STRUCTURE OF MEMBRANE HOLES IN OSMOTIC AND SAPONIN HEMOLYSIS

P. SEEMAN, D. CHENG, and G. H. ILES

From the Department of Pharmacology and the Institute of Biomedical Electronics, University of Toronto, Toronto 5, Ontario, Canada. Dr. Cheng's present address is The Rockefeller University, New York 10021.

ABSTRACT

Serial section electron microscopy of hemolysing erythrocytes (fixed at 12 s after the onset of osmotic hemolysis) revealed long slits and holes in the membrane, extending to around 1 μm in length. Many but not all of the slits and holes (about 100–1000 Å wide) were confluent with one another. Ferritin and colloidal gold (added after fixation) only permeated those cells containing membrane defects. No such large holes or slits were seen in saponin-treated erythrocytes, and the membrane was highly invaginated, giving the ghost a scalloped outline. Freeze-etch electron microscopy of saponin-treated membranes revealed 40–50 Å-wide pits in the extracellular surface of the membrane. If these pits represent regions from which cholesterol was extracted, then cholesterol is uniformly distributed over the entire erythrocyte membrane.

INTRODUCTION

It is known that membrane holes or defects appear in the erythrocyte membrane about 10 s after the onset of rapid osmotic hemolysis (1). As seen by electron microscopy these holes are approximately 200–500 Å wide (1) and can permit passage through the membrane of various macromolecules including colloidal gold (1), ferritin (1–5), dextrans (6, 7), catalase (8), albumin (7, 9), and foreign hemoglobin (10, 11). These membrane holes are only transiently patent since the membrane spontaneously reseals itself about 25–250 s after the onset of osmotic hemolysis (1).

The membrane holes tend to cluster in a region of about 1 μm in diameter (reference 1), in agreement with light microscope findings that hemoglobin exits via 1–2 μm-wide streams (12–14); freeze-fracture electron microscopy also indicates 1 μm-wide openings in the membrane (15).

The studies presented in this paper were done to find out the three-dimensional pattern of the membrane holes. The purpose was to determine whether the membrane holes were or were not connected to one another. By means of serial section electron microscopy it was found that most but not all of the membrane defects were confluent. Membrane holes produced by saponin were not confluent.

METHODS

The Fixation of the Transient Holes in the Open Position

The procedure was exactly as that used in reference 1. An aliquot of 0.04 ml of whole fresh blood (human; heparinized with 100 IU per ml of blood) was pipetted into the bottom of a test tube and 1 ml of 25 mM NaCl (in 10 mM sodium phosphate buffer, pH 7) was added to start hypotonic hemolysis. After 12 s, 3 ml of 2% glutaraldehyde (in 0.1 M sodium cacodylate-HCl buffer, pH 7.2) were added rapidly while mixing on a Vortex mixer (Scientific

Industries, Inc., Springfield, Mass.). The fixed cells were then centrifuged (10,300 *g* for 10 min at 18°C) into a pellet which was twice washed in Michaelis buffer (16). The cells were finally resuspended in 0.5 ml of ferritin or ferritin plus gold in 154 mM NaCl for 30 min (the ferritin solutions had been previously dialyzed overnight in 154 mM NaCl, 10 mM sodium phosphate buffer, pH 7). The cells were finally centrifuged into a pellet once more and 2% OsO_4 (Palade's fixative; reference 16) was added for 1 h. As explained previously (1), after glutaraldehyde fixation the 1-h period of OsO_4 fixation did not cause any artifactual translocation of ferritin or gold particles across the membrane. The pellets were gently broken into 1-mm cubes and were treated for 1 h in 0.5% uranyl acetate in Veronal-acetate-HCl buffer (17) to enhance the contrast of the membrane. The pellets were dehydrated in 100% ethanol, passed through propylene oxide, and embedded in Epon 812 (reference 17).

Horse spleen ferritin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, as cadmium-free, twice-crystallized ferritin. Colloidal gold suspension was from Abbott Laboratories, North Chicago, Ill. The stock solutions of gold and ferritin were dialyzed for 48–72 h against a dialysing medium about 40 times the volume of the dialysate; the medium was changed every 12 h. The composition of the medium was 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7.

Serial Section Electron Microscopy

Silver-grey-colored serial sections of the Epon-embedded tissue were cut with a Dupont diamond knife on an automatic MT-2 Porter-Blum microtome. The ribbon of serial sections was picked up on Formvar-coated copper single-slot grids. The grid was almost completely immersed in the knife-boat water and, after guiding the end of the ribbon to touch the edge of the grid, the grid was slowly pulled out of the water. The sections were stained with uranyl and lead (17). The lead citrate stain caused considerable loss in contrast to the ferritin particles. Higher contrast in ferritin was obtained with the Karnovsky stain (18). The sections were photographed in a Philips EM 300 at an original magnification of 31,900.

Hemolysis by Saponin

An aliquot (0.04 ml) of heparinized whole blood was added to 1 ml of 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7, and the cells were lysed by the addition of 30 μg of holothurin A (in 0.05 ml of 154 mM NaCl). Holothurin A is a saponin purified from the sea cucumber, *Actinopyga agassizi*, and was generously donated by Dr. J. D. Chanley, the Mount Sinai Hospital, New York. Around 5–6 min after the addition of the saponin the turbid erythrocyte suspension cleared. 10 min after clearing, 3 ml of 2% glutaraldehyde (pH 7, 0.1 M cacodylate buffer) were added for 5 min and the cells were centrifuged at 10,300 *g* for 14 min. The pellets were washed twice in 154 mM NaCl, pH 7, (without resuspending the cells), and the cells were finally resuspended in 0.5 ml of ferritin in 154 mM NaCl, pH 7, for 5 min. The cells were then fixed in Palade's fixative (16). The Epon-embedded sections were photographed using the Hitachi HS-7S and the Siemens Elmiskop I electron microscopes.

Freeze-Etch Procedure

The saponin-treated erythrocyte ghosts were freeze-etched using a Type II simple freeze-cleave device (19, 20). The membranes were not suspended in any cryoprotective solvents before freezing in Freon 22. Complete details on the freeze-etch procedure are described elsewhere (21). The platinum/carbon replicas were approximately 33–55 Å thick (as monitored by a "Talystep" device; see reference 21) and were photographed in a Philips EM 300 electron microscope.

RESULTS

Structure of Membrane Holes in Osmotic Hemolysis

Fig. 1 shows eleven continuous serial sections of two erythrocytes which had been fixed 12 s after the onset of osmotic hemolysis. The low magnification of Fig. 1 gives a general survey of the field chosen for detailed study.

A higher magnification of Fig. 1 f is shown in Fig. 2 where it can be seen that the erythrocyte on the right contains membrane holes as well as numerous ferritin and gold particles. The erythrocyte on the upper left does not contain any particles and the membrane is continuously sealed. The individual colloidal gold particles are readily identified by their sharp circular profile (25–300 Å in diameter) and their extreme electron opacity (e.g. particle outside cell in lower left-hand corner of Fig. 2). The ferritin particles are observed as circular, pale-grey, 90 Å-wide densities, and are very numerous in Fig. 2. Ferritin is 122 Å in diameter (22) but the iron core is about 90 Å wide and does not have any substructure (23–27); the protein shell, however, may have substructure (28, 29).

By enlarging the micrographs of Fig. 1 and constructing a lucite scale model of the cells

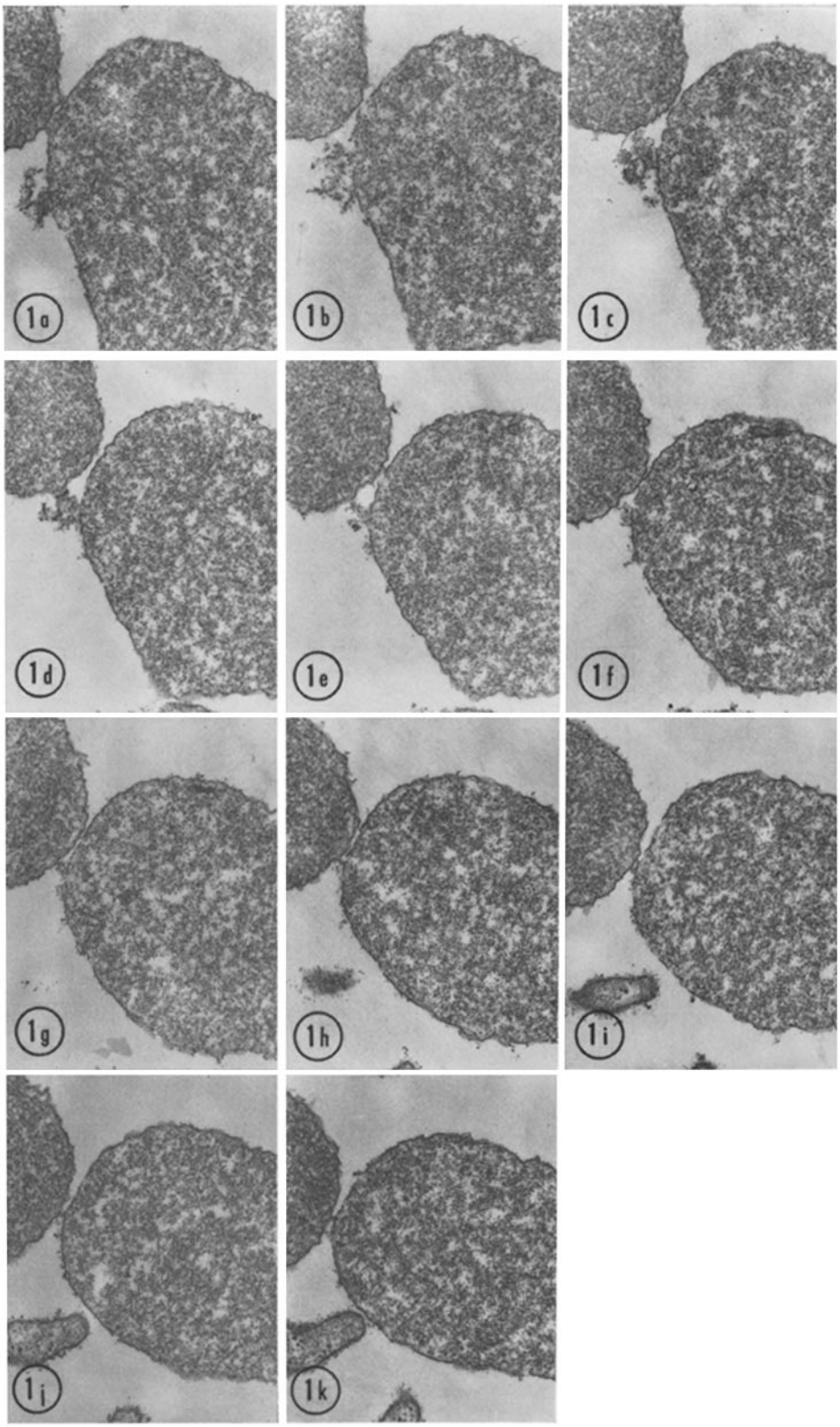

FIGURE 1 Serial sections of human erythrocytes which had been fixed in glutaraldehyde 12 s after the addition of hypotonic solution. The glutaraldehyde-fixed cells were subsequently incubated in a solution containing ferritin and colloidal gold. Each section is approximately 500–600 Å thick (silver-grey). A lucite scale model was constructed from enlargements of these micrographs in order to align the micrographs for Fig. 3. × 19,100.

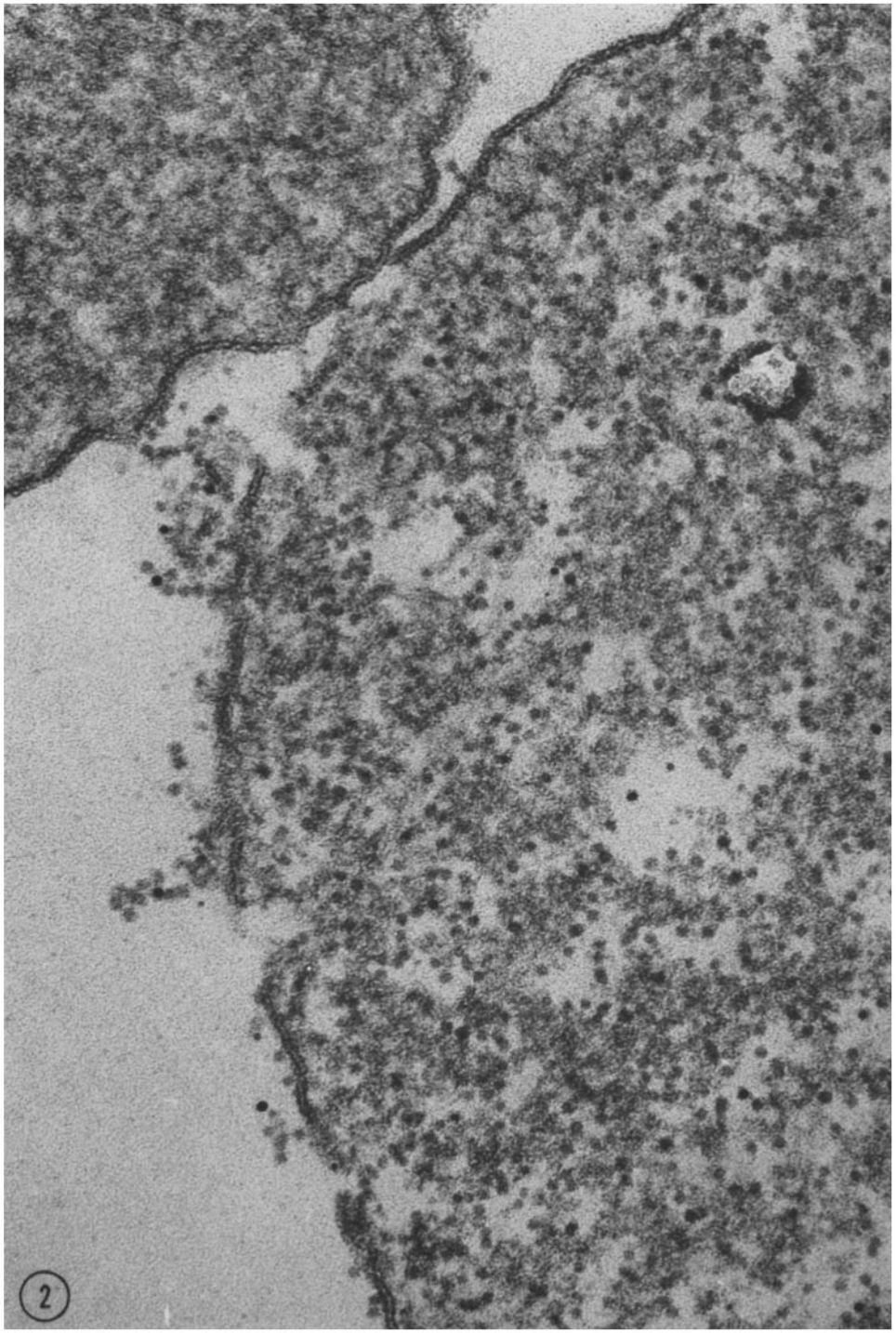

FIGURE 2 This micrograph is an enlargement of Fig. 1 f. The ferritin particles are seen as numerous grey 90 Å-wide dots. Colloidal gold particles are very electron-opaque, circular, and ranging from 25 to 300 Å in diameter. The erythrocyte membrane on the right reveals membrane holes and the cell contains abundant ferritin particles. The cell in the upper left hand corner has no membrane defects and is free of ferritin particles. × 140,000.

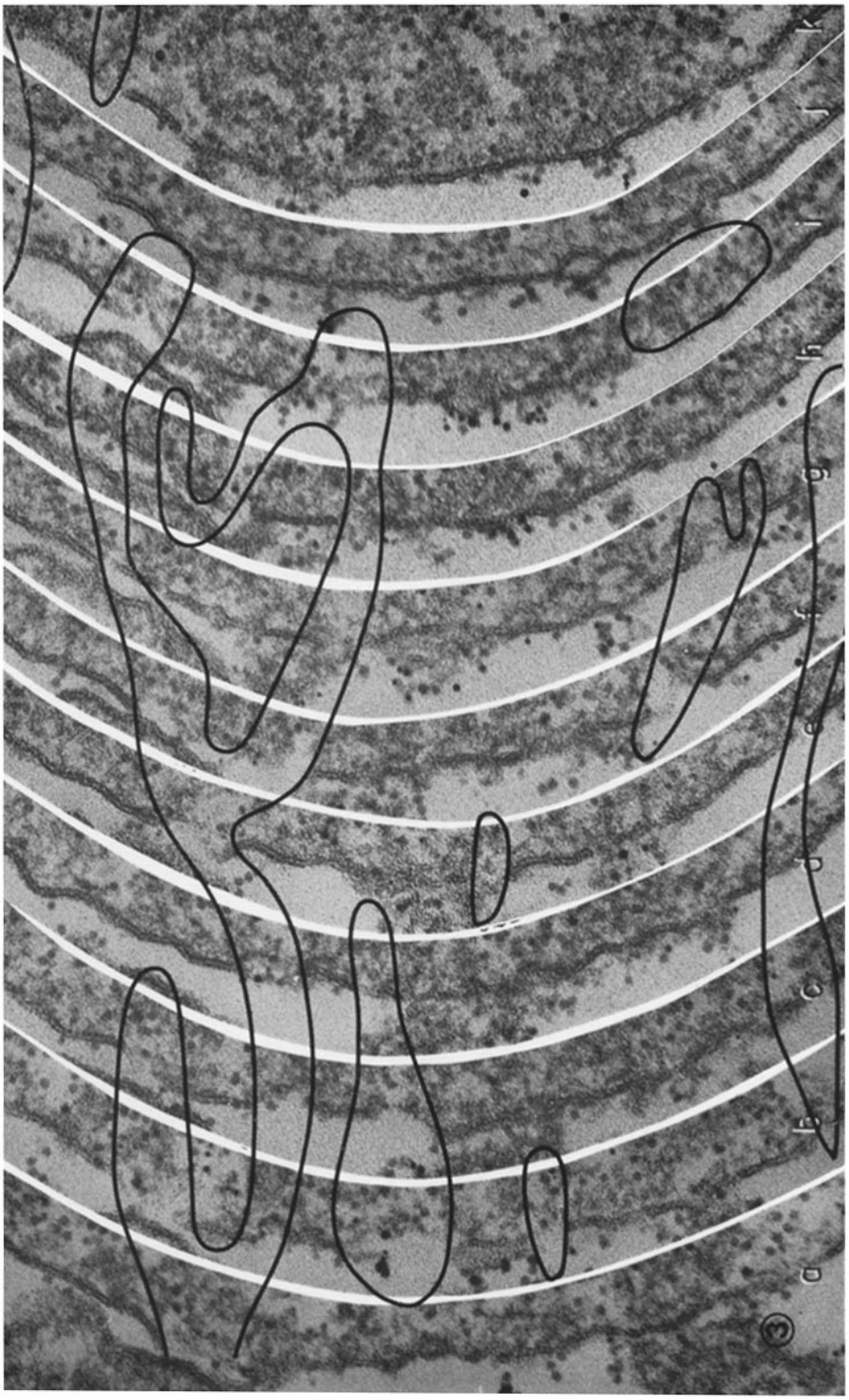

FIGURE 3 Illustrating the confluence of membrane defects, using enlarged and aligned micrographs from Fig. 1. Despite the margin of uncertainty in alignment (approximately 200–500 A), it appears that not all the membrane holes (or slits) are confluent with one another. × 111,000.

and portions of cells in Fig. 1, it was possible to align the micrographs of Fig. 1 beside each other. The aligned result is shown in Fig. 3 where it can be readily seen that many if not most of the membrane holes or defects are adjacent to one another from section to section. Each section is approximately 500–600 Å thick (silver-grey). The black lines drawn in Fig. 3 illustrate one way of drawing the pattern of confluence between these membrane defects. Although there are obviously many ways to draw the pattern of confluence, it seems unlikely that the pattern of membrane slits toward the bottom of Fig. 3 is confluent with the pattern of slits toward the top of Fig. 3. The margin of uncertainty in aligning these micrographs is between 200–500 Å in Fig. 3.

About 15 sets of serial sections of different erythrocyte membrane holes were photographed. The results presented in Figs. 1–3 are typical of all the other findings.

Structure of Membrane Holes in Saponin Hemolysis

The membrane defects just described for osmotic hemolysis disappear about 25–250 s after the onset of osmotic hemolysis (1). The membrane holes or defects produced by saponin, however, were long-lasting and presumably permanent. This is shown in Fig. 4 where it can be seen that ferritin is found on both sides of the cell membrane of ghosts which had been exposed to saponin for 10 min before fixation in glutaraldehyde and subsequently exposed to ferritin. Fig. 4 illustrates the scalloping of the membrane produced by saponin (the top and bottom of Fig. 4 a are the cytoplasmic spaces of two hemolysed erythrocytes). Fig. 5 shows the freeze-etch appearance of these cells at a final magnification of 124,000. The bottom quarter of Fig. 5 shows the extracellular water which has been frozen into ice and which has not been etched away in the vacuum. The middle part of the micrograph reveals the etched extracellular aspect of the cell membrane, in accordance with the interpretations given by others (30, 31). There are numerous pits in this extracellular surface of the membrane, each pit of the order of 40–50 Å in diameter. These pits were never seen in the absence of saponin. The top quarter of Fig. 5 reveals the cleavage plane of the membrane, exhibiting the 90 Å-wide globules residing in the intracellular leaflet of the cell membrane (see 30, 31, 21 for references and interpretations). It is difficult to discern whether there are also pits between the globules in the cleavage plane; this is because the cleavage plane is very irregular in the presence of saponin. The scalloped shape of the saponin-treated ghost was only seen by freeze-etching when the etching was very deep. The invaginations giving the scalloped outline were filled with extracellular ice which sublimed after long etching times; Fig. 5 illustrates a region of the erythrocyte membrane which is apparently between the scalloped invaginations.

DISCUSSION

The results in Figs. 1–3 indicate that the structure of the membrane holes during hypotonic hemolysis is that resembling slits or longitudinal tears in the membrane. The cells in Figs. 1–3 were fixed at 12 s which is the time when the majority of the erythrocytes are starting to open and release hemoglobin. It is also possible that the pattern of holes or slits shown in Fig. 3 represents the pattern just before the membrane seals over.

The entire area of these holes extends over the membrane surface by an order of 1 μm or so. This confined region of holes tends to suggest that the membrane may rupture as a result of a forceful blowout, rather than from a more gentle phase transformation within the membrane (see reference 32).

Since the erythrocyte ghost is a perfect osmometer (33), these membrane holes must seal over very tightly. Presumably the well-known fluidity of the membrane makes this possible (see references in 34, 35).

The 40–50 Å-wide pits in the saponin-treated membranes (Fig. 5) are smaller than the 80–90 Å-wide "holes" that are known to exist in artificial mixtures of saponin and cholesterol (36–40). This difference in dimensions suggests that the etch surface may reveal something different from a cholesterol-saponin mixture. The pits cannot be the channels by which ferritin enters the cell since ferritin is about 115 Å wide. The ferritin presumably enters via larger defects in the membrane (e.g. bottom of invaginations in Fig. 4 a).

An important finding concerning the saponin results is that the pitting on the extracellular surface was seen all over the entire surface of the erythrocyte membrane. *If* these pits represent regions in the membrane from which cholesterol

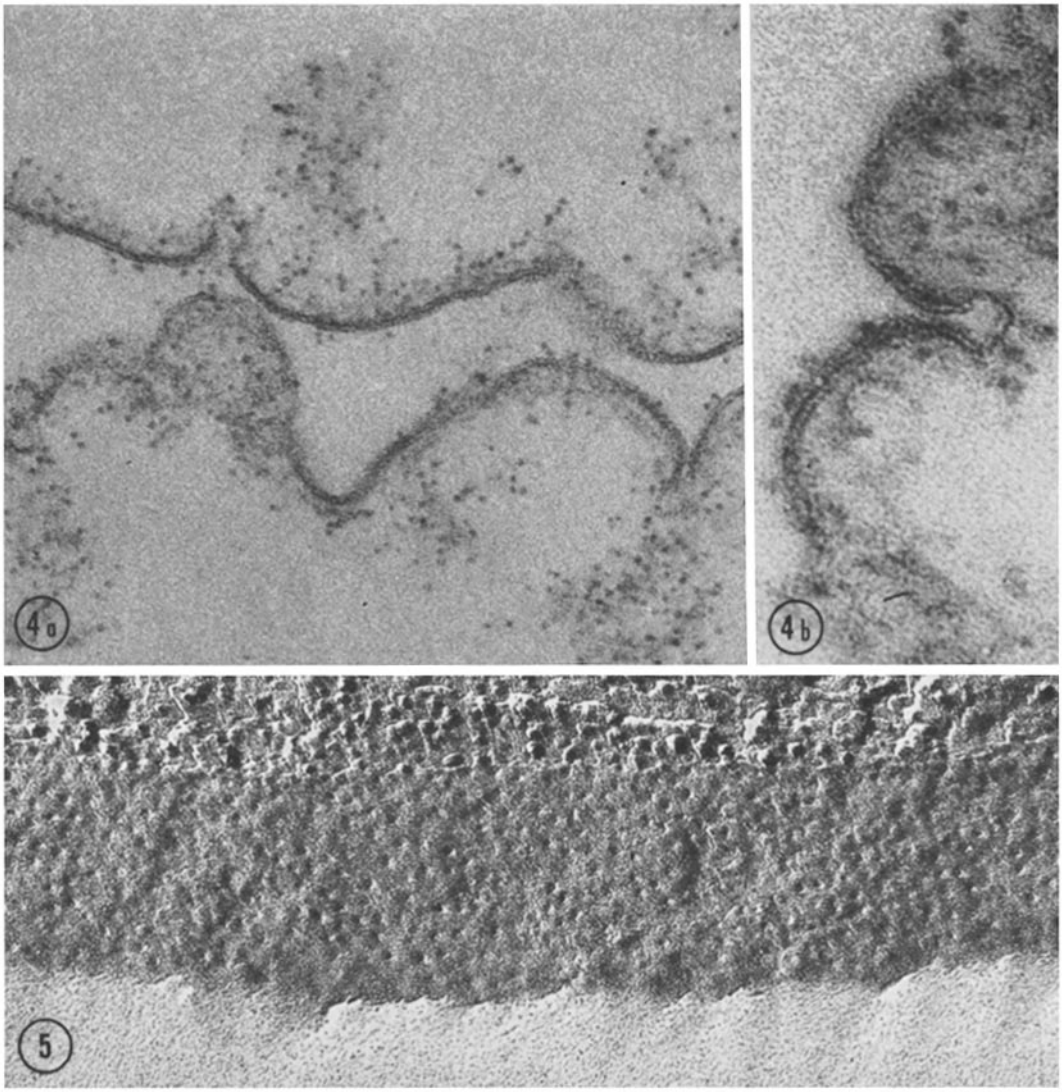

FIGURE 4 The effect of saponin on erythrocytes. The ghost membranes are scallop shaped (Fig. 4 a) with deep invaginations (Fig. 4 b). Ferritin particles, added to the glutaraldehyde-fixed ghosts, permeated into the cytoplasmic spaces. Fig. 4 a, × 124,000; Fig. 4 b, × 252,000.

FIGURE 5 Freeze-etch micrograph of the saponin-treated erythrocytes. The bottom part shows the ice, the central part reveals the etched extracellular aspect of the membrane with many small pits, while the cleavage plane reveals the membrane globules. The pits may be from where the cholesterol was extracted; such pits were seen over the entire surface of the erythrocyte, suggesting a uniform distribution of cholesterol. × 124,000.

had been extracted, this implies that cholesterol is found uniformly distributed over the entire membrane surface. Such a conclusion would be the opposite of that of Murphy's (41) wherein it was claimed that radio-cholesterol was localized as a circumferential belt or band around the erythrocyte's disc edge, and that this preferential localization explained the biconcave shape of the erythrocyte. However, since the biconcave nature of the erythrocyte is satisfactorily explained

by Canham's hypothesis of minimum bending energy (42), and since the membrane has considerable fluidity (34, 35), it appears more reasonable to conclude in the light of Fig. 5 that the distribution of cholesterol is more or less uniform over the erythrocyte membrane.

The work of Danon (13), wherein air-dried specimens were used, revealed a large circular defect in the membrane of the order of 1 μm in diameter. The ghosts observed by Danon, however, were air dried for electron microscopy many hours after osmotic hemolysis, and after extensive washing (and rehemolysis?) to remove hemoglobin. Since the membrane holes in osmotic hemolysis only last a few minutes at most, the membrane lesion observed by Danon may be of a different type than that under study here. The freeze-etch results of Huhn et al. (15) indicate that there is a membrane lesion about 1 μm wide during hypotonic hemolysis. Thin sections of glutaraldehyde-fixed cells are preferable, however, for detecting whether small pieces of membrane overlay such a wide opening; the streams of hemoglobin in the freeze-etch procedure tend to obstruct such small pieces of membrane. A second advantage is that by using ferritin it is possible to check whether the cell is physiologically patent while concomitantly locating the anatomical lesions; the ferritin particles cannot readily be distinguished from hemoglobin particles in freeze-etch microscopy. A third advantage is that it is possible to survey in detail broad regions of membrane; in freeze-etch microscopy one must select cells which have been fractured at right angle across the membrane, and this limits the amount of membrane area that can be examined in the region of the lesion.

It is possible that the glutaraldehyde used to fix the hemolysing erythrocytes caused osmotic shrinkage of the cells. There is no reason to believe, however, that glutaraldehyde altered the *structural continuity* of the erythrocyte membrane (i.e. causing membrane fusions or membrane disruptions) because (*a*) artifactual translocation of ferritin does not occur in cells fixed by glutaraldehyde (reference 1; reference 32 in reference 1); (*b*) the time course of the development of membrane holes and the sealing of these holes is approximately the same whether measured by adding hypertonic NaCl solutions at various times during hemolysis (subsequently followed a few minutes later by glutaraldehyde) or measured by adding glutaraldehyde directly during the course of hemolysis (see reference 1 for details); (*c*) glutaraldehyde does not alter the edge-to-edge thickness of 70–72 Å of the cell membrane (cf. references 22–25 in reference 1 with freeze-etch references in reference 30); (*d*) glutaraldehyde does not disrupt the membrane, since erythrocyte membrane defects were never found in glutaraldehyde-fixed erythrocytes or ghosts *which were devoid of ferritin* (reference 1); (*e*) glutaraldehyde does not appear to promote or alter membrane-membrane fusion, since identical membrane ultrastructure has been observed in nerve terminals (where presynaptic vesicle membranes fuse with the prejunctional neurolemma) whether observed by thin section of glutaraldehyde-fixed material or by freeze-etch microscopy (see reference 43); (*f*) the freeze-etch ultrastructure image of the erythrocyte membrane is invariably identical whether or not glutaraldehyde has been used, as numerous experiments in this laboratory and in other laboratories have shown (44).

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