mononucleosis produced interferon (9). Although the inducer of interferon synthesis has not been identified, preliminary electron microscopy data in our laboratory indicates the presence of unusual 22 mμ particles in all of the cell lines studied for antiviral activity (10).

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Penetration of Red Cell Membranes by Some Membrane-Associated Particles* (33013)

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A novel 100 Å particulate component associated with red cell membranes has recently been demonstrated with freeze-cleave techniques (1). Morphologically similar particles are apparently quite ubiquitous and have been reported on the surfaces of bacterial, fungal, plant, and a variety of animal membranes (2–4). The number and distribution of particles varies for different membranes and some membranes, such as myelin, are free of these particles (5,6). Thus far, speculation on their functional significance has centered on the possibility that these particles may represent multienzyme complexes (2,3) although alternative interpretations have been offered (1,2,6,7).

In preliminary studies on freeze-cleaved and replicated red cells, membrane-associated particles were observed on both outer and inner surfaces of the red cell membrane(1). However, the quality of these earlier replicas precluded examining the membranes in cross section. Replicas made with an improved freeze-cleave and replication apparatus used in this study now allow for the examination of the relationship of these particles to membrane ultrastructure as seen in cross section. These improved replicas show that some, but not all, cell membrane-associated particles penetrate through the entire thickness of the red cell membrane forming continuous structural units between the external milieu and the cell's cytoplasm.

Methods. Improved freeze-cleave apparatus. In these epxeriments, a modified Bullivant–Ames freeze-cleave and replication apparatus(7) was used to produce replicas of frozen fracture faces of intact red cell membranes and red cell ghosts. The original Bullivant–Ames specimen block ("type I apparatus") consists of a cylindrical brass block with the specimen well located on its upper face(7). A heavy brass lid covers the specimen well when the brass block is carried to an evaporator for replication with carbon and carbon–platinum following fracturing of the

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pellet of frozen blood. Frozen fracture faces are exposed to evaporated replicating materials (platinum and carbon) from many directions since no effort is made to aperture the carbon and carbon–platinum sources. The improved apparatus used in this study ("type II apparatus") was proposed as a "Note of added proof" by Bullivant and Ames in their original communication(7). In addition to the components of the type I apparatus, the type II apparatus incorporates a large central brass tier which is perforated by two long narrow channels. One channel is perpendicular and directly over the specimen well, while the other is at a 45° angle to the specimen well. Carbon–platinum shadowing material is evaporated *in vacuo* through the angled channel, and carbon for backing the shadowed surface is evaporated through the perpendicular channel. During replication procedures, the cold central tier acts as a heat sink and traps contaminating gas molecules from the bell jar atmosphere. More important, the channels in the central tier effectively aperture the carbon and carbon–platinum sources during production of replicas, eliminating much of the random scatter of replicating and shadowing materials which occurs during the production of replicas with the type I apparatus. Use of the type II apparatus has improved considerably the quality of replicas.

Preparation of replicas of red cell membranes. Red cells were collected from hematologically normal human donors into ACD or CPD anticoagulant solutions and stored for periods up to 3 weeks at 4°C in a blood bank. Small aliquots of blood were removed sterilely from plastic blood packs, washed repeatedly with saline and glycerinated by successively resuspending cells after light centrifugation in 10, 20, and 40% glycerol solutions containing 0.9% NaCl at 15-min intervals. Following glycerination, cells were again centrifuged, placed in the specimen well of the type II Bullivant–Ames apparatus so as to completely fill the well and protrude slightly above the well's mouth. The specimen was frozen by placing the apparatus in a container of liquid nitrogen (−196°C). The lid and central tier were also cooled in liquid nitrogen. After the temperature of the block equilibrated to the temperature of the liquid nitrogen, the frozen packed cells were cleaved (fractured) with a precooled razor blade held in the jaws of a hemostat as described in detail elsewhere(7,8). The brass block was covered with the cold central tier and lid and was carried under liquid nitrogen to a Kinney evaporator. The evaporator bell jar was then evacuated. When a vacuum of 10^{-5} Torr was achieved, the specimen cover was lifted with a small, externally controlled electric crane. The cleaved surface was shadowed at a 45° angle by flashing a carbon–platinum pellet and it was then backed with evaporated carbon. Deep etching, as described by Moor(2), was avoided since our experience with red cells indicated that etching may introduce undesirable artifacts (8,9). Following replication of the frozen fracture face, the brass specimen block was removed from the evaporator chamber. After the packed cells melted, they and the overlying replica were placed in strong household bleach to digest the cells away from the replica. Fragments of replica were rinsed several times in distilled water, picked up on 400 mesh uncoated copper grids, and viewed directly in Siemens Elmiskop I electron microscope.

Replicas of red cell ghosts. Since some features of inner aspects of red cell membranes are partially obscured by the granular appearing cytoplasm of intact red cells, ghosts were also prepared to afford a better view of membranes in cross section. Ghosts were prepared by lysing 4 ml of washed, packed whole cells in 50 ml of 20 milliosmols of phosphate buffer at pH 7.4(10), washing three times with fresh lysing solution and suspending the ghosts in 40% glycerol solution containing isotonic NaCl for 30 min. Then, ghosts were packed in a refrigerated centrifuge at 4°C for 20 min at 20,000*g*. Packed ghosts were pipetted into the specimen well of the type II Bullivant–Ames specimen block. The block was submerged in liquid nitrogen and after equilibration of the specimen at liquid nitro-

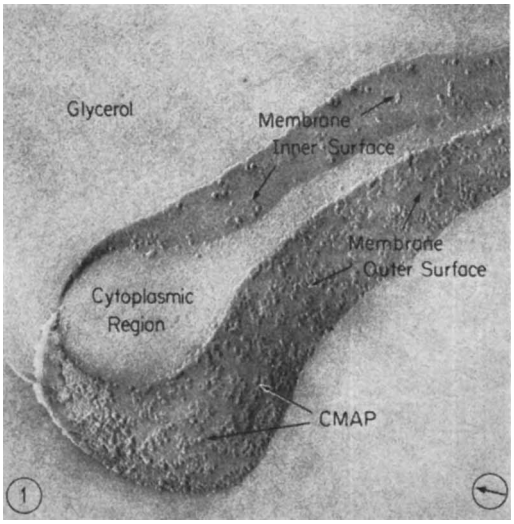
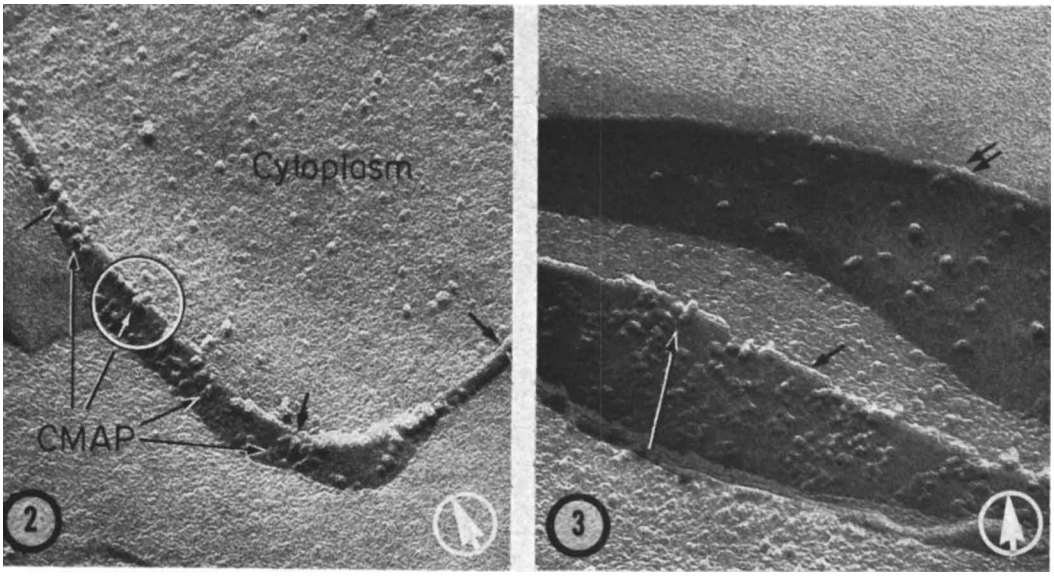


FIG. 1. Carbon–platinum replica of part of the membrane of an osmotically lysed red cell. The membrane is thin and its outer surface is covered with more cell membrane-associated particles (CMAP) than its inner surface. Most of the granular cytoplasm has been removed by osmotic lysis resulting in an empty appearing cytoplasmic region. Arrows in the lower right corners of all micrographs show the direction of carbon–platinum shadowing. × 59,000.

gen temperature, the pellet was cleaved and replicated in the same manner as described for whole cells.

Results. The type II Bullivant–Ames apparatus allows for the production of higher quality replicas than was attained in earlier efforts with freeze-cleaved red cells using a type I apparatus(1). Evaluation of these improved replicas, (Figs. 1, 2, and 3) shows more structural detail on membrane surfaces. Cell membrane-associated particles appear more discrete than in previously described replicas and it readily becomes apparent that the inner (juxtacytoplasmic) surface of the red cell membranes and ghosts (Fig. 1) are covered with fewer particles than outer surfaces. Because replicas made with the type II apparatus are thinner and made under conditions of reduced platinum and carbon scattering, replicas of individual particles appear smaller and more uniform in size than those originally reported with the type I apparatus. In an earlier description of the particles on red cell membranes, it was stated that replicas of individual particles ranged in size from 120 to 210 Å in diameter as measured in a plane tangential to the membrane surface (1). In these higher quality replicas, the particles have the same pattern of distribution but now measure from 80 to 130 Å in diameter, averaging approximately 100 Å in diameter. As seen in these replicas, particles are quite flat and are estimated to protrude, on the average, less than 50 Å above the surrounding membrane surface.

When the fracture plane passes directly from the extracellular compartment into the cytoplasm, the cell membrane can now be resolved in cross section although this was not apparent in poorer quality replicas(1). The inner edge of the membrane is still difficult to identify in replicas of whole cells with flat fractures but can be readily identified in replicas of red cell ghosts where most of the cell's granular cytoplasm has been removed by osmotic lysis. Membranes and ghosts in cross section appear thin, measuring less than 100 Å in thickness as seen in replicas. At irregular intervals along these membrane cross sections, there are round to cylindrical particles that appear to be continuous through the entire thickness of the membrane, protruding into both the cytoplasmic region, facing the inner surface of the membrane, and into the extracellular compartment, facing the membrane outer surface (Figs. 2 and 3). When a flat fracture plane passes directly from the extracellular medium into the cytoplasm, these particles may appear as focal thickenings in the thin membrane, giving the membrane edge, in cross section, a beaded appearance. When the fracture plane is first deviated for some distance by the cell membrane before breaking through the membrane into the cytoplasmic region, the relation of the penetrating membrane-associated particles to the membrane in cross section is better appreciated. These particles pass through a discontinuity in the surrounding sheet-like membrane. It is impossible to know what percentage of the membrane-associated particles represent the poles of such penetrating units since penetration is only ascertained when fortuitous frac-



FIGS. 2 and 3. Photomicrographs show replicas of small areas of red cell ghost membranes. The fracture plane has passed from the extracellular space (*bottom*) and has been deviated along the membrane outer surface which is partially covered with cell membrane-associated particles (CMAP). The fracture then breaks through the membrane (*short single arrows*) into the cytoplasmic region. Where the fracture passes through the membrane, cylindrical particles (Fig. 2, *encircled long arrow*; Fig. 3, *long arrow*) are revealed which appear to extend across the entire thickness of the membrane as seen in cross section. The poles of these cylindrical particles protrude into the adjacent compartments. In Fig. 3, the fracture continues along the inner surface of the ghost membrane and then breaks through the membrane (*double arrow*) into the extracellular compartment (*top*). Figs. 2 and 3, × 120,000.

tures happen to cut the membrane in cross section at the level of such a penetrating particle. However, it is obvious that all membrane-associated particles can not penetrate through the membrane since there are many more particles on the outer surface than on the inner surface of the membrane. The percentage of particles on inner surfaces which penetrate is unknown.

The precise dimensions of these membrane penetrating particles is impossible to determine since replicas magnify dimensions due to the added thickness of the replicas themselves. However, maximum dimensions can be estimated because actual dimensions will always be smaller. When cell membrane-associated particles are measured along the surface of the membrane, they measure up to 130 Å in diameter. Within the membrane itself, their apparent maximum cross-sectional diameter may be slightly larger, measuring up to 150 Å in diameter. Along their long axis (perpendicular to the plane of the membrane), most of the particles are judged to measure in the 200–250 Å range, although particles up to 290 Å in length have been observed.

Discussion. By means of the freeze-cleave technique, it is possible to examine large areas of outer and inner surfaces of red cell membranes as well as the membrane in cross section, when fortuitous fractures pass at right angles through frozen membranes or membrane ghosts. As seen in these replicas, some membrane-associated particles appear to penetrate through the entire thickness of the red cell plasma membrane. The vast majority of the surface area of the membrane appears as a thin sheet with finely granular or fibrillar surfaces except for the local discontinuities where particles penetrate through the entire thickness of the membrane. There are an estimated four to five times as many particles on outer sur-

faces of membranes and ghosts as compared to the inner surfaces, so that the maximum number of penetrating particles would possibly be reflected by the number of particles on the inner surface, although there is no way of knowing that *all* particles on inner surfaces penetrate through the membrane. Identification of penetrating particles is dependent on favorable breaks through the membrane precisely at the level of a penetrating particle. Since particles cover less than 5% of the total inner surface area of membranes, the number of particles that might be exposed in such breaks would be expected to be very small, as is the case. Sampling is further limited by the fact that only particles shadowed at certain angles can be correctly interpreted as passing through the membrane.

Models of plasma membrane incorporating specialized sites to account for some functions have heretofore been supported by indirect evidence(11,12,13) while direct evidence obtained by thin section electron microscopy techniques has usually been interpreted in terms of a continuous bimolecular lipid leaflet sandwiched between thin protein layers(14). Observations on freeze-cleaved membranes in this study now provide electron microscopic evidence that the red cell plasma membrane is not a continuous uniform thin sheet, but rather is discontinuous where membrane-associated particles pass through its entire thickness.

Previous failure to demonstrate focal structural specialization of red cell membranes may possibly be attributed to the limitations of the techniques used. Thin section techniques, for example, impose severe limitations on sampling and require many artifact producing steps such as chemical fixation of cells with killing agents and subsequent dehydration with organic solvents. Negative stain techniques are capable of demonstrating some structures but require that the specimen be dried in the presence of increasingly concentrated salt solution before examination in the electron microscope. Although the freeze-cleave technique also has its limitations, our ability to demonstrate structures penetrating through membranes by means of this approach may be partly due to the elimination of some traditional artifact producing steps in this study.

Summary. Replicas of freeze-cleaved and replicated red cells produced with an improved Bullivant–Ames apparatus show the plasma membrane to be thin and sheet-like with its inner and outer surface partially covered with a small particulate component. In cross section, some of these particles appear to extend through the full thickness of the membrane. Discontinuities in the thin sheet-like membrane accommodate these penetrating particles. These findings give morphologic evidence of structural specialization of the plasma membrane.

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