Unique Cleavage Planes in Frozen Red Cell Membranes[1] (34973)

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(Introduced by E. B. Taft)

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The freeze-cleave electron microscopy preparative technique is particularly useful for examining large areas of biological and artificial membranes. However, the interpretation of the relation of surfaces revealed by the technique to membrane ultrastructure has been controversial (1, 2). According to Branton (3), cleavages propagate along the interior of frozen membranes and generate previously unseen faces. Branton sugggests that a single potential cleavage plane exists in many membranes and indicates that this plane may correspond to the hydrophobic center of the lipid bilayer in the Davson–Danielli model (3, 4). According to the original Moor–Mühlethaler hypothesis, the faces revealed by freeze-cleaving represent true outer surfaces of membranes (5, 6). This implies that two potential cleavage planes exist along membranes: one along the membrane's true outer surface and a second along its true inner (juxtacytoplasmic) surface.

Branton's direct evidence for membrane splitting has come largely from studies of artificial membranes (7). Recently, Wehrli, Mühlethaler, and Moor (8) presented direct evidence of a unique cleavage along chloroplast membranes using a novel double-replica freeze–cleave technique. We have modified the Bullivant freeze–cleave protocol (9, 10) in order to perform similar double-fracture face experiments. We now document the existence of a single cleavage plane near the center of human red cell membranes and thus provide direct evidence of the applicability of the Branton hypothesis to animal cell membranes.

Materials and Methods. Blood was drawn by venipuncture from human donors, centrifuged lightly, and the buffy coat removed. The red cells were washed in 0.9% NaCl, suspended in cold 0.9% NaCl containing 20% glycerol as a cryoprotective agent, and pelleted by light centrifugation.

Small stainless-steel cartridges were columnated (Fig. 1). Packed cells were passed through a hypodermic needle into the common central channel of the cartridges. The specimen in the cartridge assembly was frozen in liquid Freon 22 at −150° and rapidly transferred to a large Styrofoam container filled with liquid nitrogen (−196°). The two cartridges were snapped apart under liquid nitrogen and placed side by side into the specimen well of a Type II simple freeze–cleave device (9, 10). The device was carried under liquid nitrogen to a Kinney PW400 evaporator where the liquid nitrogen was removed *in vacuo* by evaporation and sublimation. A carbon–platinum replica was cast of the two complementary fracture faces simultaneously. In some experiments, single fracture faces were heat-etched at −100 or −105° for brief periods of time in a Type II device. Other replicas of single heat-etched fracture faces were prepared with a Balzers BA 360 Freeze-Etch machine according to the technique of Moor (1). After thawing of the specimens, the replicas were cleaned in household bleach and distilled water and retrieved on 50-mesh Formvar-coated copper grids.

Replicas of the two complementary fracture faces were photographed separately in a Siemens Elmiskop I electron microscope. Since the freezing cartridges are relatively

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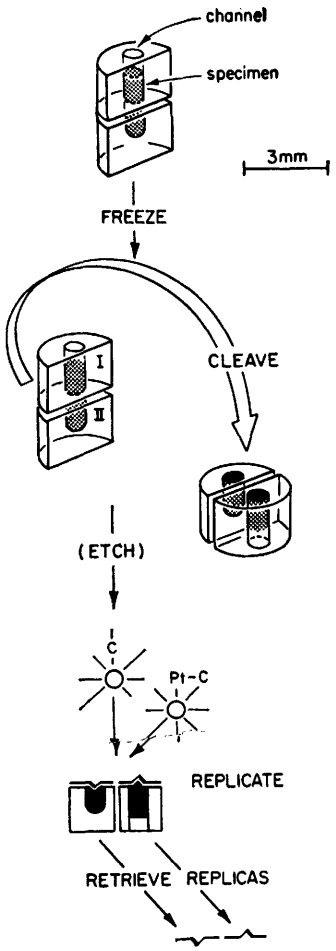


FIG. 1. Protocol for preparing paired replicas of complementary fracture faces.

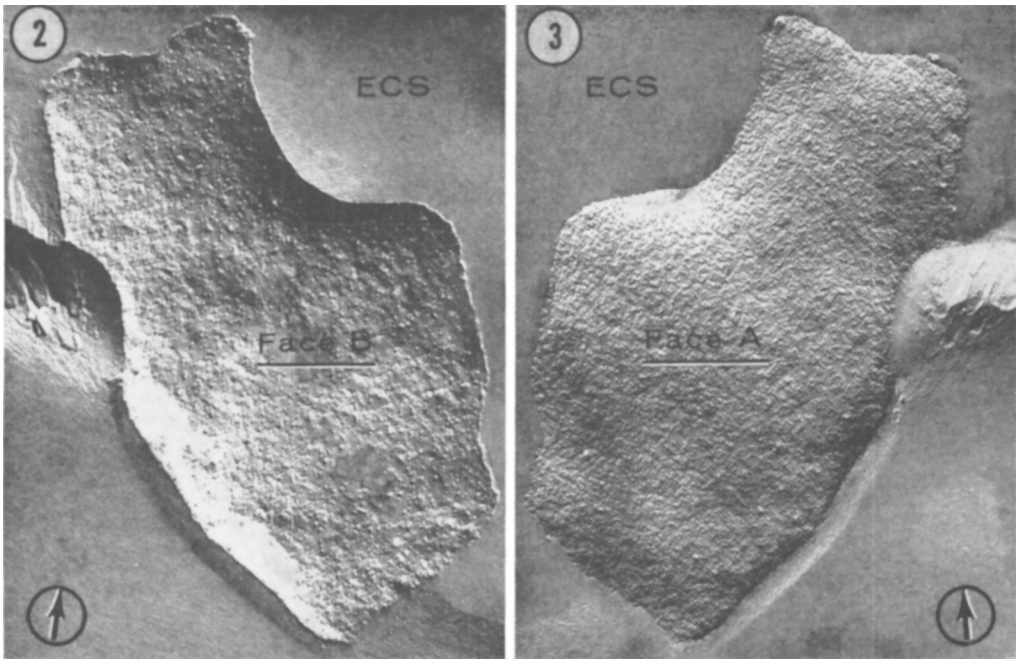
large, cooling rates are slow enough to allow the cells to become mildly distorted during freezing. This distortion facilitated finding complementary areas in the paired replicas since the cells tended assume distinctive shapes that were easily recognized.

Results and Discussion. As described elsewhere, two distinctive nonetchable membrane faces are demonstrated for red cell membranes with the freeze–cleave technique (6, 11). One face (by convention called "Face A") is oriented toward the extracellular compartment and is partially covered with many discrete 80- to 130-Å particles. The second face (Face B) has 20 – 25% as many particles as Face A and is oriented toward the cell cytoplasm.

Analysis of complementary areas of paired replicas gives new information on the relative positions of the Face A and Face B of freeze-cleaved membranes. Previously, Face A was interpreted as the true outer surface of the membrane and Face B as the true juxtacytoplasmic surface of the red cell membrane. In Figs. 2 and 3, complementary areas of paired replicas show that a single area of red cell membrane has been cleaved into two lamellae, one with the characteristic membrane Face A and the other with Face B. Since Face B (Fig. 2) and Face A (Fig. 3) have been generated by a single cleavage, they previously must have faced each other at some level within the membrane, as shown schematically in Fig. 4. If Face A had been the true outer surface of the membrane, then the complementary surface would have shown extracellular fluid originally abutting against the membrane. Likewise, if Face B had been the true juxtacytoplasmic surface of the membrane, the complementary surface would have been cytoplasm. Instead, the paired replicas invariably show that the red cell membrane is cleaved into two lamella with the distinctive Face A and Face B oriented toward each other.

The position of the cleavage plane within the membrane can be approximated from measurements of the thickness of each of the two membrane lamellae in preparations which are heat-etched (3). With etching, aspects of both lamellae are revealed whereas cleaving alone only demonstrates a single lamella at a given fracture face. The thickness of the lamellae exposed by cleaving and etching is difficult to measure accurately. However, in near cross fractures, the two lamellae appear almost equal in thickness suggesting that the cleavage passes near the anatomic center of the membrane.

Although the two faces of the membrane revealed by cleaving are grossly complementary, it is noteworthy that they are not absolutely complementary with respect to all fine structural details. This might be attributable to any of a number of factors including surface contamination of cold fracture faces *in vacuo* prior to replication, plastic deformation introduced during cleaving, and image distortion introduced during shadowing with



FIGS. 2 and 3. Electron photomicrographs of complementary areas of replicas of two fracture faces originating from a single freeze-cleaved red cell membrane. Face B in Fig. 2 (left) has fewer membrane-associated particles than Face A in Fig. 3 (right). Faults in the frozen fluid of the extracellular space (ECS) are complementary. The encircled arrows show the direction of platinum–carbon shadowing. ×30,000.

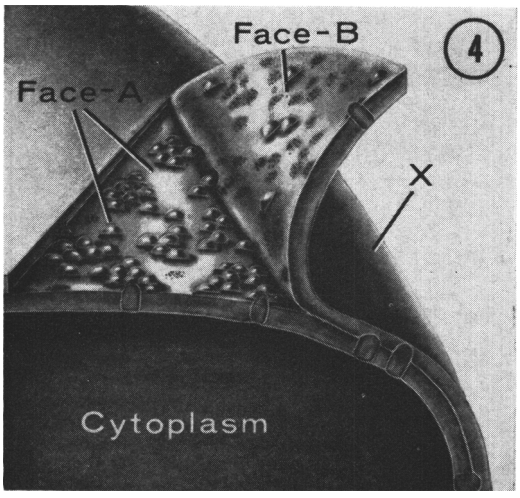


FIG. 4. Schematic representation of the position of the Face A and Face B within the membrane. Face A is oriented toward the extracellular compartment and Face B is oriented toward the cell cytoplasm. The relatively smooth true outer surface of the membrane (X →) is revealed exclusively by heat etching.

platinum. These factors have not been systematically evaluated.

The observation of Weinstein and Koo that some membrane-associated particles extend across the membrane (11) may remain valid in light of the present evidence although the face view of the membrane in their preparations is now known to represent a different level in the membrane than previously assumed. Some of the sheetlike structures which they originally believed to represent a full thickness of the membrane now appear to represent a split thickness of the membrane. The particles found extending through the full thickness of the membrane (see Ref. 11, Figs. 2 and 3) are now reinterpreted as extending across one of the two membrane lamellae. However, such particles might still penetrate into and extend through the apposed leaflet prior to its removal by cleavage. Estimates of the distance that the membrane-associated particles on Face A and Face B protrude above the smooth portion of the faces range up to 40 Å. This height is

sufficient for the particles to extend through the apposed lipid monolayer and probably to the true outer surface of the membrane.

As emphasized by Branton (3), the existence of a potential cleavage plane within membranes can be reconciled with the Davson–Danielli model if the potential cleavage plane is along the hydrophobic center of a lipid bilayer. Such cleavages have been shown for artificial lipid bilayers (7) and the further demonstration of central cleavages in biomembranes provides physical evidence for that model. The particles on Face A and Face B may represent focal areas of structural specialization within the membrane, perhaps in the form of proteins extending across the hydrophobic interior of the membrane (2, 11–14).

Summary. The Bullivant freeze–cleave technique has been modified to allow for the casting of replicas of the faces produced both above and below a single cleavage through frozen specimens. Analysis of replicas of paired fracture faces indicates that membrane faces normally revealed by cleaving are complementary and originate from the interior of the cell membrane. The thickness of the layers demonstrated by cleaving and heat-etching indicates that the potential cleavage plane from which membrane Face A and B are generated exists near the center of the membrane. The results provide physical evidence for the presence of a lipid bilayer within at least part of the red cell membrane.

Note added in proof: Recently, Da Silva and Branton (*J. Cell Biol.* **45**, 598 (1970) and Tillack and Marchesi (*J. Cell Biol.* **45**, 649, 1970) have shown that heat etching is required to demonstrate the true outer surface of the red cell membrane. Their work is interpreted as showing that the cleavage planes are within the interior of membranes but does not rule out the possibility of multiple cleavage planes in each membrane. Our observations on complementary replicas provide direct proof of a single cleavage plane.

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