# Endothelial Cell Membranes: Polarity of Particles as Seen by Freeze-Fracturing

Abstract. *Freeze-fracturing shows particles within membranes. In plasma membranes of most cells the particles are more strongly bound to the inner half. In unfixed endothelial cells, this polarity is reversed. Glutaraldehyde fixation results in conventional polarity. The reverse polarity may be related to a mechanism for preferential fusion of pinocytotic vesicles with the plasma membrane.*

In the freeze-etching (freeze-fracturing) technique, membranes are split and small (8.5 nm) particles (possibly containing protein) are revealed in their interiors (*1*). The functional complexity of membranes can be qualitatively correlated with the particle population density being observed (*1*). The particles show a polarity with respect to the membrane, the majority adhering more strongly to one fracture face than the other. Usually, when a plasma membrane is fractured the particles are preferentially associated with the inner half of the membrane (*2*), more being seen on the face of the half membrane left frozen to the cytoplasm. For convenience, we refer to this face as an A face (*3*). The face of the half membrane left frozen to the extracellular space after fracture (the B face) bears fewer particles. The particle polarity just described is the same whether the tissue is unfixed or fixed in glutaraldehyde before freeze-etching.

We have used the freeze-fracture technique to study endothelial cells in a number of different tissues. In the plasma membranes of unfixed endothelial cells, the particle polarity is the reverse of the conventional situation described above. Prior fixation in glutaraldehyde results in a change to conventional polarity. This difference of particle polarity between the plasma membranes of endothelial cells and those of other cells may be related to transport properties.

The freeze-fracturing technique that we have used has been fully described (*4*, *5*). Fracturing and replicating are carried out at such a low temperature that etching does not occur, and in this respect the technique differs from freeze-etching (*6*). Etching does not alter the appearance of membrane fracture faces. We have examined freeze-fracture replicas of endothelial cells in rat posterior pituitary; human placenta; and mouse heart, psoas muscle, diaphragm, kidney, and liver. Prior to rapid freezing, the tissue was either soaked in 25 percent glycerol in phosphate buffer in the cold for at least 30 minutes, or fixed in glutaraldehyde and then treated with the glycerol solution. Occasionally, fresh tissue, neither fixed nor glycerinated, was frozen.

When examining freeze-fracture replicas of the plasma membranes of most cells, it may be difficult to decide whether a particular face is an A or B face. To be certain, it is necessary to see either a sufficiently large area to determine the curvature of the face (the A face appears convex, the B face concave), or a region where the fracture plane leaves the membrane and passes into the cytoplasm or extracellular space. However, with endothelial cells there is no such difficulty, for they possess inbuilt reference structures, in the form of fenestrae or attached pinocytotic vesicles. The endothelial cell membranes of fenestrated capillaries will fracture to reveal a number of "holes" or broken-off "necks" (Fig. 1), which correspond to the fenestrae (circular pores) that extend from one side of the endothelial cell to the other. The attached pinocytotic vesicles of continuous capillaries fracture in a similar manner. The half membrane left adhering to the cytoplasm of the cell (the A face) will show holes, while the half left adhering to the extracellular space (the B face) will show necks. This applies to either the luminal or basal membrane of the endothelial cell.

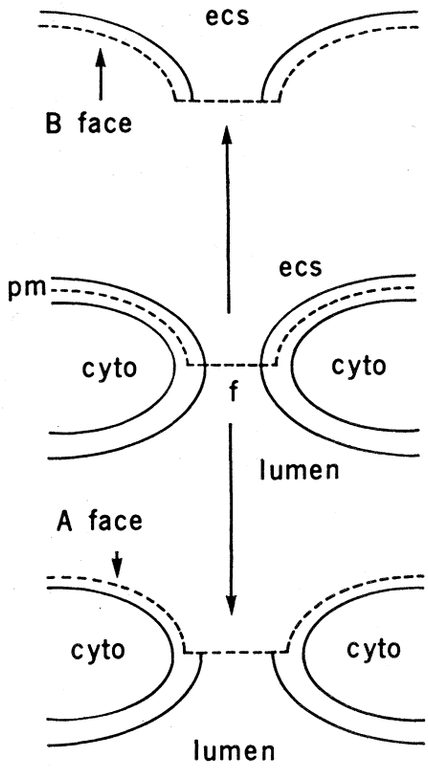


Fig. 1. Diagram to show the path the fracture plane takes within the plasma membrane of the endothelial cells of fenestrated capillaries. A portion of the intact endothelial cell is shown in the center. Cytoplasm, *cyto*; extracellular; *ecs*; fenestration, *f*; plasma membrane, *pm*. After fracturing, the B face (upper) shows a "neck," and the A face (lower) a "hole." A fracture through the basal plasma membrane is shown in the diagram, but the luminal plasma membrane will fracture in the same manner. So will the membranes of continuous capillaries, revealing the fused pinocytotic vesicles as holes on the A face or necks on the B face.

We initially studied the fracture faces of endothelial cell membranes of the fenestrated capillaries of the posterior lobes of the rat pituitary gland and noted that in unfixed tissue there were more particles on the B face than the A face (Fig. 2a), while in glutaraldehyde-fixed tissue there were more on the A face (Fig. 2b). Subsequent investigation showed that the endothelial cell plasma membranes of the continuous capillaries of human placenta and mouse heart, psoas muscle, and diaphragm; the fenestrated capillaries of mouse kidney; the sinusoids of mouse liver; and the endothelial cell lining of mouse heart all have the same fracturing properties. The plasma membrane of cells other than endothelial cells (for example, renal epithelial cells) show conventional polarity of membrane particles whether the tissue is glutaraldehyde-fixed or unfixed.

The reason for soaking in the glycerol solution is to prevent the formation of ice crystals. To check that the reverse polarity of the particles was not induced by glycerol and restored by glutaraldehyde, posterior pituitary tissue was taken directly from a rat and freeze-fractured with no fixation or glycerol treatment. Although the absence of any cryoprotective agent resulted in the formation of large intracellular and extracellular ice crystals, it was possible to observe areas of endothelial cell membrane fracture face. There were more particles on the B face than the A face, showing that the reverse polarity was not due to the glycerol treatment.

At the time that Friederici (*7*) studied fenestrated capillaries in the kidney

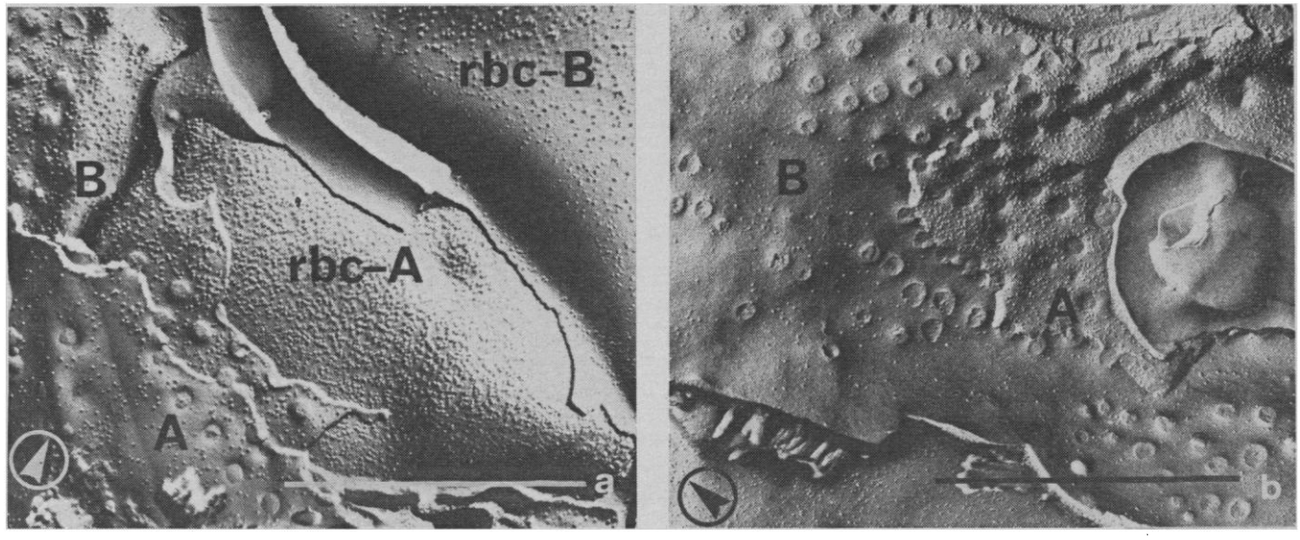


Fig. 2. Electron micrographs showing fracture faces of endothelial cell membranes of fenestrated capillaries in (a) unfixed and (b) fixed posterior pituitary tissue. The encircled arrows denote the direction of platinum shadowing and the scale marks are 1 μm. (a) The fracture has revealed the A face (*A*) of the basal plasma membrane (fenestrations represented by "holes") and has then passed through the thin cytoplasm of the cell to reveal the B face (*B*) of the luminal plasma membrane (fenestrations represented by "necks"). There are more particles on the B face than the A face. Note that the A and B faces (*rbc-A* and *rbc-B*) of the red blood cells in the lumen of the capillary have conventional polarity. (b) The fracture has revealed the A face (*A*) of the luminal plasma membrane and the B face (*B*) of the basal plasma membrane. There are more particles on the A face.

by freeze-etching, it was generally thought that the two different fracture faces seen were the true inner and outer surfaces of the membrane, rather than interior views. He worked with unfixed material and noted that, for renal epithelial cell plasma membranes, there were more particles on the "outer" surface than on the "inner" surface, while the reverse was true for the endothelial cell plasma membranes. He commented that this difference might be related to the differing functions of the two cell types. When Friederici's micrographs are interpreted in terms of the known splitting of membranes, they show more particles on the A face than on the B face for epithelial cell plasma membranes and the reverse for endothelial cell plasma membranes.

The nature of the small particles seen when biological membranes are freeze-fractured is not known with certainty. They may consist of protein or a lipoprotein complex (*8*). The path the fracture takes around these particles is that which requires the least amount of work and depends on the relative strengths of the bonding between the particles and two halves of the frozen membrane. Glutaraldehyde, which is known to cross-link proteins (*9*), must to some extent reverse these relative bonding strengths. In this connection, it has been shown in our laboratory that the protein myosin filaments of unfixed muscle deform and fracture asymmetrically with respect to the fracture plane, while in glutaraldehyde-fixed muscle the fracturing of the filaments is symmetrical (*10*). The fracturing properties of the intramembranous filaments of tight junctions are also altered by glutaraldehyde fixation (*11*). Certain types of gap junction fracture to show particles on the B face and depressions on the A face (*12*), which is the reverse of the normal situation (*3*, *13*). However, in these special gap junctions normal polarity is not restored by glutaraldehyde fixation (*14*). Our finding of this inverted polarity and its restoration by glutaraldehyde fixation refers to a whole membrane rather than a junctional region.

Thus, the particles within endothelial cell plasma membranes show special properties, and these may be related to the transport properties of the cells. Pinocytosis in endothelial cells involves the continual addition and removal of plasma membrane. It is possible that the reverse polarity of the particles is connected with the facility with which membrane material is added and subtracted. Alternatively, the reverse polarity may be the basis of a mechanism whereby fusion between pinocytotic vesicles and the plasma membrane is allowed, while fusion between intracellular membrane-bound organelles and the plasma membrane is not. In a non-endothelial cell, the A face of the plasma membrane is convex and bears many particles, while the limiting membranes of internal organelles appear the other way round, the B face (fewer particles) being convex (*2*, *5*). In endothelial cells (unfixed) the B face of the plasma membrane has more particles, and consequently, as far as we can determine with the small areas of membrane face revealed, so do the B faces (convex) of the pinocytotic vesicles. We have looked at the membranes of organelles such as mitochondria and nuclei, within endothelial cells, and have shown that the convex B faces have conventional polarity, with fewer particles. (These features are not illustrated in this report.) Consequently, the pinocytotic vesicles could be considered as "right way round" to fuse with the plasma membrane, while other organelle membranes would be "wrong way round." In actively transporting endothelial cells with a high flux of pinocytotic vesicles, this mechanism might prevent removal of essential organelles, such as mitochondria, by fusion and extrusion. We realize that other cells, such as the renal epithelial cells, also have pinocytotic activity. However, in such cells the pinocytotic flux is not so great, nor is it usually a two-way process.

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15. We thank J. B. Gavin and R. E. F. Matthews for discussion of the manuscript. Supported in part by a grant from the Medical Research Council of New Zealand.

4 August 1972; revised 10 October 1972 ■


# Mechanism of Action of Vitamin K: Demonstration of a Liver Precursor of Prothrombin


Abstract. *Extracts of sonicated liver microsomes that are prepared from rats deficient in vitamin K or from rats given vitamin K antagonists contain a factor that liberates a thrombin-like activity when it is incubated with venom from* Echis carinatus. *The amount of this factor is low in control rats and in hypoprothrombinemic rats given vitamin K 1 hour before they were killed. These data indicate that this factor is a protein precursor of prothrombin, which is synthesized in the liver.*


The only generally accepted function of vitamin K in higher animals is that of regulating the synthesis of prothrombin and the other plasma clotting factors dependent on vitamin K (factors VII, IX, and X). The vitamin regulates the rate of synthesis of prothrombin after transcription but the nature of the control site is still undetermined. Although it has been suggested (*1*) that the vitamin regulates the de novo synthesis of prothrombin, observations from a number of different laboratories (*2*) indicate that protein synthesis is not required for the step sensitive to vitamin K in the production of prothrombin; we have recently presented indirect evidence (*3*) for the conversion of a precursor protein to prothrombin in the rat.

Stenflo (*4*) has reported the presence of an antigenically active, but biologically inactive, prothrombin in the plasma of cows treated with Dicumarol. The venom from *Echis carinatus* (saw-scaled viper) (*5*) can cause generation of thrombin from purified preparations of this abnormal prothrombin (*6*). This observation, and that of Josso *et al.* (*7*) suggested that the postulated prothrombin precursor from the liver might also be activated by such non-physiological means.

Male Holtzman rats (200 g) were made hypoprothrombinemic by treating them with the vitamin K antagonists warfarin or 2-chloro-3-phytyl-1,4-naphthoquinone (chloro-K) (*8*), or by feeding them a diet deficient in vitamin K for 7 days, while they were in cages that prevented coprophagy (*9*). Blood was drawn by cardiac puncture from starved rats anesthetized with ether; a liver microsomal extract was also prepared from these animals (*10*, *11*). Prothrombin concentrations were measured in plasma by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (*12*), and in the microsomal extracts by methods previously described (*11*).

The microsomal extract from rats treated with warfarin for 18 hours before they are killed has previously been shown (*11*) to contain no detectable prothrombin activity. However, when the thrombin-generating step of the two-stage assay was replaced by an incubation with *Echis carinatus* venom, a thrombin-like activity that would clot the fibrinogen mixture in less than 15 seconds was generated in extracts from the rats treated with warfarin. When the mixture containing fibrinogen was incubated with venom alone, or with microsomal extract alone, no clot was formed in 120 seconds. The clotting activity, generated from some factor other than prothrombin in the microsomal extract, will be subsequently referred to as precursor activity.

The amount of precursor activity in the liver microsomal extract is inversely related to prothrombin concentrations (Table 1). The data show that two different vitamin K antagonists, as well as a nutritional deficiency of the vitamin, all result in a decrease in prothrombin concentrations in the plasma and

Table 1. Activities of prothrombin and precursor in microsomal extracts of rat liver. Prothrombin in plasma and microsomal extracts was assayed, and results were expressed as Iowa units. To measure precursor activity, I incubated 0.5 ml of microsomal suspension with 0.1 ml of *Echis carinatus* venom (1 mg/ml) at 37°C. After 15 minutes, 0.1 ml of the treated suspension was added to a mixture of 0.1 ml of fibrinogen (1 percent clottable protein in 25 m*M* imidazole (*p*H 7.4) and 150 m*M* NaCl), and 0.3 ml of a buffered acacia solution containing 50 m*M* imidazole (*p*H 7.4), 120 m*M* NaCl, 5 m*M* $CaCl_2$, and 3 percent acacia. The mixture was tipped gently in a small tube until a visible clot formed. These clotting times were converted to thrombin units by comparison to a standard curve prepared by dilution of NIH standard thrombin. Sodium warfarin (5 mg/kg, intraperitoneal) or chloro-K (5 mg/kg, intracardial) was given 18 hours before the experiments, and vitamin K (5 mg/kg) was given 1 hour before the animals were killed. The vitamin was given intramuscularly to rats deficient in vitamin K, and intravenously to rats treated with warfarin. Cycloheximide (5 mg/kg, intraperitoneal) was given 30 minutes before the vitamin. Results are expressed as means ± standard errors. The number of animals used in each experiment are given after the treatment in column 1.

| Treatment | Prothrombin concentration (unit/ml) in: | | Precursor activity | |
|---|---|---|---|---|
| | Plasma | Microsomal extract | Clotting time* (seconds) | Thrombin equivalent in microsomal extract (unit/ml) |
| Control (7) | 218 ± 12 | 2.1 ± 0.1 | 30 (22–46) | 1.9 ± 0.3 |
| Warfarin (10) | 32 ± 6 | <1 | 12 (11–14) | 8.7 ± 0.6 |
| Chloro-K (4) | 34 ± 4 | <1 | 11 (10–11) | 11.8 ± 0.3 |
| Deficient in K (6) | 81 ± 20 | <1 | 20 (18–24) | 3.2 ± 0.2 |
| Deficient in K + K (6) | 175 ± 27 | 2.2 ± 0.1 | 77 (65–120) | 0.7 ± 0.1 |
| Warfarin + K (4) | 136 ± 3 | 1.9 ± 0.1 | 97 (96–120) | 0.6 ± 0.1 |
| Warfarin + K + cycloheximide (4) | 104 ± 3 | 2.0 ± 0.1 | 52 (34–62) | 1.1 ± 0.2 |

* Expressed as median with range in parentheses.