Complementary Freeze Fracture Methods Applied to Cattle Disk Membranes*

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Summary. The rod-outer-segment disk membranes were studied in cattle retinas by electron microscope observation of ultrathin resin sections and complementary freeze replicas. In sections, the membranes are characterized by discontinuous electron densities along the unit membrane structure, in which electron-less-dense globular substructures, forming an intermediate layer, are enhanced. Such substructures are estimated as 60 Å in diameter. In freeze replicas, the membranous particles of 77 Å in diameter are distributed quite densely in the P-face and scattered in the E-face. Like a number of preceding investigators who have failed to obtain convincing findings to correlate similar profiles with the visual pigment molecules, we should also raise a question about preparation involving chemical and thermal procedures affecting the dimensions of the final images.

Our constant results obtained from the two methods, however, will suggest that the thin-section substructures and freeze-replica particles represent two different phases respectively of the same molecular existence rather than a complete profile of the visual pigment. It is also uncertain yet whether one membranous particle should be in fact associated to one molecular pigment or an aggregation of several molecules. The last problem will be resolved to some extent by topographical analyses of the complementary replicas in progress in our laboratory.

Electron microscopists have paid attention to the irregular, globular substructures of the disk membranes of mammalian photoreceptors which appear in various thin sections (NILSSON, 1964, 1965; HIROSAWA, 1971; ROSENKRANZ, 1973; NIR and HALL, 1974; NIR and PEASE, 1975). Single or combined artifacts produced by fixation (COHEN, 1971; KORENBROT, BROWN and CONE, 1973; JONES, 1974), dehydration (NIR and PEASE, 1973), staining (GODFREY, 1973) and/or sectioning (ROBERTSON, 1966) have been examined as possible factors which might cause such globular images. Even though some distortions may possibly result from such different treatment, it seems likely that the disk membrane actually contains the globular substructure. These globular components have not been directly compared with freeze-fracture (FF) particles because both types of electron microscope images have not been fully trustworthy

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yet (TONOSAKI, WASHIOKA and MIZOGUCHI, 1978). Nonetheless, their potential relationship should be explored.

Recently, remarkable progress has been made by analytic electron microscopy, in particular, the three-dimensional model of bacteriorhodopsin polypeptides (HENDERSON and UNWIN, 1975). The relationship between its single protein molecule, composed of seven α helices, and the subunits of purple membrane FF particles has been evaluated by FISHER and STOECKENIUS (1977). In FF replicas image resolution and complementarity are reported to be enhanced by lowering the specimen temperature while maintaining the vacuum at 10^{-9} Torr (GROSS, BAS and MOOR, 1978). With regard to the morphology of an individual membrane particle, potential elastic and plastic deformations during FF procedure have been reviewed (SLEYTR and ROBARDS, 1977). The present paper will deal with the progress of complementary FF methods and the results obtained by the use of a simple device adapted to a conventional vacuum system (TONOSAKI and YAMAMOTO, 1974; YAMAMOTO, TONOSAKI and WATANABE, 1974). Remarkable textual non-complementarity is observed between complementary fracture surfaces at higher magnification, in contrast to the complete matching of the cellular outlines. Terminology of plasmic fracture (PF) and exoplasmic fracture (EF) faces is employed after BRANTON et al. (1975).

MATERIALS AND METHODS

1. *Preparation*

Cattle eyes were obtained from freshly killed animals (160 eyes in total for 23 experiments) at the regional slaughterhouse, and stored in ice-cold physiological saline for 30–120 min for both epoxy-resin sections and FF specimens. The retinae were fixed for 24 hr at 3°C with 2.5% glutaraldehyde in cacodylate buffer adjusted to pH 7.3 (SABATINI, BENSCH and BARNETT, 1963). For some FF specimens the retinae were fixed in phosphate-buffered glutaraldehyde at pH 7.4 for 3–16 hr at 3°C (MILLONIG, 1962). Such alteration did not result in any recognizable variations of findings.

2. *Epoxy-resin sectioning*

The fixed retinae were further treated with 3.3% osmium tetroxide solution adjusted to pH 7.3 with cacodylate for 2 hr at 3°C. After dehydration with a graded ethanol series, the specimen was embedded in epoxy-resin (LUFT, 1961). All sections were mounted on carbon-coated micro plastic grids (FUKAMI and ADACHI, 1965), and stained with 2% uranyl acetate and saturated lead citrate (WATSON, 1958; REYNOLDS, 1963).

3. *FF instruments and procedures*

Prior to FF experiments, the specimens were cut into rectangular pieces $2 \times 8 \text{ mm}^2$ and incubated in the fixative containing 30% glycerol for 3 hr at 0°C. The strip of retina was then placed in the specimen holder (Fig. 1, 2) and frozen with liquid nitrogen without preliminary freezing with other coolant. The complementary FF device consisting of the specimen holder and cooling socket was used (TONOSAKI and YAMAMOTO, 1974). It has been modified in this laboratory to protect the specimen holder from frost during the transfer from the nitrogen bath onto the stage of the

evacuator and preliminary pumping. A copper cover was placed over the cooling socket while it was in nitrogen and was removed after 10^{-4} Torr was reached. The covered parts were thus kept clean throughout the preparation. The thermal stability of the device is shown in the graph (Fig. 2). The specimen underwent fracturing at the lowest temperature (-160°C) at $1-3 \times 10^{-6}$ Torr after 30 min of evacuation. The device was adapted to a conventional evacuator with a simple 4-inch- ϕ oil diffusion pump and liquid nitrogen-cold trap (Fig. 3). Evaporation of platinum

Fig. 1. Cooling socket, left, and parts of specimen holder, right. A specimen grid, 3 mm in diameter, is shown for scaling.

Fig. 2. Specimen holder (SH), before and after fracturing, inside the cooling socket (CS), adapted to the cold stage (S) of the evacuator. The parts of SH, A and B, are detailed on the left. C carbon source, cc copper cover, Pt platinum source. **Graph:** Thermal stability of SH and CS after initial cooling to liquid- N_2 temperature measured with thermocouple inserted and held in the position of SH in the course of evacuation without addition of coolant. D.P. high vacuum phase with diffusion pump, R.P. preliminary phase with rotary pump. Note SH maintained at 4.9 mV or -160°C around the time of fracturing ($\nearrow$).

Fig. 3. Schematic diagram of complementary freeze-fracture device situated in the evacuator equipped with Twin Hearth Electron Bombarded Source (EBS). DP diffusion pump, ES external shutter, IS internal shutter, S specimen stage of evacuator.

and carbon was made using the Twin Hearth Electron Bombarded Source (Edwards High Vacuum, The British Oxygen Co., Crewley, England). Direct current (3 kV, 50 mA) was applied for 7–10 sec for Pt shadowing during which time a stripped tip of 1 mm ϕ wire embedded in the carbon rod was evaporated. For C backing, 4 kV and 50 mA were used for 12 sec, and the tip of 1 mm ϕ rod was spent. The specimen halves carrying Pt-C replicas were rinsed in absolute ethanol at room temperature, which reduced deformative forces exerted in thawing the tissues. After 60 min of cleaning in sodium hypochlorite and distilled water, the replicas were scooped up with 250 or 300 mesh copper grids.

Using our device, the vertically-laid retina was transversely fractured and the two halves were arranged back to back with the fractured faces oriented horizontally. A gap of 6 mm between two halves was considered small enough to produce roughly equivalent shadowing angles for both specimens for the shadowing distance of 115 mm, and for angles of 80° for shadowing and 90° for backing.

4. Electron microscopy

A JEOL-200B-ASID electron microscope was used at accelerating voltage of 150 kV for replicas, and 200 kV for epoxy sections. All of the specimen grids were mounted on a specimen carrier specialized for horizontal rotation which permitted precise matching of the orientation between complementary replicas and stereoscopic pairs. A goniometer was also used for inclination. The beam current was set at 100–110 μ A and the spot size was 2 μ m ϕ . An object aperture of 120 μ m ϕ was regularly used.

OBSERVATIONS

The cattle rod outer segment is uniform in shape and size, and is about $18.0\mu\text{m}$ in length and $1.5\mu\text{m}$ in diameter as measured on electron micrographs of thin sections. The disk membrane, when observed at higher magnifications, resembles a beaded string. Its thickness is approximately $60.5\text{\AA}$ (Fig. 4). The staining heavy metals are deposited onto the outer and inner leaflets forming discontinuous lines. Electron lucent to moderately opaque globular substructures, about $60\text{\AA}$ in diameter, are prominent especially near the periphery of disks. The microvillous plasma membrane of the pigment epithelial cell, on the other hand, appears as a continuous, trilaminar unit membrane composed of heavily stained outer and inner leaflets (ca $30\text{\AA}$ in thickness) separated by a continuous middle stratum (ca $28\text{\AA}$) with an overall thickness of $88\text{\AA}$ (Fig. 4, PM).

When the outer segments are laid slanting across the fracturing line of the specimen holder, they are fractured nearly-transversely (Fig. 5). The disks thus exposed on the replica have a diameter of $1.5\mu\text{m}$ ($N=51$) which is in good approximation with the figure obtained from thin sections. The pair of membranes limiting a disk are distinguished as an unit of PF and EF faces separated by a narrow intradisk

Fig. 4. Stereoscopic high-power view of a thin section of cattle outer segment, arranged for unaided cross vision. Substructures are indicated with arrowheads. *D* disk membrane, *OM* plasma membrane of outer segment, *PM* plasma membrane of the pigment epithelial cell process. $\times 600,000$

space (Fig. 6). The PF face is characterized by 77 Å diameter particles ($N=342$) as measured with the criteria applied to the purple membrane (FISHER and STOECKENIUS, 1977). The density of the particles is $31.7/10^{-6} \text{ Å}^2$ on the average. The free edge of the PF face is remarkably serrated with a series of projections extending out over vertical cliffs of the underlying interdisk cytoplasm (Fig. 7, arrows). The EF face which has been often described as smooth or particle-free is in fact characterized by sporadic particles with densities of $1.3/10^6$ or $9.0/10^6 \text{ Å}^2$ in randomly selected areas. Such textures are often characterized as a cobblestone pattern, particularly at low shadowing angles (Fig. 5). The EF face does not present adequate numbers of depressions to receive the PF particles. Only uniquely shaped aggregations of particles can be correlated with their complementary counterparts (Fig. 7).

Fig. 5. Typical FF faces containing an entire cross section of a rod outer segment. A small part of PF face of the plasma membrane (*P*), cytoplasm (*C*), and PF faces of two neighboring disks (*D*₁, *D*₂) are distinguished. $\times 100,000$

Fig. 6. Complementary replicas from a fractured disk. Note different surface density of bumps in the EF face depending upon its orientation towards the shadowing source (encircled area). Marked faces in this figure are those of two membranes belonging to a single disk. $\times 60,000$

Fig. 7. **a** and **b.** Stereographs of complementary PF, EF, and fracturing edges of cattle disks, arranged for unaided cross vision. Major topological relationships are almost self-evident. Correlations between surface details are made by tracing certain landmarks one by one as indicated by the numerical sequence. Note the serrated, free edges ($\nearrow$).

DISCUSSION

In the preparation of specimens embedded for thin sectioning, considerable molecular rearrangements presumably occur within biological membranes regardless of the exact preparatory method employed. It seems most probable that the globular substructures represent the proteins surrounded by lipid aggregations, all of which are stained en masse by heavy metals. Since FF procedures appear to involve less drastic treatments, they may preserve more information of molecular arrangement. It is thus important to determine whether a PF particle represents one protein molecule plucked off the outer leaflet, or a mass of molecules resulting from the collapsed lipids and proteins. In the *Halobacterium* purple membrane (FISHER and STOECKENIUS, 1977), omatidial rhabdomeric membrane (YAMADA and USUKURA, 1978) and dark-adapted frog disk membrane (OLIVE and RECOUVREUR, 1978), the PF particles have been described to be packed so densely that they form remarkable geometric arrays. In cattle we have failed to find PF particles organized with such a high degree of regularity, as shown with optical diffraction methods. The higher lipid content, 40% in vertebrate disk membranes compared with 25% in the purple membrane (BLAUROCK, 1972), may restrict the size of clustering PF particles.

According to the recent hypothesis that rhodopsin is spindle-shaped, 75 Å or more in length, and penetrating the membrane transversely, its diameter should be considerably less than 40–45 Å, as estimated for a spherical molecule of 28,000–40,000 Dalton molecular wt (WU and STRYER, 1972; RAUBACH, NEMES and DRATZ, 1974). On the other hand, the molecular conformation of the membrane phospholipids has been defined as a hydrocarbon chain with a van der Waals cross section of 5 Å diameter (BENNETT, 1975). The PF particles, which have a wide range of diameters (e.g., 77 Å in cattle), may represent protein-lipid complexes rather than specific protein molecules. A report that rhodopsin loses its regenerability upon the complete removal of the associated phospholipids (ZORN and FUTTERMAN, 1971) would emphasize structural associations between the protein and lipids. Such interactions in no way imply a rigid architecture that might impede lateral diffusion of rhodopsin in the plane of the disk membrane (POO and CONE, 1974). On EF faces, there is no compelling evidence for deep holes which should be present if proteins have been removed. The only possible explanation for this fact may be that such a hole is either deformed by rearrangement of lipids (CORLESS et al., 1976) or made shallow by shadowing effects.

As shown in several types of synthetic materials (SLEYTR and ROBARDS, 1977), elastic and plastic deformations may produce significant changes in FF surfaces. Resolution of fine structure including such post-fractured changes will become complete only through some complementary FF methods. Clean complementary surfaces can be obtained by fracturing in a high vacuum, using either hinged (STEERE and MOSELEY, 1969; MÜHLETHALER, WEHRLI and MOOR, 1970) or unhinged devices (WEHRLI, MÜHLETHALER and MOOR, 1970; TONOSAKI and YAMAMOTO, 1974). It is probably not advisable for high resolution to transfer FF specimens into a bell jar after preliminary fracturing outside (CHALCROFT and BULLIVANT, 1970; SLEYTR, 1970) because of possible contaminating deposits during pumping. Our complementary methods have here proved that the specimen holder will be adaptable to a variety of

tissue or cell preparations, with good reliability and reproducibility. It has been recently emphasized that the quality of an FF replica depends upon the extent to which one can maintain a high vacuum and a low temperature during the procedure (GROSS, BAS and MOORE, 1978). We are currently planning to incorporate such considerations to further improve our apparatus. Our efforts in this regard will be the subject of a future report.

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双面凍結レプリカ法のウシ円板膜への応用

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ウシ網膜の杆状体細胞外節円板膜を, 薄切樹脂切片と双面凍結レプリカの電顕観察により研究した. 薄切法においては, 円板膜が そのユニット膜構造に沿って 不連続的暗調部を示し, その結果, 中間層を形成する やや明調な小球状構造が顕著に認められる. それらの直径は約 60Å と見積られる. 一方, 凍結レプリカ法においては, 直径 77Å の膜粒子が P面には密に, E面には散在性に分布する. すでに多数の研究者たちが 上記のような電顕像を視物質分子に結びつける 十分な所見を得ることに失敗しているが, われわれもまた化学的ないし熱的過程を含む試料作製過程が, これら最終電顕像の形状に影響を与えているのではないかと疑わざるを得ない.

しかし, われわれが異なった2方法によって得た再現性の高い結果は, 薄切切片上の小球状構造と凍結レプリカ上の粒子とが, 視物質の完全な姿よりはむしろ, 同一の分子的实际の相異なる形相に それぞれ対応することを暗示するであろう. また1個の膜粒子が果たして1個の分子的視物質に相当するか,あるいは数個の集合体に相当するかの問題も依然として不明である. この最後の問題は, 目下われわれが行なっている双面レプリカの局所地形学的分析によって 一定の限界まで解決されるであろう.

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