Periodicity in the Cytoplasm of Freeze-Cleaved Sheep Erythrocytes.* (32007)

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Individual hemoglobin molecules are not visualized in conventional thin sections of red blood cells, as seen in the electron microscope, although the known dimensions of these molecules indicate that they are well within the microscope's resolution limit. Recent studies with freeze-cleave and replication techniques have, however, demonstrated the cytoplasm of these cells to be filled with small particles, some of which may represent replicas of individual hemoglobin molecules(1,2). Bradley found in carbon-platinum replicas of fracture faces of cleaved crystalline sucrose what he interpreted as a form of molecular organization(3,4). In this study, fracture faces of frozen, cleaved sheep red blood cells were examined in an effort to find evidence of molecular packing in the cytoplasm in these cells.

Materials and methods. Blood samples were drawn by venipuncture from adult Shropshire sheep, collected in an equal volume of Alsevers solution(5), and centrifuged. The packed cells were rinsed in isotonic phosphate buffer and saline, and passed stepwise through graded glycerol solutions containing phosphate buffer(2). Several drops of packed, glycerinated red cells were placed in the well of a brass block, as described elsewhere in detail by Bullivant and Ames(6). In order to freeze the cells rapidly, the brass block was submerged in liquid nitrogen (—196°C). The portion of the drop of blood protruding above the mouth of the well was then planed off (cleaved) with a precooled razor blade, thus exposing a freshly fractured surface for replication. The brass block was then covered with a tight fitting brass lid and, still under a protecting layer of liquid nitrogen, carried to a Kinney evaporator (PW 400). Following exication of the evaporator bell jar to a pressure of 10^{-5} torr or less, the specimen lid was lifted with an externally controlled electric crane. A carbon replica was made of the cleaved surface after shadowing with a platinum-carbon pellet evaporated at a 45° angle to the cleaved surface. Following replication, the specimen was removed from the evaporator and allowed to melt. Then, the specimen and the overlying replica were placed either in hot nitric acid or strong household bleach where the blood dissolved. Fragments of replica were then rinsed several times in distilled water, picked up on copper grids and examined in a Siemens Elmiskop I electron microscope.

Results and discussion. The fracture through the packed frozen red blood cells often passed directly through the cell cytoplasm as a flat cleavage plane, exposing large areas for replication. The replicas of these areas typically reveal a pattern of packed granularity with each particle appearing fairly uniform in size (approximately 120A° in diameter). As has been described elsewhere for mouse and human erythrocytes(1,2), these particles in sheep erythrocytes appear to fill the cytoplasm without evidence of organization in large areas (Fig. 1). However, in sheep red blood cells, individual particles are occasionally ordered, forming long linear arrays. These arrays, made up of many particulate subunits, may be either straight lines (Fig. 4) or curved (Fig. 3). Typically, these individual lines are parallel to one another, and the parallel rows are packed together forming extensive patterns which may fill much of the cytoplasm in a single fracture face of a sheep erythrocyte. The lines always appear oriented in relation to one another within a local area and never appear randomly distributed in the cytoplasm. However, several areas, consisting of many parallel lines each, may co-exist in a fracture face of a single erythrocyte, with the individual groups of lines oriented in different

* This investigation was supported in part by USPHS Grant 5 T01 GM 00192-08 and CA-07368-03 from Nat. Inst. Health.

directions with respect to one another. Areas adjacent to the parallel lines show a random granularity and there is no evidence of a membrane separating the areas of organization from the areas of randomness (Fig. 4). The lines tend to be peripheral in distribution although they may extend into the center of the cell.

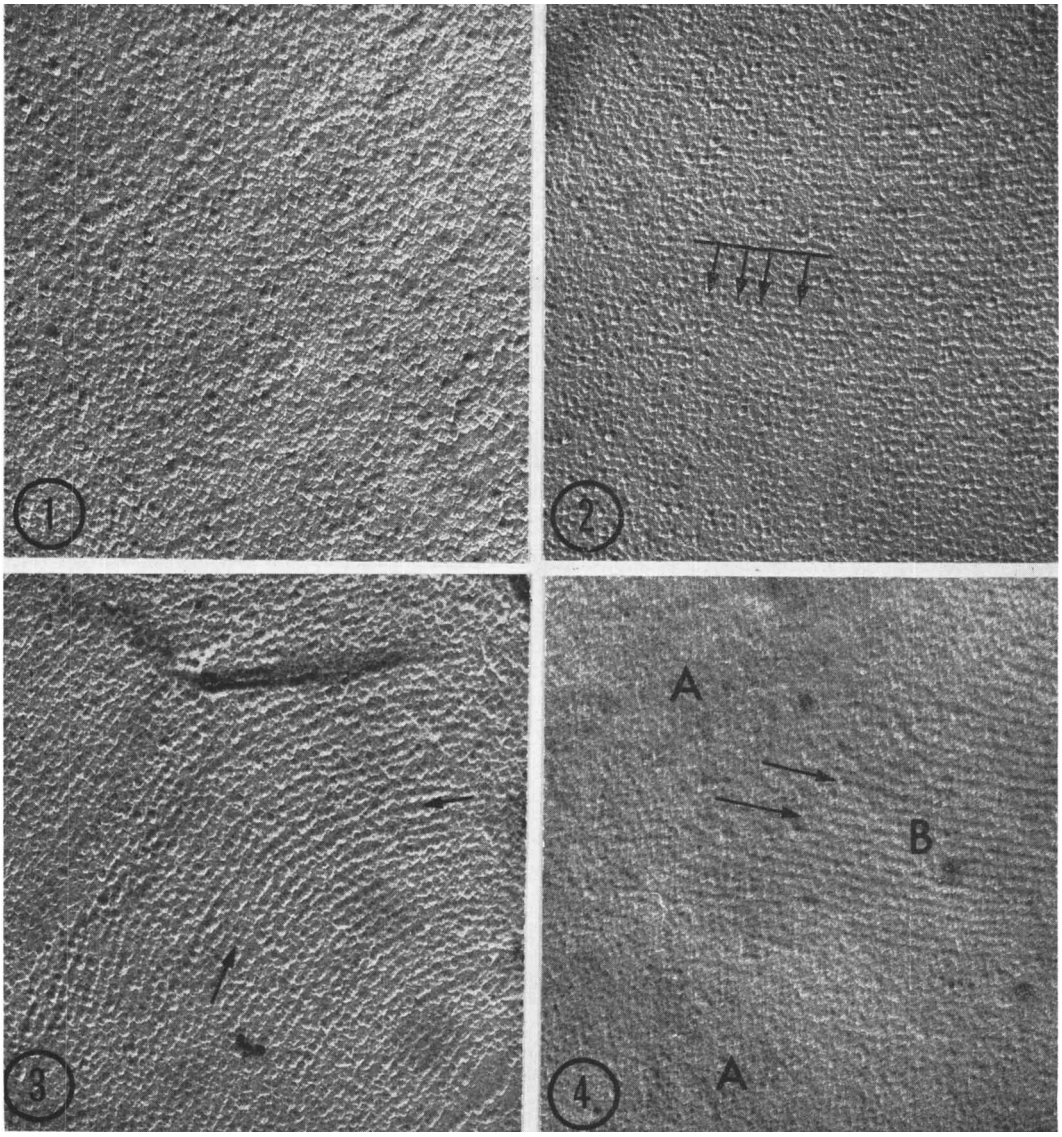

FIG. 1. Carbon-platinum replica of a fracture face produced by a flat plane of cleavage passing through the cytoplasm of a frozen sheep erythrocyte. The cytoplasm appears to be filled with randomly distributed small particles. There is no evidence of ice crystal formation. × 56,000.

FIG. 2. Replica of a sheep erythrocyte showing extensive intracytoplasmic patterning. Individual particles (*arrows*) form long linear arrays. These lines are parallel to one another and appear tightly packed. × 67,000.

FIG. 3. Replica revealing packed curved lines (*arrows*) made up on individual particulate subunits. The micrograph is printed as a positive so that particles appear dark and shadows produced by platinum shadowing appear white. × 66,000.

FIG. 4. One region (*area B*) in this replica shows many parallel straight lines surrounded by cytoplasm (*area A*) filled with randomly distributed small particles. × 50,000.

Individual hemoglobin molecules in human erythrocytes are spheroid and measure approximately 64A° in their greatest dimension (7), and sheep hemoglobin is probably of comparable size. Replicas of individual molecules would be larger than the molecules being replicated due to the added thickness of the replica and might correspond to the size of the individual particles seen in these fracture faces of sheep red blood cells. The extensive areas of patterning seen in the cytoplasm of many cleaved sheep red blood cells could represent areas of molecular packing. It is postulated, on the basis of the appearance of these replicas, that the fracture plane passes through certain areas revealing monomolecular steps which appear as parallel lines. In earlier studies on fractured erythrocyte cytoplasm, it was stressed that the random appearing granularity, as seen in replicas of mouse and human erythrocyte cytoplasm, could possibly represent a contaminant adsorbed from the evaporator chamber onto the freshly cleaved cold surface of the tissue prior to replication and that these contaminants could conceivably be replicated(2). However, it seems unlikely that the extensive patterning seen in the cytoplasm of these sheep erythrocytes could represent a random contaminant. It is suggested that these areas of periodicity represent areas of molecular organization within the sheep cell cytoplasm. The biological significance of these patterns is unknown. The packing, *per se,* may have been induced by collection, glycerination, or freezing procedures, or may represent a kind of organization present in areas in the cytoplasm of these sheep erythrocytes in *in vivo* conditions.

Summary. Sheep red blood cells have been freeze-cleaved and the exposed fracture faces have been replicated with carbon, shadowed at a 45° angle with carbon-platinum, and examined in the electron microscope. The cytoplasm appears to be filled with tightly packed particles, the replicas of which measure approximately 120A° in diameter. In large areas, these particles occasionally form extended linear configurations which may be straight or curved. Typically, large numbers of these lines are parallel to one another. It is suggested that the lines in these replicas may represent areas of molecular packing within the cytoplasm of sheep erythrocytes. The possibility that the individual particles may represent a contaminant adsorbed onto the cold freshly cleaved surface prior to replication or that the orderly pattern seen in replicas may be an artifact introduced during glycerination, freezing, or cleaving cannot be entirely eliminated by this data.

The authors thank Mrs. Marilyn Schaefer for typing the manuscript and Miss Joyce Jakobsen for preparing the illustrations.

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Received December 16, 1966. P.S.E.B.M., 1967, v125.