness of each slice was measured by focusing on the upper and the lower surface of the slice and converting the units of fine focus travel to actual thickness. The fine focus control was calibrated by using thin quartz plates. The slices attached to the coverslip were then immersed in 0, 20, 40, 60, 80, 100% aqueous glycerol solutions, and the birefringence was measured after each change of solutions at the same location in the slice. Refractive index of the various glycerol solutions was determined by using a Bausch & Lomb refractometer.

Results

Pelleted FSR vesicles after two hours of drying are densely packed and consist mostly of circular, bilayer-bounded profiles (Fig. 1 *). The diameter of these vesicles ranges from 0.06 – 0.2 μm with the vast majority in the 0.08 – 0.1 μm range. This diameter agrees with that reported by Hasselbach and Elfvin [12]. The distance across a single bilayer is 65 – 75 Å. Although the vesicles are closely packed, it is important to note that the individual integrity of each vesicle is maintained even though 30 – 40% of the starting pellet weight has been lost. After four to five hours of drying, distance between membranes decreases still further and the fusion is just beginning. A mixture of flattened, circular profiles and flattened pairs of unit membranes (arrows) is seen (Fig. 2). Such pellets typically have lost 55 – 65% of their starting weight. The bilayer structure is clearly visible.

Drying under vacuum overnight at 2 – 5° causes the fusion process to continue until few circular profiles remain (Fig. 3 **). The main structural feature seen is double bilayers which are profiles of the outer membranes of two adjacent flattened, fused vesicles with their long axes parallel to the diameter of the pellet. An average of ten measurements indicates that the diameter of these flattened profiles, *i. e.*, the distance one is able to follow a single bilayer before it curves away from the neighboring vesicle, is 0.6 μm. Measurements across a single bilayer after drying (Fig. 3), however, show that it retains the same width as that observed in the membrane surrounding the original vesicles before drying (Fig. 1). This finding agrees with DuPont, *et al.* [8] who also observed that drying did not change the x-ray spacing attributable to the membrane width.

Careful examination of these double bilayers reveals that outer surfaces of each pair of bilayers are relatively free of electron-opaque material in contrast to the space between the two bilayers which does contain this material. Because of the docu-

* Figs 1 and 2 see Plate on page 704 a.
** Figs 3 and 4 see Plate on page 704 b.

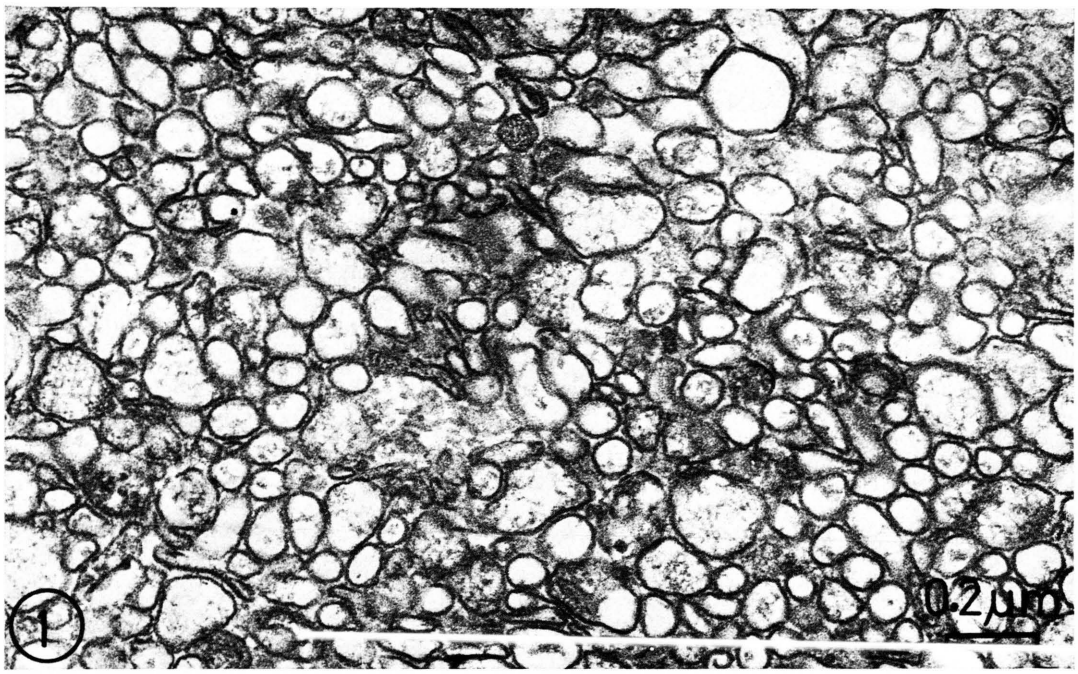

Fig. 1. Section through a pellet of FSR vesicles, pelleted through a 5% glycerol solution and dried for two hours. Vesicles are approximately circular, maintain their individual integrity and are bounded by the usual bilayer structure. X 60,000.

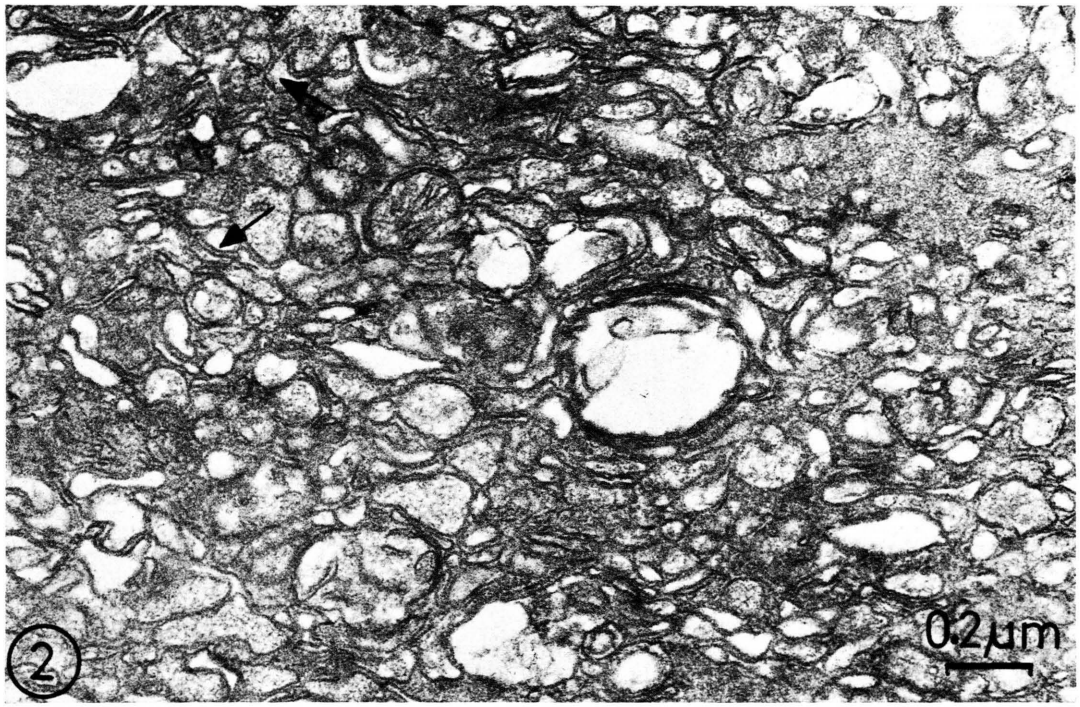

Fig. 2. Drying the FSR pellet for four hours results in somewhat flattened vesicle profiles, a further decrease in distance between vesicles and limited areas of contact between vesicles (arrows) which resemble the double bilayers seen in later samples. X 54,000.

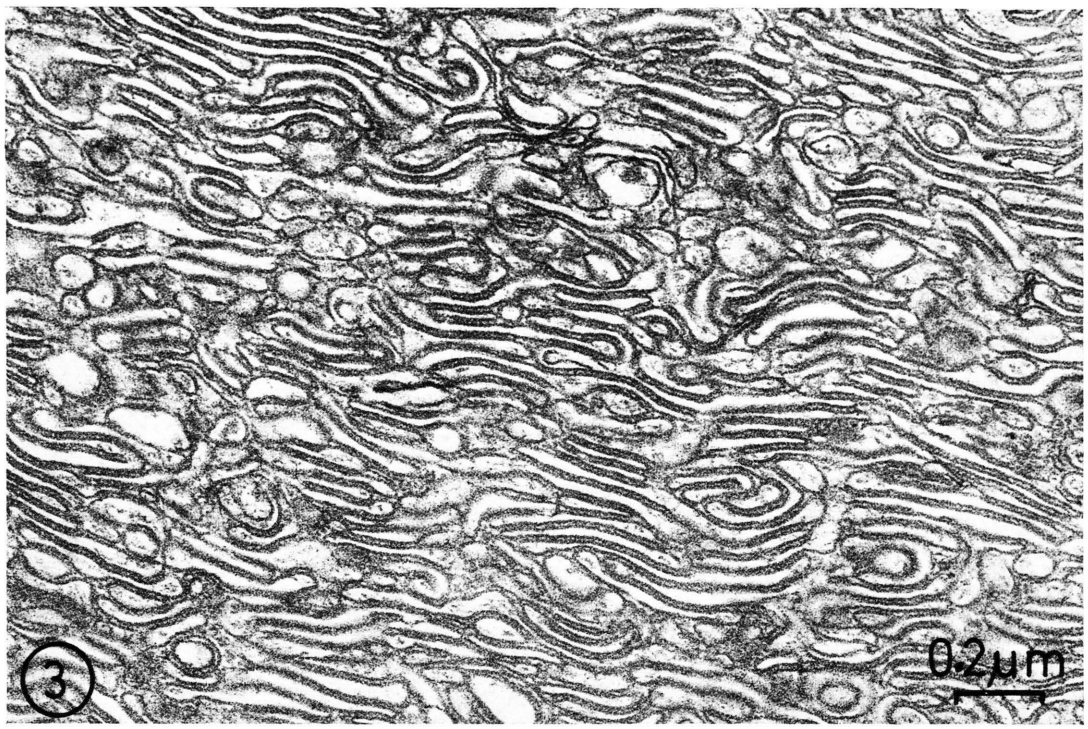

Fig. 3. A FSR vesicle pellet which has been dried overnight in the cold and has lost 75% of its starting weight. Comparatively few circular profiles remain. Instead, flat, sheet-like double bilayers which measure 165–175 Å across are seen. The space of uniform width at the center of each bilayer pair is opaque compared with the outer edge of each pair. X 60,000.

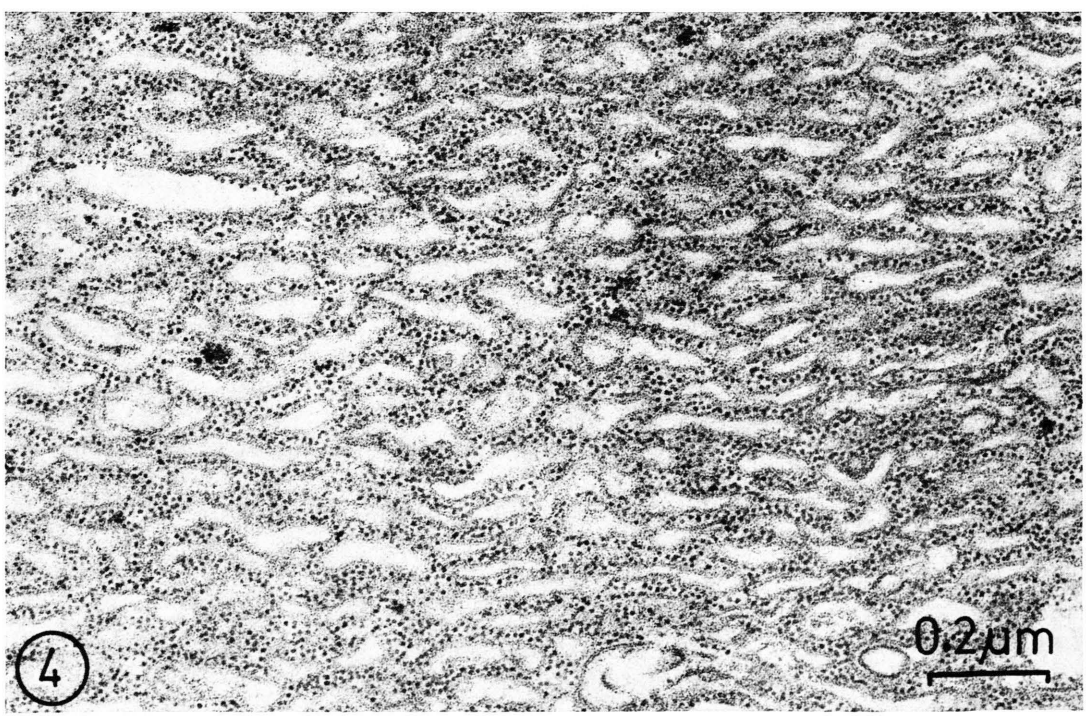

Fig. 4. This very thin section of FSR vesicles heavily labeled with Hg-phenyl azoferritin shows that the inner space between the two halves of the double bilayer originates from the extracellular space. Although this sample is in the early stages of fusion, the long rows of attached ferritin granules differentiate the intra- and extravesicular compartments. X 80,000.

mented presence of knobs and stalked knobs on the outer surface of native FSR vesicles, we first assumed that the inner, opaque space between the two bilayers was the extravesicular space and that the outer, clear surface of each double bilayer originated from the intravesicular space. To verify this assumption, Hg-phenyl azoferritin was coupled to the outer surface of native FSR vesicles, and the fusion process was observed. The very thin section shown in Fig. 4 clearly indicates that the ferritin particles are located only in the space between the two bilayers and never on the outer surface of the double bilayers. The means that the outer surface of the original FSR vesicle is located between the two bilayers, *i.e.*, in the electron opaque space. Although this particular sample was dried only two hours and has lost 50% of its starting weight, the heavily labeled vesicles are already flattening and starting the fusion process. The very extensive fusion and the reduction in space between adjacent vesicles usually seen after longer drying make the interpretation of such images less straightforward than with shorter drying times.

Because of the general similarity between our flattened fused FSR vesicles and the stacked lamellae in rods of the eye, we compared the birefringence of our fused vesicles during glycerol imbibition with

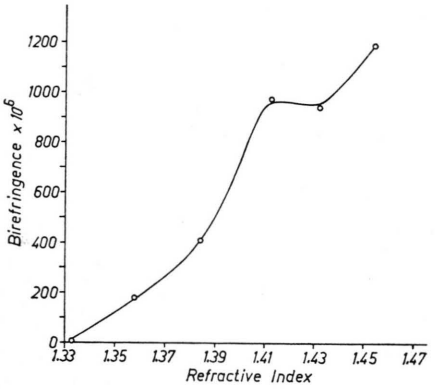

Fig. 5. Quantitative measurements of birefringence of slices from an overnight-dried pellet are plotted *versus* the refractive index (η) of increasing concentrations of glycerol solutions (0, 20, 40, 60, 80, 100%). A steady increase in birefringence preceeds a plateau between $\eta = 1.41 - 1.43$. From this plateau, the amount of birefringence again increases. The refractory indices are designated at $\eta_{||}$ and $\eta_{\perp}$, respectively, when the electric field component of the polarized light is parallel or perpendicular to the normal of the membrane lamellae. For birefringence measurements, the preparation was positioned diagonally between the crossed polarizer and analyzer of the microscope. The birefringence $\Delta\eta = \eta_{||} - \eta_{\perp}$ is defined as positive when $\eta_{||} > \eta_{\perp}$.

that reported for the frog retina [15]. The birefringence measurements (Fig. 5) show a steadily increasing amount of birefringence with increases in glycerol concentration until the 60 – 80% range is reached ($\eta = 1.41 - 1.43$). In this range, birefringence remains nearly constant or may even decrease slightly presumably due to matching of refractive indices of the glycerol solution and some component of the membranes. After this plateau, the birefringence continues upward if the pellet slice is immersed in higher concentrations of glycerol. Like the disks in the rods of the eye, these highly ordered FSR membrane preparations are characterized by a negative form and a positive intrinsic birefringence with reference to the normal of the disk plane. In contrast to the situation in the frog retina whose static birefringence curve can be fitted by the Wiener equation [15], the curve obtained from fused FSR is more complex.

Discussion

A comparison of starting vesicle circumference with distances that uninterrupted, flattened membranes can be traced indicates that fusion of vesicles is the only possible explanation for the changes observed. Because most vesicles have starting diameters from 0.08 to 0.1 μm, multiplying π by the diameter indicates that the circumference of these vesicles would be 0.25 to 0.31 μm. If these vesicles had simply flattened instead of fusing, the maximum distance that one could expect to follow double bilayers would be one-half this value or 0.125 to 0.155. Fig. 3 and the average of our measurements show clearly that flattened sheet-like pairs of membranes can be followed uninterrupted for distances 3 – 4 times greater than would be expected if fusion had not occurred.

The fusion of individual functional FSR vesicles into flattened, sheets of membranes is apparently strongly influenced by loss of water from the pellet. At the first sampling time, pellets typically had lost 30 – 40% of their starting weight. Because a larger proportion of this early water loss is, of necessity, loss of water adhering to the outer surface of the pellet rather than loss from within the pellet, the actual water loss from the interior of the pellet is undoubtably somewhat lower. At the second sampling time, a weight loss of 55 – 65% was usually observed and, after overnight drying, 65 – 75% loss had occurred. When the extent of fusion

is releated to weight loss, it becomes clear that water loss alone is not sufficient to explain the fusion process. Although comparatively little weight loss occurs between the second and final sampling times, the extent of fusion is much greater in the final sample than in the second. This implies that the fusion process requires a time-water loss interaction. In instances where very little additional weight loss occurred overnight, extent of fusion was much more advanced than that observed in the preceeding sample. That water loss rather than packing force or duration of centrifugal force was the primary agent responsible for fusion was determined by centrifuging vesicles for 14 h at 35 000 rpm. Prolonged centrifugation did not result in formation of flattened, extended double bilayers. It has already been reported that this drying of FSR vesicles and accompanying time interval result in little or no loss of Ca^{2+}-stimulated ATPase activity and, in addition, Ca^{2+}-uptake activity is retained [8]. This is strong evidence that the fusion of FSR vesicles reported here involves membranes which retain their biological activity.

The effect of glycerol concentration on the fusion process could not be fully explored because, as the starting glycerol concentration was increased above 5%, the stability of the pellet dropped rapidly so that it was usually not possible to remove the supernatant or the pellet from the tube without disruption of the pellet. In related experiments, however, (Stromer and Hasselbach, unpublished results), it was found that fusion was blocked if higher starting concentrations of glycerol were used. If one assumes that the evaporation of glycerol is negligible, then the initial 5% glycerol concentration in the pellet should increase to $7-8\%$ at the time of the first sampling, to $11-14\%$ at the time of the second sampling and to $14-20\%$ after overnight drying. Although higher glyceral concentrations were used to study intramembranous particle aggregation in lymphocytes [16] and to study fusion of human erythrocytes [17], it is difficult to relate these results with heterologous systems to the FSR results reported here. That fusion of FSR vesicles is not strictly dependent on glycerol is shown by the fusion produced by other fusogenic compounds (Stromer, The and Hasselbach, manuscript in preparation).

The mechanism of FSR fusion in glycerol involves first a decrease in distance between vesicles (*cf.* Fig. 1 and Fig. 2) which results in formation of areas of contact which resemble short segments of subsequent-appearing double bilayers. The second step is for the vesicles to flatten and to decrease the intravesicular volume and for the flat, sheet-like double bilayers to form. This flattening of vesicles produces regions at their edges where the curvature would be extremely small and, in agreement with the hypothesis of Lucy [2, 3], would permit fusion of one vesicle with an adjacent one. This accounts for the transition from individual flattened vesicles with the same orientation as the final sheet-like membranes. This mechanism differs from that proposed previously by DuPont *et al.* [8] which was based on x-ray diffraction data. These workers proposed that the first step was vesicle flattening followed by a decrease in distance between vesicles or bilayers. It is logical to assume that the distances obtained from the x-ray diagrams and assigned to intravesicular space should have been assigned to extravesicular space.

The plateau in the birefringence curve is presumably due to a virtual matching of the refractive indices of the glycerol solution and some membrane component. Based on published refractive index data [18] for fatty acids and mixed triglycerides present in FSR membranes [19–21], it is difficult to relate the slightly lower refractive index at the plateau to the higher refractive index of these compounds. One possible explanation for this plateau comes from the work of Weber [22] who observed that penetration of the imbibing liquid into the space between molecules affects the observed birefringence. In the case of the fused FSR, the presence of water in the bilayer would lower its refractive index below that of pure lipids. When water but not glycerol penetrates the bilayer, the birefringence tends to decline when the refractive index of the glycerol-water mixture exceeds that of the hydrated bilayer. The curve continues upward when, with increasing glycerol content of the imbibing medium, the positive intrinsic birefringence of the protein predominates. This contribution to the positive intrinsic birefringence with reference to the normal of the flattened membranes indicates the presence of highly ordered protein domains in the transport molecule perpendicular to the plane of the membranes.

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