TRANSIENT HOLES IN THE ERYTHROCYTE MEMBRANE DURING HYPOTONIC HEMOLYSIS AND STABLE HOLES IN THE MEMBRANE AFTER LYSIS BY SAPONIN AND LYSOLECITHIN

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ABSTRACT

Ferritin and colloidal gold were found to permeate human erythrocytes during rapid or gradual hypotonic hemolysis. Only hemolysed cells contained these particles; adjacent intact cells did not contain the tracers. Ferritin or gold added 3 min after the onset of hypotonic hemolysis did not permeate the ghost cells which had, therefore, become transiently permeable. By adding ferritin at various times after the onset of hemolysis, it was determined that for the majority of the cells the permeable state (or interval between the time of development and closure of membrane holes) existed only from about 15 to 25 sec after the onset of hemolysis. It was possible to fix the transient "holes" in the open position by adding glutaraldehyde only between 10 and 20 sec after the onset of hemolysis. The existence of such fixed holes was shown by the cell entry of ferritin and gold which were added to these prefixed cells. Membrane defects or discontinuities (of the order of 200–500 A wide) were observed only in prefixed cells which were permeated by ferritin subsequently added. Adjacent prefixed cells which did not become permeated by added ferritin did not reveal any membrane discontinuities. Glutaraldehyde does not per se induce or create such membrane defects since cells which had been fixed by glutaraldehyde before the 10-sec time point or after the 180-sec time point were never permeable to added ferritin, and the cell membranes never contained any defects. It was also observed that early in hemolysis (7–12 sec) a small bulge in one zone of the membrane often occurred. Ghost cells produced by holothurin A (a saponin) and fixed by glutaraldehyde became permeated by ferritin subsequently added, but no membrane discontinuities were seen. Ghosts produced by lysolecithin and fixed by glutaraldehyde also became permeated by subsequently added ferritin, and many membrane defects were seen here (about 300 A wide).

INTRODUCTION

The work in this paper is concerned with the disruption and repair of the erythrocyte cell membrane during hypotonic hemolysis and with the time course of these events. The ultrastructure of the membrane defects involved in osmotic hemolysis and in the hemolysis induced by two surface-active agents, lysolecithin and holothurin A, is also described.

HYPOTONIC HEMOLYSIS: After hypotonic hemolysis the cell subsists as a distinct cellular corpuscle (1) and retains osmotic properties characteristic of semipermeable membranes (2–4). The

experiments of Teorell (4) and of Hoffman (5) demonstrated that erythrocytes hemolysed in 5 parts water (such that the final concentration of the hemolysate was 20 mM Na and K; Teorell, reference 4) or in 9 parts of 18 mM NaCl (5) spontaneously recover their low permeability to Na, K, glucose, and sucrose.

While the *untreated* ghost can be impermeable to low molecular weight substances, it is known that during the course of hypotonic hemolysis the erythrocyte becomes highly permeable to substances of low and high molecular weight (6–8). Some time after the completion of hemolysis, however, the cells recover their normally low permeability. Exceptions to this impermeability of ghosts have been reported (9, 4, 5).

The size and number of holes that develop during hemolysis are not known. Various estimates for the size have been made and range from less than 100 A in diameter (10) to over 100 A (9) and even up to diameters of the order of 1 μ (11, 12). In order to check on the size of the hole, ferritin (molecular weight of around 10^6; diameter of about 120 A total; diameter of electron-opaque core about 80 or 90 A; Haggis, reference 13) and colloidal gold (25–300 A diameter) were used in the experiments to be described.

LYTIC HEMOLYSIS: Saponin may produce holes in the erythrocyte membrane (14–17). The work reported in the present paper indicates that there are stable holes or defects in saponin- and lysolecithin-treated erythrocytes.

MATERIALS AND METHODS

Collection of Blood

The human erythrocytes were obtained from a fasting human volunteer and the blood was heparinized (100 units per milliliter blood).

Preparation of Gold Suspensions and Ferritin Solutions

Horse spleen ferritin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, as cadmium-free, twice-crystallized ferritin. Colloidal gold suspension was obtained from Abbott Laboratories. Chicago. These stock solutions of gold and ferritin were then dialysed for 48–72 hr against a dialysing medium about 40 times the volume of the dialysate; this medium was changed every 12 hr. The composition of the dialysing medium was either 154 mM NaCl or 25 mM NaCl in 10 mM sodium phosphate buffer, pH 7.0. The hypotonic ferritin plus gold solution was prepared by dialysing the stock ferritin and gold suspensions individually against 25 mM NaCl (pH 7) and mixing 1 volume of each just before the experiment.

Hypotonic Hemolysis Experiments Involving Ferritin and Gold

1. STANDARD RAPID HEMOLYSIS PROCEDURE: An aliquot of erythrocytes (either 0.02 ml of packed cells or 0.04 ml of whole heparinized blood) was added to either 0.5 ml or 1.0 ml of 10% ferritin (and/or colloidal gold each at half the stock concentration) containing 25 mM NaCl in 10mM sodium phosphate buffer, pH 7.0. The cells were mixed by means of a vortex mixer and after 5 min they were fixed in suspension by the addition of 2 or 3 ml of either 2% osmium tetroxide (Palade's fixative; reference 18) or 2% glutaraldehyde (in 0.1 M sodium cacodylate–HCl, pH 7.2). Erythrocytes that had been fixed only in 2% OsO_4 were exposed to the OsO_4 no longer than 20 min total time. This short fixation time was chosen to preclude or diminish the possibility of artifactual translocation of ferritin or gold across the cell membrane. (The definition of, and the problem of, artifactual translocation is presented in the Discussion.) The duration of glutaraldehyde fixation was from 20 to 30 min, which includes the time required for centrifugation. After the cells were fixed in suspension with glutaraldehyde, the erythrocytes were centrifuged at about 11,000 *g* for 8–10 min to form a pellet. These glutaraldehyde-fixed pellets of cells were then fixed in 2% OsO_4 for 30 min to 2 hr; it was found that after glutaraldehyde fixation such long periods of OsO_4 fixation did not cause any artifactual translocation of particles. The pellets were then gently broken into 1-mm pieces and in most experiments were treated for 1 hr in 0.5% uranyl acetate in Veronal-acetate–HCl buffer (19). This uranyl treatment did not cause artifactual translocation of particles in glutaraldehyde-OsO_4–fixed cells, and greatly enhanced the contrast of the membrane. The pellets were dehydrated, embedded in Epon 812, and sectioned (silver sections); the sections were stained with uranyl and lead (19). The lead citrate stain causes considerable loss in contrast of the ferritin particles and was, therefore, kept to 1 min or less. Much higher ferritin contrast is obtained by following the uranyl with the Karnovsky stain (20), and many sections were stained in this way. The sections were examined in the RCA-EMU 3F, the Hitachi HS-7S and the Siemens Elmiskop I electron microscopes.

Individual colloidal gold particles were readily identified by their circular profile (25–300 A in diameter) and their extreme electron opacity. Ferritin particles were also easily identifiable when the sections were stained with uranyl and Karnovsky B stains. In agreement with Haggis (13), ferritin was observed and photographed as circular and usually uniformly

dense-cored particles about 90 A in diameter. As mentioned above, ferritin in sections stained with lead citrate had less electron opacity. In these sections the density of ferritin approached the low density of the irregular and dispersed cytoplasm. The distinction between ferritin and isolated pieces and strands of cytoplasm was, however, virtually never in doubt. Firstly, with the exception of the chemically *fixed hole* experiments, there were usually hundreds of intracellular particles all about 90 A in diameter. No erythrocyte hemolysed in the absence of ferritin ever exhibited uniform dense dots of this sort. Second, a routine procedure was to make several micrograph prints of each microscope plate, one of which was a high contrast and underdeveloped print. This print would usually show up the existence of ferritin to advantage while suppressing the low density cytoplasmic remnants.

2. PROCEDURE OF GRADUAL HEMOLYSIS BY DIALYSIS: An aliquot of whole blood (0.04 ml) was added to 1.0 ml of ferritin (at half the stock concentration) in 154 mM NaCl, pH 7, in a dialysis bag. The thin dialysis bag was then dialysed against 250 ml of 25 mM NaCl, pH 7, for 20 min, during which time the turbid erythrocyte suspension cleared completely; clearing occurred at about 6–8 min after the onset of dialysis. An aliquot (0.05 ml) of 2.3 M NaCl was then added and the cells were fixed in suspension with Palade's fixative (18). The control for this experiment was as follows. Erythrocytes were added to 0.5 ml of 154 mM NaCl and dialysed for 10 min. After that time, 0.5 ml ferritin in 25 mM NaCl was added and dialysis carried on for another 10 min; 2.3 M NaCl (0.05 ml) was then added and followed by Palade's fixative (18).

3. TIME COURSE OF THE DEVELOPMENT OF HOLES: An aliquot of 0.02 ml packed cells was pipetted into the bottom of a test tube; 1 ml of ferritin (at half the stock concentration) in 25 mM NaCl in 10 mM sodium phosphate buffer, pH 7, was added for varying periods of time (5, 10, 20, 30, and 60 sec); after these time periods, 0.05 ml of 2.3 M NaCl was added (to make the final solution isotonic and to close the presumed holes) for corresponding time periods (5 min, 55 sec; 5 min, 50 sec; 5 min, 40 sec; 5 min, 30 sec; and 5 min, respectively). The erythrocytes, now having been exposed to ferritin for a total time of 6 min, were fixed in suspension with 1 ml of 2% OsO_4 (Palade's fixative made isotonic with NaCl instead of with sucrose) for 5 min. Photographs of the erythrocytes in thin section were taken in the RCA-EMU-3F microscope. Between 50 and 70 cells for each experimental time point were randomly photographed.

4. TIME COURSE OF THE CLOSING OF HOLES: An aliquot of 0.02 ml packed cells was pipetted into the bottom of a test tube and 0.5 ml of 25 mM NaCl (pH 7) was added for varying periods of time (15, 30, 60, 120, and 180 sec); 0.5 ml of ferritin in 25 mM NaCl (pH 7) was then added for 3 min. An aliquot (0.05 ml) of 2.3 M NaCl was added to make the cell suspension isotonic and, after 3 min, 1 ml of 2% OsO_4 (Palade's fixative made isotonic with NaCl) was added to the suspension for 5 min. The ferritin, therefore, was in contact with the erythrocytes for 11 min in all the tubes. Approximately 50 cells for each time point were photographed in the electron microscope.

5. THE FIXATION OF THE TRANSIENT HOLES IN THE OPEN POSITION: The procedure was as follows. An aliquot (0.04 ml) of whole blood was pipetted into the bottom of a test tube and 1 ml of 25 mM NaCl (pH 7) was added to start hypotonic hemolysis; after 7, 12, 18, 22, 60, and 300 sec, 3 ml of 2% glutaraldehyde were added. The fixed cells were then centrifuged (10,300 *g* for 10 min at 18°C) into a pellet which was washed twice in Michaelis buffer (18). The cells were finally resuspended in 0.5 ml ferritin or ferritin plus gold in 154 mM NaCl for 30 min; the cells were then centrifuged into a pellet once more and OsO_4 was added.

6. EFFECT OF SOLUBLE HEMOGLOBIN ON FERRITIN ENTRY INTO CELLS: To test the possibility that the released hemoglobin adsorbs and thereby restricts the entry of ferritin, the following experimental procedure was carried out. An aliquot (0.08 ml) of whole blood was added to 1 ml of 25 mM NaCl (pH 7) and, after 5 min, the ghosts were centrifuged down at 10,000 *g* for 10 min; the hemoglobin-containing supernatant was saved and the ghosts were discarded. An aliquot (0.04 ml) of whole blood was now added to 1 ml of ferritin-hemoglobin solution (0.5 ml 10% ferritin in 25 mM NaCl plus 0.5 ml of the hemoglobin in 25 mM NaCl) and, after 5 min, an aliquot (0.05 ml) of 2.3 M NaCl was added (to restore isotonicity) followed by the addition of 2 ml of Palade's fixative (18).

7. HEMOLYSIS BY LYTIC AGENTS: Holothurin A (a saponin preparation purified from the sea cucumber, *Actinopyga agassizi*) was kindly donated by Dr. J. D. Chanley, the Mount Sinai Hospital, New York. Lysolecithin was obtained from K & K laboratories, Jamaica, N. Y. The experiments involving hemolysis by lytic agents were carried out in the following manner. An aliquot (0.04 ml) of heparinized whole blood was added to 1 ml of 154 mM NaCl, pH 7, and the cells were hemolysed by the addition of 30 μg of holothurin A (in 0.05 ml of 154 mM NaCl) or 0.2 ml of 3×10^{-3} M lysolecithin. The turbid erythrocyte suspension cleared at around 5 to 6 min in the presence of holothurin and at around 14 min with lysolecithin. 10 min after clearing, 3 ml of glutaraldehyde fixative 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 finally resuspended in 0.5 ml ferritin in 154 mM NaCl, pH 7, for 5 min. The cells were then fixed in Palade's fixative (18).

RESULTS

Entry of Ferritin into Erythrocytes during Hemolysis

Ferritin was found inside erythrocytes which had been hypotonically hemolysed in the presence of ferritin (Methods, part 1). This is shown in Fig. 1 *a*. The ferritin appears as extremely dense dots about 90 A in diameter when the thin sections have been stained by means of Karnovsky B lead stain. These black ferritin dots are seen against the irregular gray background of residual intracellular material. Fig. 1 *a* also shows the corner of an erythrocyte which did not undergo hemolysis, as indicated by the homogeneous dense cytoplasm; such nonhemolysed cells never contained any ferritin particles. Many hundreds of cells were examined during the course of 25 experiments and every hemolysed cell contained ferritin. Varying the osmotic pressure or the pH of the solution did not affect the location of ferritin, other than change the proportion of hemolysed cells.

The relationship between per cent hemolysis and the per cent of cells containing ferritin was also studied. An osmotic fragility curve was obtained in the usual way by adding erythrocyte aliquots to a series of varying hypotonic NaCl solutions and measuring the hemoglobin released into the cell-free supernate at 543 mμ. A duplicate series of hypotonic solutions containing ferritin was treated in the same way, except that the cells were fixed and examined for intracellular ferritin (Methods, part 1). (A duplicate series was necessary since the intense optical density of ferritin interfered with the spectrophotometry of hemoglobin.) It was found that the proportion of cells containing ferritin exactly paralleled the per cent hemolysis.

No membrane discontinuities were observed in these cells (which had been fixed 5 min after the onset of hemolysis). A sample of the intact membrane is shown in Fig. 1 *b*; here the middle part of the photograph is the intracellular space and the ferritin dots appear very gray with low contrast (since the lead citrate stain was employed). The portion of the cell shown in Fig. 1 *b*, although demonstrating the intact membranes to advantage, does not contain abundant numbers of ferritin particles in the thin cytoplasmic region; this particular cell does, however, contain hundreds of particles in the wider expanses of cytoplasm situated 1–2 μ above and below the selected zone shown.

Entry of Colloidal Gold into Erythrocytes during Hemolysis

Colloidal gold particles (25–300 A in diameter) were also found to enter erythrocytes which had been hypotonically hemolysed in the presence of the gold. This is shown in Fig. 1 *c* and *d*, where the ferritin molecules appear gray and the gold dense black.

The Impermeability of Erythrocyte Ghosts to Ferritin or Gold Particles

When ferritin or ferritin plus gold in hypotonic solution was added 5 min after the onset of hemol-

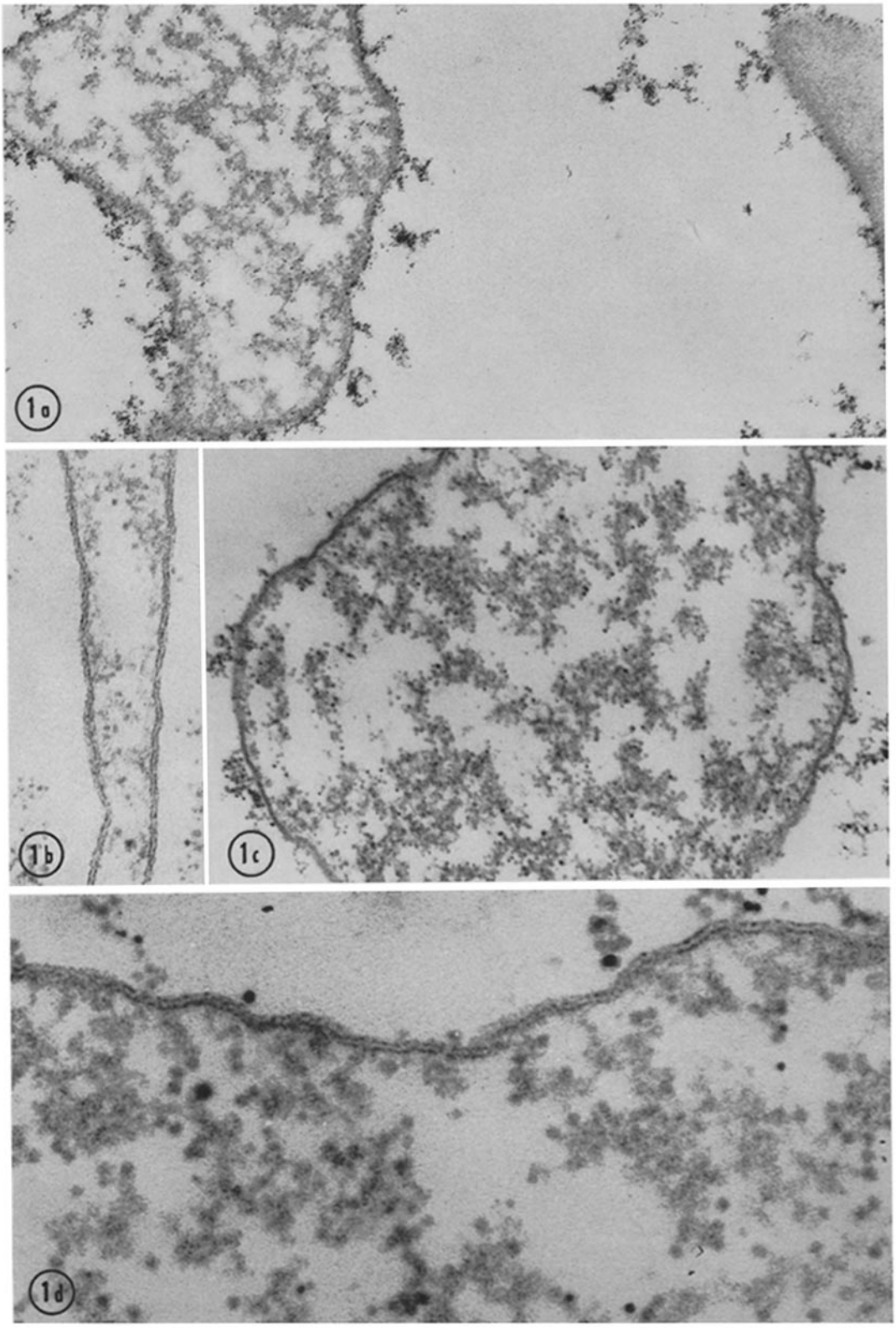
FIGURE 1 Showing that ferritin and colloidal gold enter erythrocytes during the 5-min period of hypotonic hemolysis.

FIGURE 1 *a* The cells were hypotonically hemolyzed in the presence of ferritin and then fixed in 2% OsO_4. The hemolyzed cell contains ferritin (seen as black dots, 90 A wide) while the intact cell does not. RCA-EMU-3F. Uranyl and Karnovsky B stains. × 41,000.

FIGURE 1 *b* The cells, hemolyzed in the presence of ferritin (stained as pale gray dots here), were fixed in glutaraldehyde, then OsO_4, and treated with uranyl acetate. Ferritin is inside (center of photo) and the unit membranes are not disrupted. Elmiskop I. Uranyl and lead citrate stains. × 128,000.

FIGURE 1 *c* Same as Fig. 1 *b*, except that the cells were hemolyzed in the presence of both colloidal gold (black dots) and ferritin (gray dots). × 68,000.

FIGURE 1 *d* Showing continuity of the membrane in a cell hemolyzed in the presence of gold (black dots) and ferritin (gray dots). Procedure as in Fig. 1 *b*. × 206,000.


1a
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1d

ysis, then no particles entered the cells. Examples of such results are shown in Fig. 2 *a* and *b*. Restoring tonicity by the addition of an aliquot of hypertonic NaCl solution to make a final concentration of 150 mM before adding the ferritin also gave the same results.

There are two possible interpretations of the fact that no ferritin was found inside the erythrocyte when the ferritin was added 5 min after the onset of hypotonic hemolysis. One possibility is that the holes have spontaneously closed and that the ghost membrane has become impermeable to the ferritin.

A second conceivable situation is as follows. When ferritin is added 5 min after the onset of hemolysis, the ferritin may become quickly and totally adsorbed to the abundant hemoglobin which has been released to the extracellular space during the 5-min period. Such adsorption would markedly reduce the extracellular concentration of free ferritin particles. In this case, virtually no ferritin would enter the erythrocyte ghosts even if the holes in the membrane still existed. In this event, therefore, the absence of intracellular ferritin would not indicate anything about the permeability of the membrane to ferritin. To test this second possibility, erythrocytes were hemolysed in the presence of ferritin and hemoglobin (Methods, part 6). By electron microscopy it was observed that all the hemolysed cells contained ferritin. The presence of extracellular hemoglobin, therefore, does not prevent the permeation of ferritin into the cell.

Gradual Hemolysis

Erythrocytes were hemolysed in the presence of ferritin by gradual hemolysis (references 11, 21; Methods, part 2); all the hemolysed cells contained ferritin. None of the erythrocyte ghost control cells contained any ferritin.

Gradual lysis was also done in the presence of colloidal gold. Gold particles less than 150 A in diameter were found inside the cell; although larger particles were not found in the cytoplasm it was not possible to say whether these large particles were excluded because of their size or because there were simply not enough particles greater than 200 A in diameter.

Time Course of the Development and Closing of Holes in Hypotonic Hemolysis

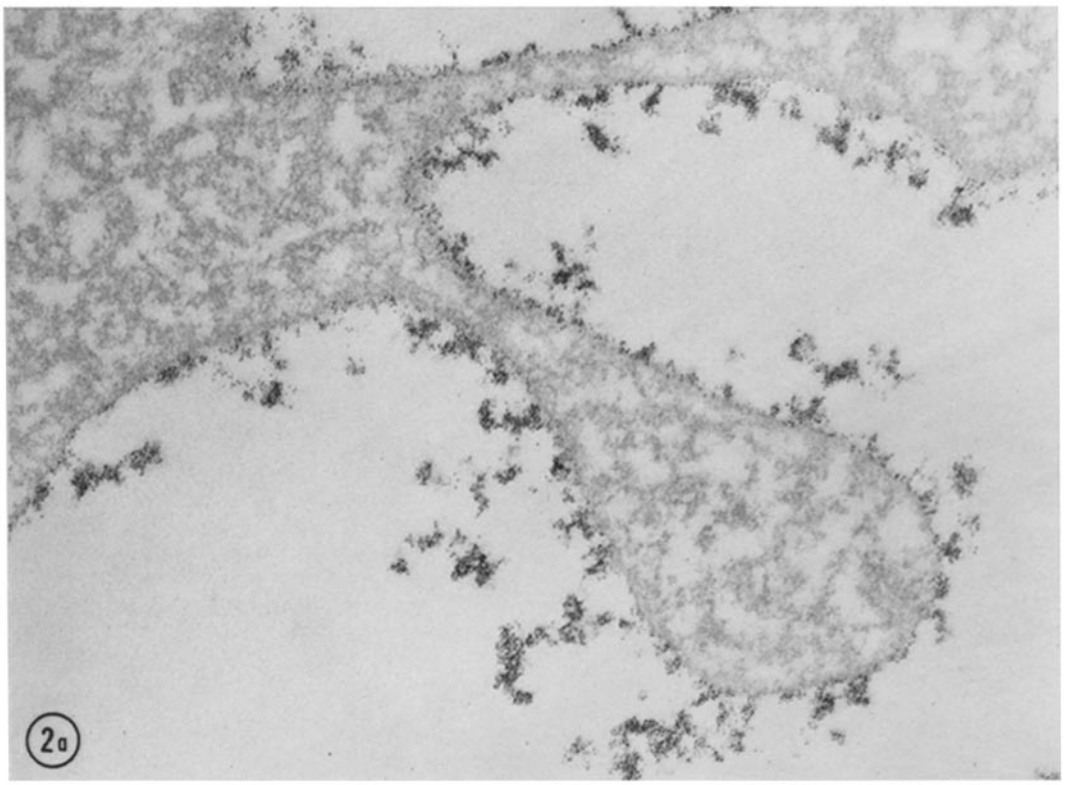
The time course of the development and closing of membrane holes was investigated as follows.

1. THE DEVELOPMENT OF HOLES: The rationale here was to time the development of the holes by inducing hypotonic hemolysis in the presence of ferritin and by restoring the isotonicity of the medium (thereby presumably leading to a rapid closure of the holes) at short intervals thereafter (Methods, part 3). Only the hemolysed cells (with mottled intracellular texture) contained ferritin. The number of cells containing ferritin was counted and plotted as a per cent of the total number of approximately 60 cells for that time point. The result is shown in Fig. 3, curve A. It is seen that 50% of the cells has been permeated by ferritin at around 15 sec. At 60 sec, all the cells have been permeated. Curve A represents the time course of the development of the holes or pores, therefore. It should be mentioned that the location of ferritin at the time points studied here was virtually an all-or-none phenomenon; that is, either there was not one single particle to be found in the hemolysed cell or else the cell was richly loaded with ferritin.

FIGURE 2 The impermeability of erythrocyte ghosts to ferritin or gold particles. Erythrocytes were first hypotonically hemolysed for 5 min in the absence of ferritin or colloidal gold.

FIGURE 2 *a* After hemolysis the medium was made isotonic with NaCl, OsO_4 was added, and then ferritin. No ferritin (black dots) permeated the fixed ghosts. Identical results were obtained if the ferritin was added before the NaCl or before the OsO_4. RCA-EMU-3F. Uranyl and Karnovsky B stains. × 44,000.

FIGURE 2 *b* After hemolysis ferritin (pale gray dots) and gold (black dots) in hypotonic solution were added for another 5 min before fixing in glutaraldehyde (followed by OsO_4 and uranyl solutions). No cytoplasmic particles are seen. Elmiskop I. Uranyl and lead citrate stains. × 92,000.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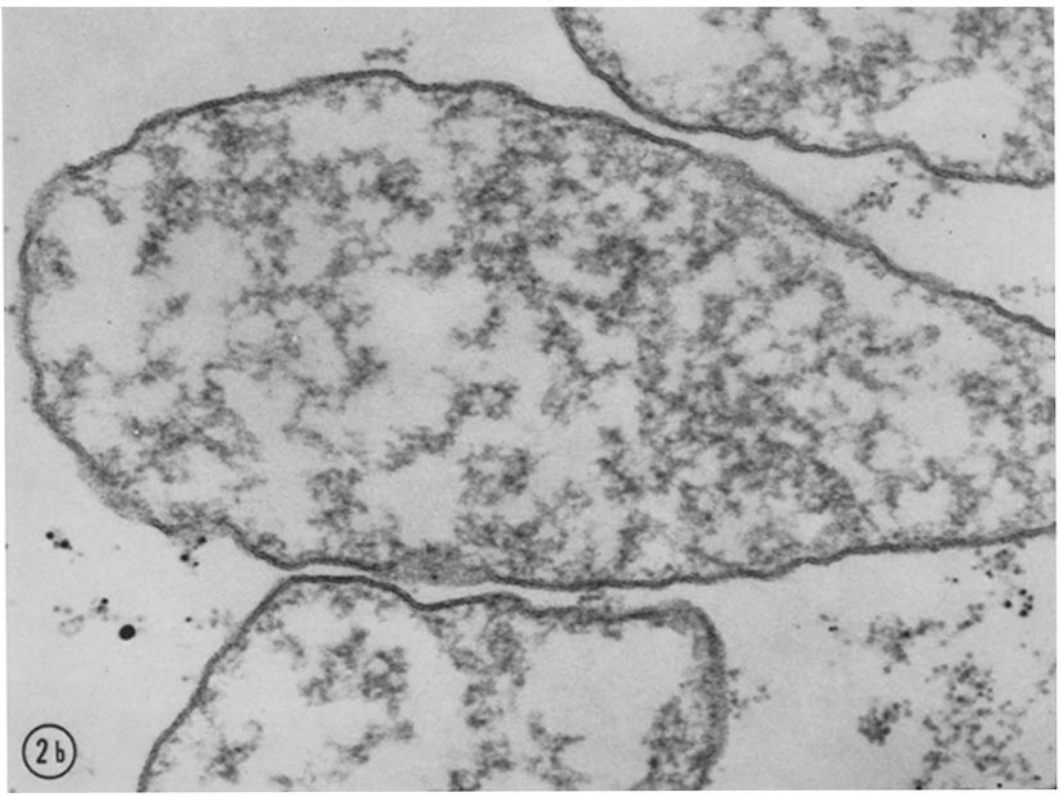
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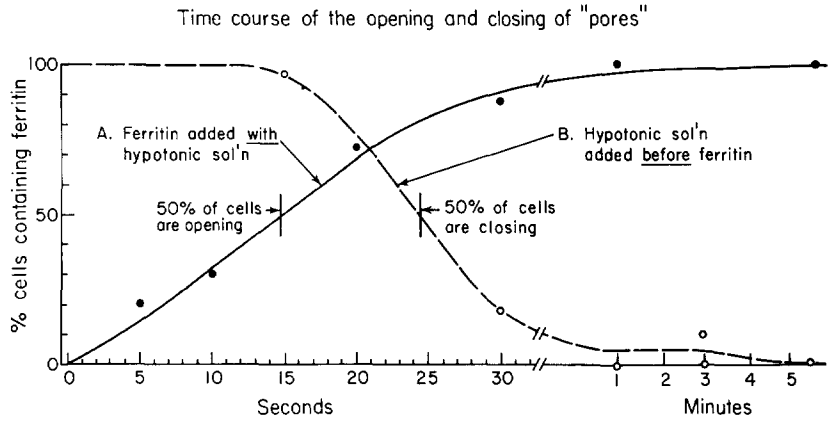


FIGURE 3 Data delineating the transient permeable state of the hemolysing erythrocyte. A *Cell-opening series* The cells were placed in hypotonic solution containing ferritin, and after different time intervals NaCl was added to make the medium isotonic. The cells were fixed in OsO_4. Each point represents the fraction (of around 60 cells) which contain ferritin. 50% of the cells has admitted ferritin at 15 sec. B *Cell-closing series* The cells were put into hypotonic solution *not* containing ferritin; at varying times after the onset of hypotonic hemolysis ferritin was added and the cells were later fixed in OsO_4. Most of the cells have closed at 30 sec. Considering A and B together, the majority of the cells have "holes" or "pores" existing for a 10-sec interval between 15 and 25 sec.

2. THE CLOSING OF HOLES: The rationale here was to add ferritin at various times after the onset of hemolysis (Methods, part 4); since the holes are known to close spontaneously (Fig. 2 *b*), ferritin should not enter if it is added late in the hemolysis period. Although all the cells were hemolysed, not all contained ferritin. The proportion of cells containing ferritin at each time point is graphed in Fig. 3, curve B. It is seen that, when the ferritin was added at 15 sec, all the cells picked up the ferritin. Only 18% of the cells was permeated by ferritin if the latter was added 30 sec after the onset of hemolysis. This indicates that most of the cells have closed at 30 sec. Interpolating, it can be seen that 50% of the cells is spontaneously closing at around 25 sec. Ferritin added 1, 3, and 5 min after the onset of hemolysis did not enter the ghosts; in one experiment, however, it was observed that ferritin added 3 min after the onset of hemolysis entered about 10% of the erythrocytes that were hemolysed. This is indicated in Fig. 3, curve B.

It is interesting to note that, among those cells examined at the 15-sec time point, two were hemolysed but did not contain any ferritin. This indicates that these two cells hemolysed and closed in the 15-sec interval before the ferritin was added. 15 sec, therefore, represents the upper limit for the duration of the existence of the transient holes of these two cells.

3. THE FIXATION OF TRANSIENT HOLES IN THE OPEN POSITION: The results shown in Fig. 3, curve A, indicate that 50% of the cells is developing holes at around 14 or 15 sec; Fig. 3, curve B, indicates that 50% is closing between roughly 24 and 25 sec. These data imply that the majority of the cells have holes at about 19 or 20 sec and that the holes remain open for about 10 sec. It was necessary to check these two points by some different procedure. In the hope of fixing the transient holes in the open position and having ferritin enter the chemically-fixed holes, OsO_4 or glutaraldehyde fixative was added to the cell suspension at various time points during hypotonic hemolysis, and this was followed by the addition of ferritin. Fixation by OsO_4 was tried with both isotonic and hypotonic fixatives added at 21 sec after the onset of hemolysis; although many if not all of the cells were hemolysed, none contained ferritin. The OsO_4 apparently failed to fix the transient holes in the open position or to keep them open long enough for ferritin to be admitted.

It proved possible, however, to fix the transient holes in the open position by means of glutaraldehyde (Methods, part 5). Ferritin and gold added to cells which have been fixed during the first

minute of hemolysis are found to permeate many of the cells (see Fig. 4). Approximately 70 cells were photographed for each time point and the proportion of cells containing ferritin was plotted as a per cent of these 70 cells. In Fig. 5 it is seen that the majority of the cells are open at around 15 sec, and this agrees fairly well with the value of about 19 or 20 sec obtained in the *hole-development* and *hole-closure* series of experiments. The time interval between the ascending and descending limbs of Fig. 5 is about 10 sec (at the 50% to 90% level of the ordinate), and this represents the duration of the existence of the holes for the majority of the cells. At 60 sec, there are a number of cells (8%) which have persisting holes or holes which have developed late in the hemolysis period (see Fig. 4 *b*).

Anatomy of the Membrane Defects or Holes Developed during Hypotonic Hemolysis

A large number of cell profiles in the *hole-fixation* series revealed membrane breaks or discontinuities. Three examples are shown in Fig. 6. The membrane defects are of the order of 200–500 A wide. It seems that the leaflets of the unit membrane at the edge of the defect end abruptly and do not join one another (except at the double-shafted arrow in Fig. 6 *a*—see legend to Fig. 6 and Discussion). Only those cells which contained ferritin exhibited membrane defects. Adjacent ghosts, free of ferritin, had intact membranes (Figs. 4 *b* and 6 *b* and *c*). The defects were not found all around the perimeter of the cell profile but were clustered in one zone (about 1 μ long), for any particular cell profile. Two clusters of defects in different parts of the same cell membrane profile were not observed, although in Fig. 6 *b* a profile is shown where two defects are separated by about 1 μ. It is interesting to note that in these experiments the cytoplasmic ferritin was often seen to be located near the defect zones. For example, ferritin is seen near the holes in the cell in Fig. 6 *b* but the rest of the cytoplasm (not shown) was entirely devoid of ferritin.

Bulging of the Cell Membrane before the Development of Membrane Holes

Many of the cells fixed at 12 sec contained small patches of membrane which ballooned out (Fig. 7). Such profiles were also seen at 7 sec but were most numerous at 12 sec and nonexistent at later times. Such ballooning may represent the precursor state of the membrane before the defects appear, since there was only one such bulge per cell profile and since these cells never had any ferritin in them.

Stable Defects in the Membrane after Lysis by Surface-Active Agents

While transient holes exist during hypotonic hemolysis, it was found that there were holes or membrane defects persisting long after hemolysis (10 min) by holothurin A (a saponin) and lysolecithin. The results with lysolecithin (see Methods, part 7) are shown in Fig. 8. The cell contour is irregular and scalloped; ferritin is found inside the cells. At the base of the indentations caused by lysolecithin there seems to be a discontinuity of the membrane (Fig. 8 *b*). The results with the saponin are almost identical insofar as it creates ghosts which have scalloped contours and which are penetrated by subsequently added ferritin. The membrane pits or indentations created by the saponin are, however, only about one-fourth as deep and the membrane was always observed as continuous, even at the base of the pits; this continuity was seen despite the fact that ferritin permeated these glutaraldehyde-fixed ghosts.

Membrane Thickness

The mean value for 40 ghost membranes was 71 A (cf. references 22–25). The measurements were made on photographic enlargements and refer to the over-all distance from edge to edge of the unit membrane, the edges of which were judged by eye.

DISCUSSION

The general observation of this study is that erythrocytes become momentarily permeable to ferritin and colloidal gold during hypotonic hemolysis, but persistently permeable after lytic hemolysis.

Artifactual Translocation of Ferritin

The intracellular location of ferritin, added in *isotonic* solution to the extracellular space, is generally considered to result from a permeation of ferritin during the process of fixation, embedding, and staining, and this process is commonly referred to as artifactual translocation of ferritin. Such artifactual translocation of ferritin is induced by prolonged OsO_4 fixation and has been seen by

some investigators (26–30) but not by others (31). No cytoplasmic free ferritin was noticed by Huxley (32) who used 2 hr of glutaraldehyde fixation (followed by OsO_4).

Under the conditions employed in the present series of experiments there was no evidence of artifactual translocation of ferritin into the cell. Ferritin added in isotonic solution to cells which were subsequently fixed and treated as outlined in Methods never permeated any of the erythrocytes. Further experimental support for the absence of artifactual translocation is shown by the ferritin-free cells in Figs. 1 *a*, 2 *a*, 2 *b*, 4 *a*, 4 *b*, 6 *b*, and 6 *c* as well as by those ferritin-free cells observed in other control experiments reported in Results (see sections on *gradual hemolysis*, and on the unsuccessful attempt of osmium tetroxide *fixation of transient holes in the open position*).

The Concentration and Location of Tracer

A comparison of all the micrographs indicates that the final extracellular concentration of ferritin or gold is highly variable. The reason for this is unknown. Presumably these tracers are extracted in varying degrees depending on the number of changes of OsO_4 fixative (usually 2 changes when the cells had been prefixed in glutaraldehyde), of uranyl solution (1 or 2 changes), and of dehydrating solutions (5 to 7 changes of absolute ethanol and 4 to 6 changes of propylene oxide). A second point is that ferritin is usually but not invariably associated with dense material. This may represent adsorbed ferritin which is not easily extracted.

In the experiments on *hole-fixation* (Figs. 4, 5, and 6), wherein ferritin was added to glutaraldehyde-fixed cells, it was observed that in most cases the intracellular presence of ferritin was all-or-none (Fig. 4) as it was in the experiments of Fig. 1. In other experiments it was observed that ferritin was not uniformly distributed throughout the cell but was located near the zones of holes. Whether a cell with chemically fixed holes contains few or many particles presumably depends, among other factors, on (*a*) the moment selected for fixation (since this determines the size and number of holes), (*b*) the density and viscosity of the fixed cytoplasm through which the ferritin must diffuse, and (*c*) the length of time which the cells have been exposed to ferritin.

The Time Course of Development and Closure of the Transient Holes

In comparing the two sets of results wherein the time course of the development and closure of the holes was examined (Figs. 3 and 5), it is seen that there seems to be a discrepancy of around 5 sec or so. In Fig. 3 the maximum rate of rise is at 15 sec whereas in Fig. 5 it is at about 10 sec; likewise, the maximum rates of decline differ by 5 or 10 sec. The discrepancy is not serious if one considers that the procedures are vastly different and that the pipetting and mixing accuracy are no better than plus or minus 2 or 3 sec. It should be pointed out that, although the absolute values of the abscissae of Figs. 3 and 5 may be compared, there is no meaning in a comparison of the absolute values of the ordinates.

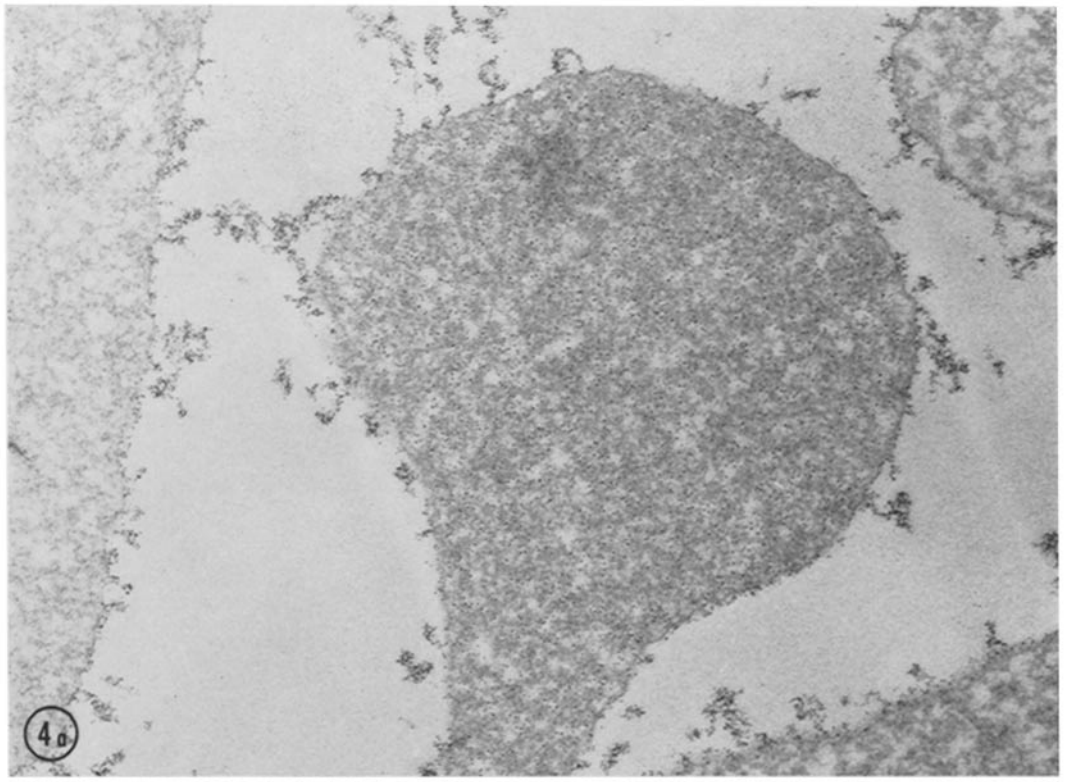
Gradual Hemolysis Compared to Rapid Hemolysis

It has been shown by Katchalsky et al. (21) that erythrocytes undergoing a gradual hemolysis (11) hemolyse at lower extracellular tonicities com-

FIGURE 4 Demonstrating the penetration of ferritin and colloidal gold into cells which have been fixed in glutaraldehyde at 22 and 60 sec after the onset of hypotonic hemolysis.

FIGURE 4 *a* At 22 sec after the cells were placed in hypotonic solution free of ferritin, the cells were fixed in glutaraldehyde, centrifuged, washed, and exposed to ferritin (seen here as black dots) which permeated 15% of the cells in the field (see Fig. 5); the cells were then fixed in OsO_4 but not given the uranyl treatment. RCA-EMU-3F. Uranyl and Karnovsky stains. × 35,000.

FIGURE 4 *b* Same as in Fig. 4 *a*, except that both ferritin (gray dots) and gold (black dots) were added to cells which had been fixed at 60 sec. This cell is unusual, in that either the "holes" have persisted for a long time (over 45 sec) or the cell has hemolysed late. Uranyl treatment was given after the fixation. Elmiskop I. Uranyl and lead citrate stains. × 160,000.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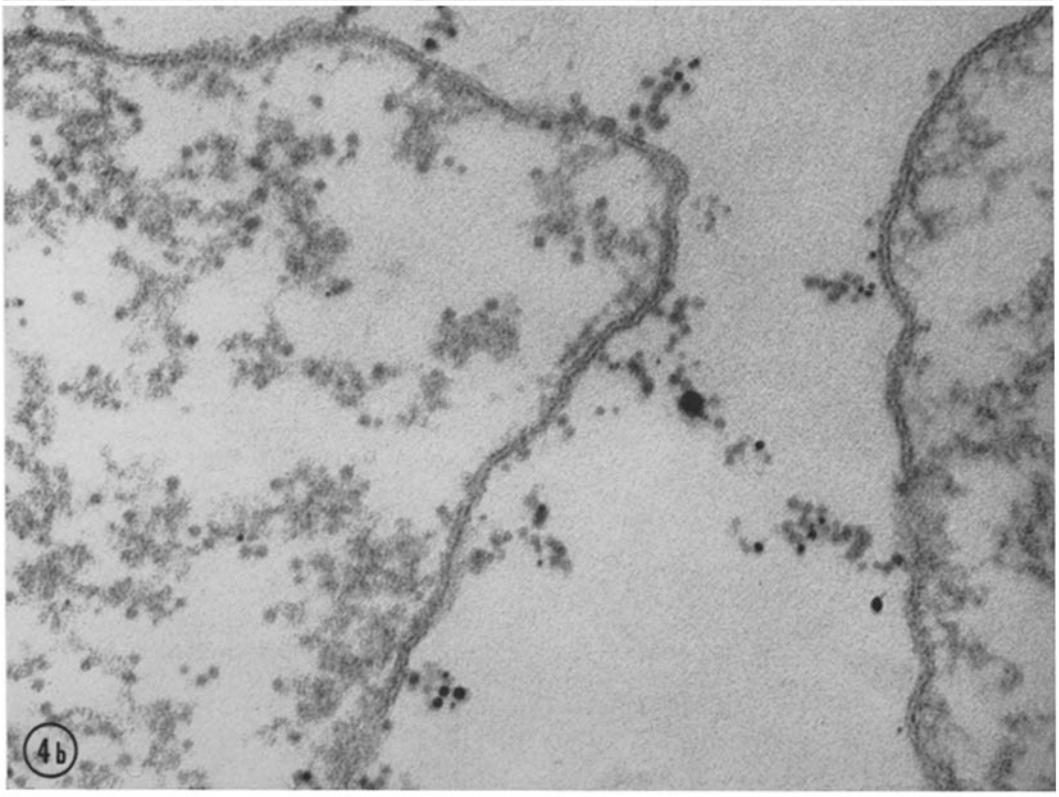

4b

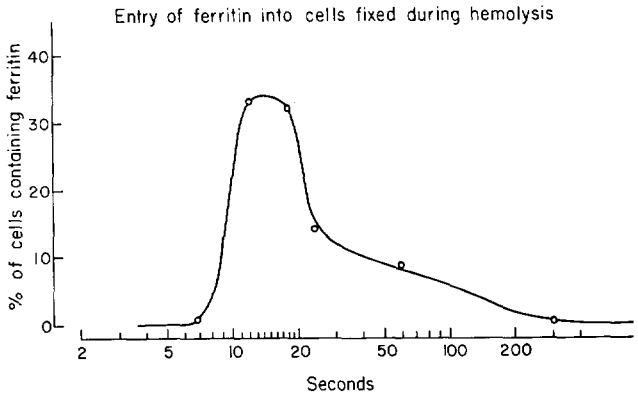


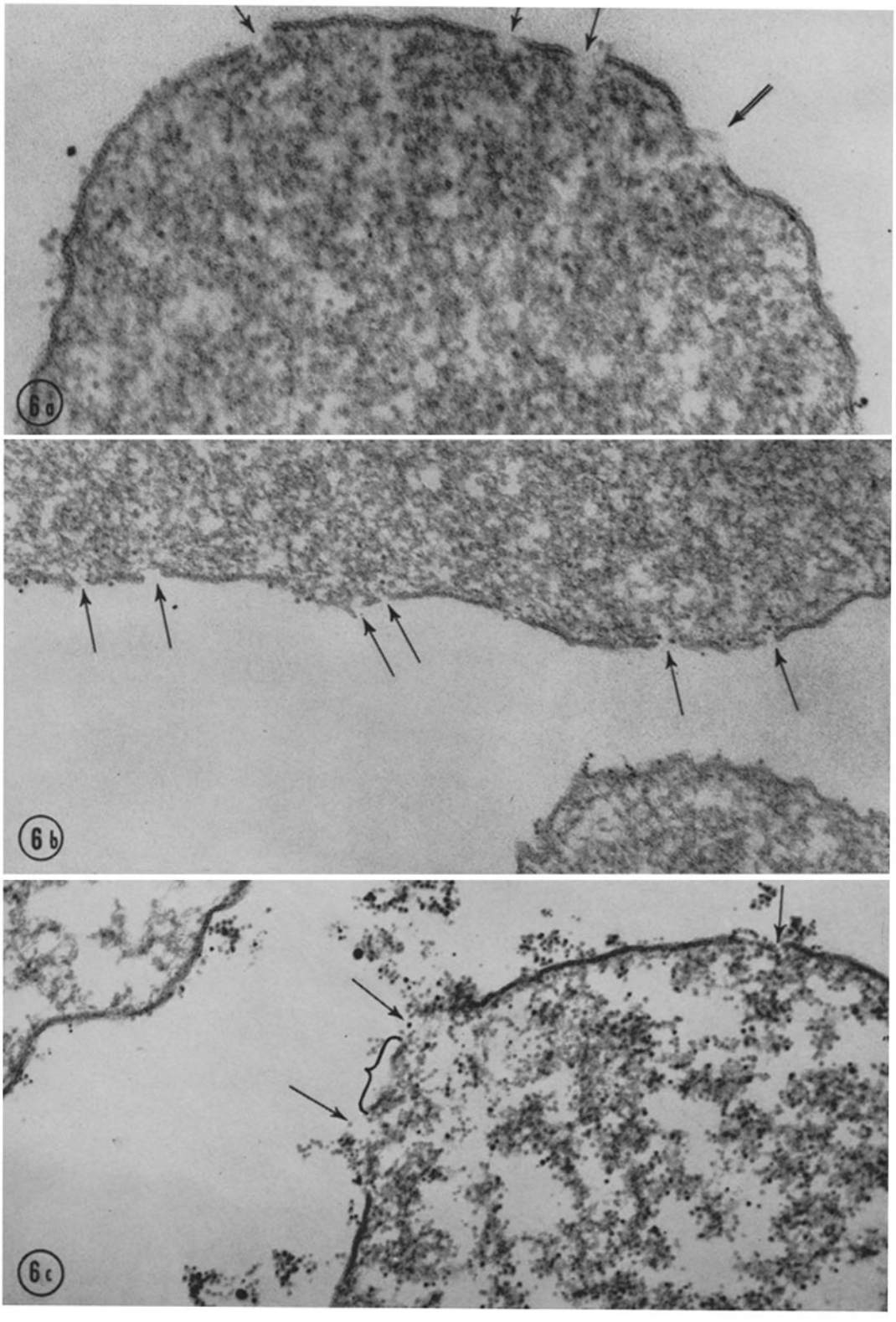
FIGURE 5 Showing the time course of the permeable state of hemolysing erythrocytes to ferritin. At various times after the onset of hypotonic hemolysis in a solution free of ferritin, glutaraldehyde was added, and the cells were centrifuged and exposed to ferritin; the cells were then fixed in OsO_4. Each point represents the fraction of cells (out of 70 cells) containing ferritin. It is seen that the majority of cells are patent between 10 and 20 sec after the onset of hemolysis. There are some cells (at 60 sec) which have either persisting holes or holes that have developed late in hemolysis.

pared to the routine rapid hemolysis; Katchalsky et al. attributed this difference to a more gentle stretching of the cell membrane by the dialysis procedure. It was conceivable, therefore, that erythrocytes hemolysed by means of the gradual hemolysis technique would not contain ferritin. The fact that ferritin does permeate into cells during gradual hemolysis indicates that the stretch is not so gentle as to sieve out ferritin. It will be necessary to fix cells at various times during gradual hemolysis in order to check the existence of membrane slits, holes, or pores.

Anatomy of the Membrane Openings

It is possible that the discontinuities or openings in the cell membrane profiles shown in Fig. 6 are all really part of an irregular star-shaped defect and that the thin section has merely passed through the points of the star-shaped lesion. This possibility is only one of other conceivable geometrical configurations, the existence of which will have to be examined by other means and techniques. Useful information would come from study of these membrane defects by (*a*) stereoscopic electron microscopy, (*b*) negative staining techniques, (*c*) serial section microscopy, and (*d*) photographing areas of the cell wherein the membrane has been grazed in section. A second point is that whereas the cell openings measure around 300 A in width they must also be at least 500 or 600 A in depth, for this is the thickness of the Epon section. In three dimensions, therefore, the membrane openings may be shaped like slits. The dashed arrow in Fig. 6 *a* points to a part in the membrane profile which may be the edge of a hole, because this spot in the membrane is not entirely devoid of membrane density. Third, it is possible that the membrane defects observed in this study are smaller than they really are; the glutaraldehyde may have caused a certain amount of shrinkage. Fourth, the clusters of defects or holes would tend to go along with light microscope observations (11, 12) that some cells bulge in one region before hemolysing.

FIGURE 6 Showing abrupt discontinuities or holes in the membrane of the hemolysing erythrocyte when the cells are fixed by glutaraldehyde during the first minute of hypotonic hemolysis. The erythrocytes were added to hypotonic solution free of ferritin, and 12 sec (Fig. 6 *a* and *b*) or 18 sec later (Fig. 6 *c*) glutaraldehyde was added. The cells were centrifuged, washed, and a solution of ferritin and colloidal gold was added. Membrane defects (arrows) were seen only during the first minute of hemolysis and only in those ghosts which contained ferritin (which appear as pale gray dots about 90 A in diameter) or gold (black dots). Ferritin-free ghosts (Figs. 6 *b*, *c*, and also 4 *b*) revealed intact membranes. The defects are between 200 and 500 A wide. The zone of membrane marked off by the bracket in Fig. 6 *c* probably does not contain a defect, the middle part of this piece of membrane having been sectioned tangentially. These membrane defects occur in clusters, one cluster to a cell profile. The opening at the double-shafted arrow may be an edge of the "hole" or "pore" (see Discussion). Elmiskop I. Uranyl and lead citrate stains. Fig. 6 *a*, × 136,000; Fig. 6 *b*, × 88,000; Fig. 6 *c*, × 80,000.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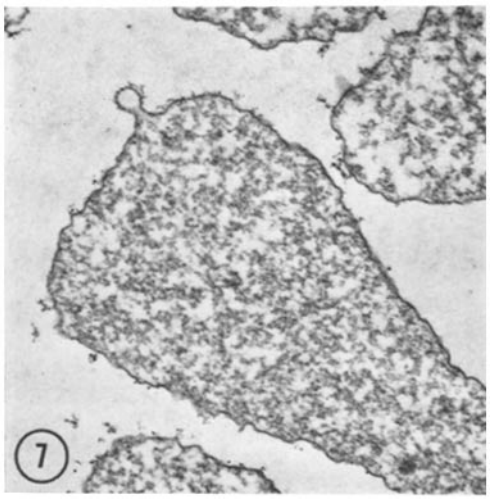

FIGURE 7 An example of bulging of the cell membrane that occurs before the permeation of ferritin particles. These cells were fixed in glutaraldehyde 12 sec after the onset of hypotonic hemolysis. Many cell profiles revealed a single bulge, as shown above; ferritin added to these fixed cells did not permeate them. These small bulges were never seen at later time points. RCA-EMU-3F. Uranyl and lead citrate stains. × 24,000.

The Duration of Existence of the Transient Holes

That most of the transient holes are open for approximately 10 to 15 sec is in reasonable agreement with the fading time of hemolysing erythrocytes (33). It is possible that the 2-μ-wide streams of hemoglobin diffusing from hemolysing erythrocytes (12) may be a consequence of hemoglobin diffusing through the cluster of tiny holes observed in the present study. By estimating the number and the size of the defects of the hemolysing cell, the diffusion coefficient of hemoglobin (34, 12) is calculated as 7.3×10^{-7} cm^2/sec (see Appendix). Polson (reference 37; see also references 38, 39) has found that the diffusion coefficient of hemoglobin is 6.9×10^{-7} cm^2/sec. This agreement seems almost fortuitous, considering the uncertainty and range in the chosen values (see Appendix).

Are the Observed Defects the Same as Those Through Which the Hemoglobin has Been Released?

Considerable evidence has been presented in this paper that glutaraldehyde does not per se induce or create artifactual membrane defects. It has been explained, in connection with the *hole-fixation* experiments, that the membrane defects were observed only in cells which were fixed between approximately 10 and 20 sec after the onset of hemolysis and which were permeated by subsequently added ferritin. *Membrane defects were never found in a glutaraldehyde-fixed erythrocyte or ghost which was devoid of ferritin.* Cells which had been fixed by glutaraldehyde before the 10-sec time point or after the 180-sec time point were never permeable to added ferritin (Fig. 5), and the cell membranes never contained any defects. This indicates that glutaraldehyde does not of its own accord induce membrane defects.

The defects, therefore, must develop in association with the process of osmotic hemolysis. Secondly, the holes are most frequently detected between 10 and 20 sec which is when the rate of hemoglobin release is highest. This fact further suggests that the transient holes are intimately associated with the hemolytic process. Third, the size and number of the transient holes are roughly adequate to account for the 10-sec fading time in osmotic hemolysis (see reference 40), assuming the hemoglobin is released by diffusion.

For these three reasons it is likely that hemoglobin has been released through the observed defects which apparently admit the ferritin particles. It is, of course, also possible that, while the "single-region openings" may have been the path of hemoglobin egress, there may be a second population of cells which rather develop "multiple-region openings" (with 150-A-wide holes) diffusely over the entire membrane (see also reference 41). Such cells could also be permeated by ferritin, but such small 150-A defects might not be detected in a 500 A–thick Epon section.

Membrane Pores Created by Surface-Active Agents

The lysolecithin-treated cells have holes about 300–400 A wide in the plane of the thin section. The saponin ghosts do not show membrane discontinuities although ferritin (120 A in diameter), subsequently added, was admitted into the fixed ghosts; the membrane holes, therefore, must be smaller than the section thickness (500–600 A).

APPENDIX

The equation used for calculating the diffusion coefficient is (34, 12) $D = \frac{4}{3} \pi r^3 \ln (C_0/C)/t\ 0.96\ d\ N$, where D is the diffusion coefficient of hemoglobin, $\frac{4}{3} \pi r^3$, the critical volume of the

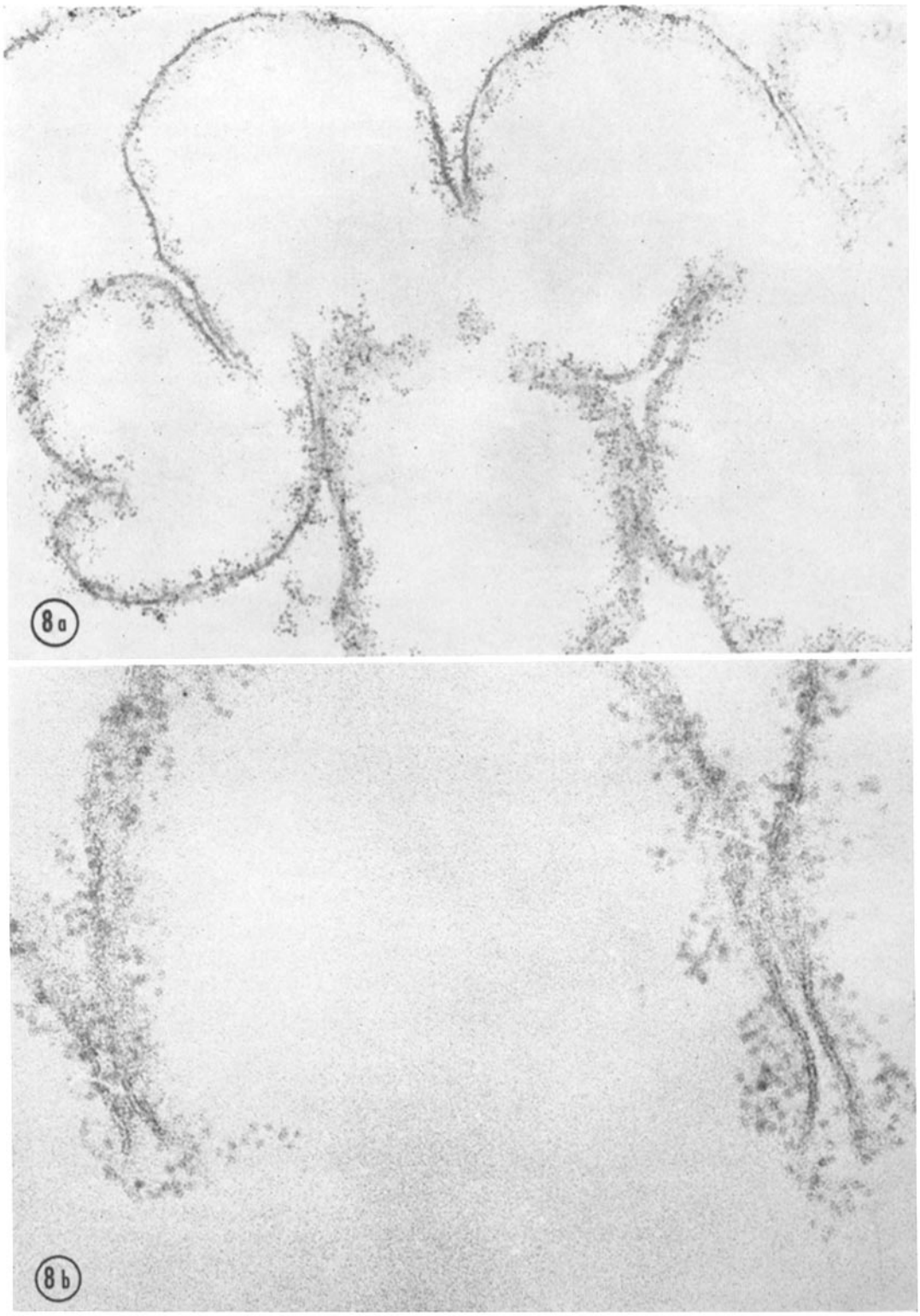

FIGURE 8 Demonstrating the existence of membrane defects with prolonged patency after hemolysis induced by lysolecithin.

FIGURE 8 *a* The cells were hemolysed in isotonic solution by lysolecithin, fixed in glutaraldehyde, washed, and then exposed to ferritin. The ferritin can be seen (as gray dots) on both sides of the cell membrane which appears scalloped. Elmiskop I. Uranyl and lead citrate stains. × 64,000.

FIGURE 8 *b* High magnification of another cell from the experiment of Fig. 8 *a*. The membrane at the base of each indentation appears discontinuous. Although the indentations created by saponin are similar and although ferritin permeates the saponin ghost, no such membrane discontinuities were observed. Elmiskop I. Uranyl and lead citrate stains. × 158,000.

cell just before the onset of hemolysis, C_o, the cell hemoglobin concentration at the critical volume, C, the final concentration in the supernatant and the ghost (which are the same; reference 7), t, the fading time, d, the diameter of an average hole (around 300 A), and N, the number of holes per cell. The average length or diameter of the zone or cluster of defects was 11,000 A; the number of defects, occurring every 1800 A or so, within a circle of diameter of 11,000 A is around $(11{,}000)^2\ A^2/(1{,}800)^2\ A^2$ or 37 holes. Since only one zone of holes was always seen per cell profile, it may be assumed that 37 ($\pm 50\%$) represents the holes per cell. The critical volume is roughly 2×10^{-10} cm^3 (35); this is 102% over normal (35) and will have a C_o of about 17.5 g% (36). For the *hole-development* and the *hole-fixation* series of experiments the dilution of the packed erythrocytes was 1:50 in the hypotonic medium; the final equilibrium concentration of hemoglobin, C, in both the ghosts and the supernatant would be 35/50 g (see reference 36). For t, the fading time, we may choose a value of 10 sec (see reference 40), which is roughly the same time that the transient holes remain open.

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