Membrane Fusion Through Point Defects in Bilayers

Abstract. *Fusion between bilayers of mixed egg phosphatidylcholine and soybean phosphatidylethanolamine was induced by freezing and thawing. Contact points between bilayers were observed by freeze fracture electron microscopy, and isotropic molecular motional averaging was detected by phosphorus-31 nuclear magnetic resonance under fusion conditions. A molecular model of point defect structure is proposed as an intermediate stage of fusion.*

Fusion between membranes is an important step in many cellular processes such as secretion, endocytosis, fertilization, and mitosis. Membrane fusion may also be induced by virus (*1*), polyethylene glycol (*2*) and other membrane-disrupting agents, cations (*1, 3–5*), and freezing and thawing (*6, 7*). These methods are widely used in cell hybridization and membrane reconstitution experiments. Yet the molecular mechanisms of fusion are still not understood. Increasing evidence points to the fact that, prior to the fusion process, membrane proteins are excluded from the site of fusion (*3, 4, 8, 9*); the lipid bilayers of the apposed membrane are then able to form close contacts through local dehydration (*2, 5, 9*). Transient destabilization of the bilayer is subsequently required to connect the two membranes (*8, 10, 11*). Various models have been proposed for the molecular arrangement in this destabilized state (*3, 8, 11, 12*). Perhaps because of the transient nature of the fusion event, direct morphological observations of an intermediate stage of fusion have been rare.

Since lipid bilayers play an important role in membrane fusion, we used lipid vesicles as a model to study induced fusion events. We monitored fusion between small unilamellar vesicles by electron microscopy (negative staining and freeze fracturing) and by light absorption. Under the same conditions, fusion between bilayers within multilamellar vesicles were also observed. Changes in lamellar spacing were measured by x-ray diffraction, while molecular motional averaging was monitored by ^{31}P nuclear magnetic resonance (NMR), which is sensitive to motional averaging of phospholipid molecules and useful for monitoring changes in molecular organization (*13*). Experimental procedures have been described in detail elsewhere (*13*).

As reported by us (*13*) and by others (*14, 15*), membrane protrusions described as lipid intramembrane particles (LIP) exist in a number of lipid mixtures, including mixed phosphatidylethanolamine (PE) and phosphatidylcholine (PC). These LIP have been associated with fusion events (*16*) without direct proof. In order to induce extensive fusion, we froze and thawed pellets of small vesicles containing 10 to 40 mole percent of egg PC in soybean PE. Large LIP-containing vesicles were obtained as a result of this process. In cross-fractured vesicles LIP appear as "rivets" between adjacent bilayers (Fig. 1, a and b). Numerous sites of close membrane contact are seen after repeated freezing and thawing (Fig. 1b). At some of the contact points the hydrophobic fracture faces of the neighboring bilayer are seen to be connected. Fusion between bilayers also created edge dislocations (*17*), as shown in Fig. 1c. When vesicles of PC and PE were mixed with proteins extracted by Triton X-100 from human erythrocyte membranes (*7*), frozen at and thawed from −70°C, larger vesicles containing many proteinaceous intramembranous particles were obtained (Fig. 1, d and e). These particles are smaller than LIP, which are usually associated with an indentation of the surrounding fractured face. Attachment of adjacent lamellae in multilamellar vesicles is readily observable regardless of the presence of protein (Fig. 1, a, d, and e). Because of the extensive fusion between lamellae, the shell structure of the multilamellar system is disrupted; this results in illusive (Escher-type) topology as shown in Fig. 1d, where points *X* and *Y* are on the same

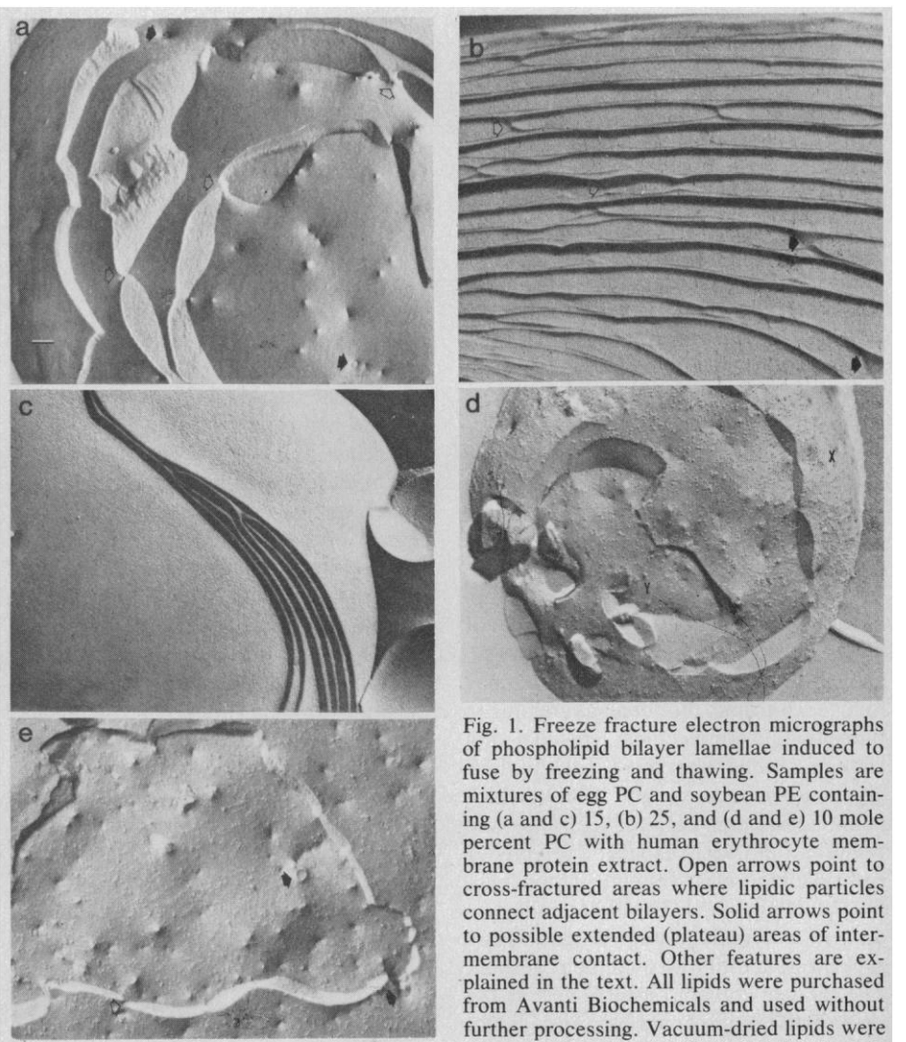

Fig. 1. Freeze fracture electron micrographs of phospholipid bilayer lamellae induced to fuse by freezing and thawing. Samples are mixtures of egg PC and soybean PE containing (a and c) 15, (b) 25, and (d and e) 10 mole percent PC with human erythrocyte membrane protein extract. Open arrows point to cross-fractured areas where lipidic particles connect adjacent bilayers. Solid arrows point to possible extended (plateau) areas of intermembrane contact. Other features are explained in the text. All lipids were purchased from Avanti Biochemicals and used without further processing. Vacuum-dried lipids were suspended in one-tenth strength Hanks balanced salt solution, pelleted at 4000*g* for 20 minutes, and twice frozen and thawed from −70°C. The samples were rapidly frozen without cyroprotectant from room temperature to −190°C (*24*). Parallel experiments in which 30 percent glycerol was used as a cryoprotectant and samples were conventionally frozen in liquid Freon 22 produced similar results. Freeze fracture was performed in a Polaron E7500 unit; the carbon-platinum replicas were examined in a Siemens 101 microscope. Scale bar, 100 nm.

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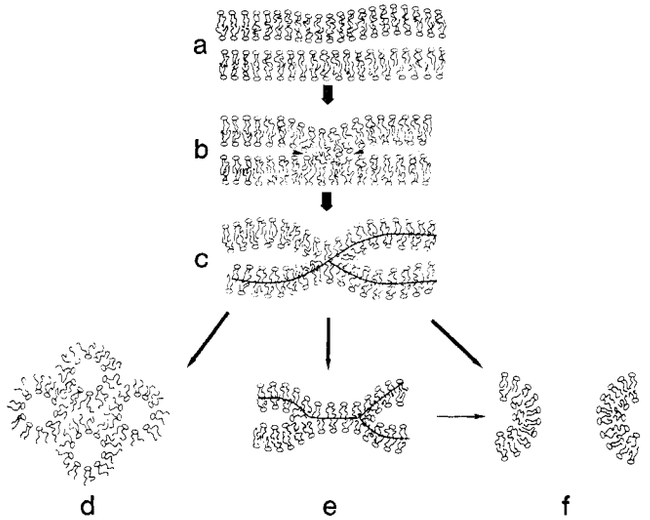

Fig. 2. Model of molecular rearrangement during the membrane fusion process. Ellipses represent the polar heads of phospholipids; wriggly tails represent apolar fatty acyl chains. Two bilayers were initially separated by a reduced water space as depicted in (a). Local dehydration or mechanical forces (such as ice crystal formation) deform the bilayer to make contact points (b). The high surface curvature (arrows) renders the structure unstable, and it can either relax back to (a) or proceed to (c). The structure in (c) probably gives rise to the pairs of LIP seen in electron micrographs (Fig. 1b). In membranes consisting of phospholipids that prefer a bilayer structure, the process will continue to (f), completing the fusion process. With abundant wedge-shaped molecules, the structures in (c), (d), or (e) may be stable. Solid lines indicate possible freeze fracture planes. Structure in (d) represents a hexagonal H_H phase (*13*).

fracture plane but are also separated by two layers along the direct distance. X-ray diffraction study of these multilayer structures gave a lamellar repeat spacing of 52 to 57 Å, depending on the PC content (*13*); this is significantly less than the typical lamellar repeat spacing of 62 Å in pure egg PC vesicles. Isotropic ^{31}P NMR signals were also observed in these samples, indicating that a significant portion of the bilayer structure was disrupted.

It is noteworthy that a similar reduction of interlamellar spacing, indicative of dehydration, was also observed during fusion induced by calcium ions (*18*) or by polyethylene glycol (*19*). However, dehydration alone is not sufficient to cause fusion, since dehydration by evaporation or by addition of dextran or glycerol does not cause membrane fusion (*19–21*), nor does dehydration alone induce isotropic motional averaging in phospholipid molecules (results not shown). On the other hand, polyethylene glycol (molecular weight, 6000), which facilitates fusion between cells (*2*) and between lipid vesicles (*19*), does induce isotropic motional averaging in phospholipid molecules (*19*). Thus bilayer disruption is a required step in the fusion process in addition to local dehydration. The bilayer contacts established at the sites of disruption could be transient. By mixing a highly unsaturated PE with PC, we may have preserved the transient bilayer contacts that occur during the fusion processes. These bilayer contacts apparently are sufficiently abundant and stable in our multilamellar samples to allow observation by freeze fracture electron microscopy.

The coincident events of local dehydration, bilayer disruption, appearance of isotropic molecular motion, and fusion lead us to propose a fusion model for the systems we studied. This is depicted in Fig. 2. Initially, the adjacent bilayers are separated by water layers. Some bilayers may have irregular undulations or defects (Fig. 2a), partly due to the presence of wedge-shaped molecules such as highly unsaturated PE or other fusogens (*11, 13, 14*). When two of these potential fusion points are brought together, as by cationic bridges or ice crystal growth in the freezing-thawing process, the high curvature (see Fig. 2b) renders this structure energetically unfavorable. The configuration can either retract to that shown in Fig. 2a or proceed to that shown in Fig. 2c. The minimization of curvature of the water "lens" in Fig. 2c tends to force the attached bilayers apart and press unattached bilayers together, creating unevenness or asymmetry in lamellar spacing. The pairing in Fig. 2b is an example, although it is not a typical configuration. Motion of lipid molecules in the contact area in Fig. 2b or diffusion over the cusp surface of approximately 20-nm diameter (Fig. 2c) is sufficient to produce an isotropic ^{31}P NMR signal. Ordinarily, as a result of osmotic swelling or cytoskeletal contraction (*22*), the fusion sites in Fig. 2c would quickly form an open channel, as in Fig. 2f. In bilayers containing wedge-shaped molecules curved surfaces (Fig. 2c) are permissible, allowing LIP to be observed by freeze fracture electron microscopy and isotropic molecular motional averaging to be observed by ^{31}P NMR. The recession of water surrounding the cusp may also cause expansion of the fused area to what may look like a plateau in a freeze fracture plane (Fig. 2e), resulting in extensive bilayer contact (Fig. 1, a, c, and e). With an excess of wedge-shaped molecules, a hexagonal tubular structure (Fig. 2d) may be preferable (*11, 13, 15*). Thus, in our model, the fusion point is a point defect in a smectic liquid crystal (*17*), which may propagate or relax into different forms.

Fusion through bilayer defects was suggested previously (*10, 23*). Since the intermediate stages of fusion are difficult to observe, many of the proposed models remained unproved. There may be more than one fusion mechanism in nature, hence our model may not be universal. However, it could represent a pathway for naturally occurring fusion events that have so far escaped detailed microscopic observation.

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25. Supported in part by grant BC-248 from the American Cancer Society and grant GM-28120 from the National Institutes of Health. S.W.H. was an awardee of NCI career development grant CA00084. We thank C. Jung and J. Cuppoletti for help in protein incorporation experiments.

31 July 1980; revised 3 February 1981

Tumor Shedding and Coagulation

Abstract. *Three syngeneic carcinomas from two species shed plasma membrane vesicles when cultured in vitro or grown in the ascites tumor form in vivo. Shed vesicles carry procoagulant activity that can account for the activation of the clotting system and the fibrin deposition associated with these and many other types of malignancy in animals and man.*

Clotting abnormalities are commonly observed in patients with malignant neoplasms (*1*), and fibrin deposits have been reported to surround many primary and transplanted tumors in man and animals (*2, 3*). However, the biological significance of tumor-associated coagulation and its pathogenesis are not understood. Among other possibilities, a fibrin gel deposited about tumors could serve as a physical barrier or "cocoon" that isolates tumor cells from the host's immunological defense mechanisms (*4*). Also, there is evidence that fibrin itself, or perhaps certain of its breakdown products, may induce angiogenesis, a characteristic and essential feature of solid tumor growth (*4*).

We report that guinea pig syngeneic line 1 and line 10 hepatocarcinomas (*5*) and mouse breast TA3-St carcinomas (*5*) release procoagulant activity (PCA) in tissue culture and in vivo. In all three tumors PCA is associated with sedimentable, ultramicroscopic plasma membrane–derived vesicles. Others have reported that tumor cells shed membrane fragments that contain various cell surface antigens and, in murine systems, virus-like structures (*6*), but such shedding has not previously been related to activation of the clotting system.

Earlier studies (*7*) demonstrated that guinea pig line 1 and line 10 hepatocarcinomas, when cultured for 4 hours in serum-free medium, release at least four distinct mediators with important biological activities: PCA, plasminogen activator, a factor that enhances vascular permeability, and an inhibitor of macrophage migration. Mediator release decreased 67 to 98 percent when cells were cultured at 4°C instead of 37°C. Puromycin ($2 \times 10^{-5} M$) decreased the release of plasminogen activator and vascular permeability factor by 66 and 45 percent, respectively, but had little effect on PCA (< 10 percent), suggesting that PCA release does not depend significantly on new protein synthesis.

In the present experiments, ascites tumor cells were harvested from strain 2 guinea pigs (tumor lines 1 and 10) or A/Jax mice (tumor line TA3-St), washed three times in Hanks balanced salt solution, and cultured at a density of 5×10^6 cells per milliliter in serum-free, antibiotic-supplemented Hanks minimal essential medium under a humidified atmosphere containing 5 percent CO_2. After 4 hours, the cultures were centrifuged for 10 minutes at 160*g* and then 10,000*g* and the cell-free supernatants were collected for procoagulant studies.

Substantial PCA was found in the cell-free culture supernatants of all three tumors (Table 1). Whereas plasminogen activator and other mediators secreted by these tumors remained soluble, little or no PCA remained in the supernatant after ultracentrifugation for 90 minutes. Recovery of PCA in the pellets varied from 50 to 95 percent. The PCA from line 10 tumors (PCA_{10}) could also be concentrated by membrane ultrafiltration (Amicon XM-50) without significant loss of activity. When passed over Sephadex G-200 columns, PCA_{10} emerged in the void volume with nearly complete recovery and with a 20- to 40-fold increase in specific activity over that of cell-free culture fluids.

Procoagulant activity was not inhibited by up to 10 m*M* diisopropyl fluorophosphate (DFP) under conditions that totally inhibited trypsin (37°C, 2 hours, *p*H 8.0). DFP (10 m*M*) completely inactivated 30 μg of trypsin (in a reaction volume of 1.0 ml), whereas PCA containing 25 μg of total protein was not inactivated. In addition, PCA was relatively heat-stable, retaining 60 percent of its activity after 30 minutes at 70°C. PCA function was not reduced by proteolytic digestion with either trypsin or papain.

To determine whether PCA was released in vivo, ascites tumor fluid was collected from guinea pigs and mice and rendered cell-free by low-speed centrifugation. Ascites fluid from both species contained abundant PCA, all or most of which sedimented at 100,000*g* (Table 1). That PCA may have biological significance is suggested by the extensive fibrin deposits found in the peritoneal walls of guinea pigs bearing ascites tumors (Fig. 1a).

Table 1. Procoagulant activity shed by tumor cells in short-term tissue culture and in ascites fluids. Data were obtained from four to seven separate experiments. Cell viability was 95 to 99 percent, as judged by trypan blue exclusion and by lack of release of succinate dichlorophenol-indophenol oxidoreductase. Tumor cells accounted for ≥ 98 percent of nucleated cells, the remainder being lymphocytes or macrophages. Identical results (not shown) were obtained with line 10 tumor cells carried in continuous culture. For assay of ascites fluids, citrated Hanks balanced salt solution was injected intraperitoneally into strain 2 guinea pigs bearing 7-day-old tumors (20 ml) or into A/Jax mice (5 ml). The peritoneal fluid was harvested and cell-free fluids, ultracentrifuge pellets, and supernatants were prepared as for culture fluids. Aliquots of cell-free culture or ascites fluids were centrifuged at 4°C for 90 minutes at 100,000*g*, the supernatant was harvested, and the pellet was reconstituted to the original volume. PCA (0.1 cm^2) was assayed by its ability to shorten the recalcification time of citrated, platelet-poor guinea pig or mouse plasma.

Tumor cells	Mean shortening of recalcification time (%)		Sedimentable PCA (%)*
	Cell-free fluids	Reconstituted pellets	
Four-hour tissue culture			
Line 10	72.4 ± 1.1	63.8 ± 5.7	97
Line 1	37.6 ± 2.6	30.4 ± 2.7	67
TA3-St	40.9 ± 3.6	22.4 ± 3.7	64
Ascites fluids			
Line 10	52.8 ± 3.8	47.2 ± 2.6	100
Line 1	29.2 ± 6.1	16.6 ± 4.1	61
TA3-St	27.0 ± 3.4	26.4 ± 1.8	98

*Determined by multiplying by 100 the quotient of the amount of PCA in the centrifuge pellet divided by the total amount of PCA (pellet plus supernatant).

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