Osmotic Hemolysis of Chemically Modified Red Blood Cells

By Richard F. Baker and Naomi R. Gillis

The mechanism of osmotic hemolysis of red cells has been a subject for discussion for many years. Much of the discussion has centered on the question of whether hemoglobin diffuses out of the cell by way of a single large break in the membrane, or by way of many small openings. Kochen using light microscopy and a dye which formed a precipitate with hemoglobin, observed what appeared to be a single stream of hemoglobin emerging from the cell as well as a recoil of the cell in the opposite direction.

Danon using an ion exchange resin to effect a gradual decrease in salt content, made phase contrast cine pictures of hemolyzing red cells, and also examined shadow cast ghosts produced by gradual and rapid osmotic shock. It was concluded that slow hemolysis was characterized by hemoglobin diffusion over the entire surface of the cell, while rapid hemolysis probably resulted in a gross rupture. It was shown by Seeman however, using electron microscopy, that holes in the membrane of the order of 200 AU in diameter opened and closed during hypotonic hemolysis. The holes were localized in a circular zone about one micron in diameter.

The work reported here had its origin in an attempt to arrest hemolysis at various stages by the device of dialysis against a fixative in hypotonic solution. It was soon apparent, however, that the hemolytic phenomenon observed was the reaction of a red cell whose properties had been modified by exposure to the fixative. The phenomenon appeared to be enough interest to warrant the report which follows.

Materials and Methods

Human red blood cells were used throughout this study. They were obtained either by finger puncture or from the Hyland Blood Bank (Hyland Division, Travenol Labs. Inc., Los Angeles, California) in ACD-plasma. The cells were washed three times in 10 volumes of 0.15 M sodium chloride before use, except when the effect of multiple washes was studied. Glutaraldehyde (25 per cent stock, Fisher Scientific Company) was distilled before use, and diluted with either distilled water or with 0.15 M sodium chloride. Osmolarities were measured on a freezing point osmometer (Osmette Precision Systems Company, Framingham, Massachusetts).

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This work was supported by Grant HE-07976 from the National Heart Institute, U.S. Public Health Service.

First submitted April 2, 1968; accepted for publication August 5, 1968.

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Fig. 1.—Slide-coverslip preparation of red cells after six minutes dialysis against 0.2 per cent glutaraldehyde in distilled water. Five of the cells show well developed crowns; a small crown is seen on cell at lower right, and a detached crown at upper right. Phase optics—× 700

Dialysis

Dialysis was carried out by suspending 0.05 ml packed cells in 1 ml. of isotonic saline in a cellophane dialysis bag (Van Waters and Rogers, Inc., Los Angeles, California). The bag was immersed in 5 ml. glutaraldehyde in distilled water at room temperature. In individual experiments the glutaraldehyde concentration was varied between 0.05 per cent and 1 per cent. Samples of red cell population were withdrawn as a function of elapsed time from the start of dialysis. Slide coverslip preparations were examined by phase microscopy (Reichert). Pellets were made of larger aliquots which were postfixed with 1 per cent osmium tetroxide and embedded in Vestopal W for thin sectioning and electron microscopy.

Two-Step Method

0.05 ml. packed cells in 1 ml. of isotonic saline was mixed with 1 ml. of glutaraldehyde in isotonic saline. The glutaraldehyde concentration was variable. Samples were withdrawn as a function of time from the moment of mixing, and these cells then were osmotically shocked by introducing them to 5 volumes of distilled water. After 1 minute of exposure to distilled water the cells were effectively fixed with an excess of 5 per cent glutaraldehyde. Phase and electron microscopy were done as described above.

Separation of Cells by Age

A method designed for small blood samples was used for most of the determinations. Washed red cells which had been in ACD less than 6 days were centrifuged for 5 minutes at 11,500 rpm. in a microhematocrit centrifuge (Clay-Adams) following which the upper and lower 10 per cent of the column were isolated, corresponding to young and old fractions. The more elaborate and precise method of separation according to buoyant density segregation in a linear density gradient of bovine serum albumin was used on one occasion. The two fractions at either end of the column were taken as representative of young and old cells.

Results

With the glutaraldehyde concentration fixed at 0.5 per cent samples were taken at one minute intervals and examined by phase microscopy. Typically after 4-6 minutes of dialysis the sample consisted of 3 classes of cells: intact cells, ghosts, and partially hemolyzed cells. Many of the partially hemolyzed cells showed a precipitated mass of hemoglobin exterior to the cell but in contact with it over an area approximately one micron in diameter (Fig. 1). For the sake of convenience we have termed these protrusions “crowns.” All stages of crown development are seen in a typical preparation, from a slight

*The authors wish to thank Dr. R. C. Leif for assistance with this procedure.
Fig. 2.—Section through red cell showing characteristic features of hemolysis by way of a single membrane break. Hemoglobin concentration is highest at the break and lowest at the opposite pole of the cell. Note the broken membrane curled back on itself. × 23,400 Insert (corner) shows the membrane loop at higher magnification. × 40,000

dimple in the cell surface to a large mass of hemoglobin nearly detached from the cell. Detached crowns are often seen.

An electron micrograph of a thin section through a typical cell is seen in Figure 2, which illustrates the high concentration of hemoglobin near the break, with a corresponding low concentration at the opposite side of the cell. The broken membrane is seen curled around on itself on one side of the crown. Serial sections support the impression gained from phase microscopy that a single, approximately circular area is torn out of the cell membrane. Stages in crown development are seen in Figures 3a, b, c, d. Initial rupture takes place over an area a few hundred Angstrom units in diameter (Fig. 3a). The diameter of the maximum area of membrane disruption is of the order of one micron. In the late stages the crown is barely attached to the cell (Fig. 3d) and the membrane appears nearly intact. The small amount of hemoglobin remaining in the ghost is clumped, and is seen by phase
Fig. 3.—(A) Earliest stage of membrane disruption in red cell after glutaraldehyde treatment followed by osmotic shock. A membrane component is lifting off in the manner of a flap. A homogeneous material lies between the flap and the cell surface. \( \times 56,500 \). (B) Hemoglobin has started to emerge through an opening 0.5\( \mu \) in diameter. \( \times 32,700 \). (C) Large precipitated mass of hemoglobin is seen in this thin section both inside and outside the red cell. The membrane break is about 2\( \mu \) in diameter. \( \times 11,450 \). (D) Late stage of hemolysis. The hemoglobin mass is barely attached to the ghost. The membrane is nearly intact in the plane of this section. Note (arrow) the loops of membrane inside the ghost, in the vicinity of the break. \( \times 17,800 \).

microscopy as an intercellular granularity. Holes were never seen in ghosts which lacked crowns, either by high power phase microscopy or electron microscopy of serial sections.

Two Step Method

Results obtained by first exposing the red cells to glutaraldehyde in saline, then to osmotic shock, were similar to those obtained by dialysis. Using this method the variables of fixative concentration, time of fixation, and salt concentration around the cells could be varied independently. Figure 4 relates the efficiency of crown production in per cent of cells showing crowns to the time of fixation with a specified concentration of glutaraldehyde. The fixation time which corresponds to the maximum number of crowns is shown in Figure 4 as \( t_{\text{max}} \). For times of fixation less than \( t_{\text{max}} \) many of the cells hemolyze without producing a crown; for longer times the number of crowns decreases toward zero with prolonged fixation. Variations in glutaraldehyde concentration merely resulted in shifting the curve of Figure 4 to the right or left on the time scale without change in shape.

Repeated Washings

One volume of blood from ACD storage (6 days in ACD) at 4 C. was washed with 10 volumes of 0.15 M sodium chloride repeatedly at room tem-
Fig. 4.—Percentage of red cells showing crowns plotted as a function of time in minutes of fixation in 0.2 per cent glutaraldehyde. The fixation time corresponding to the maximum of the curve is denoted as $t_{\text{max}}$. The efficiency of crown production is low on the left side of the curve because ghosts are being formed; low on the right side because fixation has progressed too far to allow hemolysis.

Temperature, and a determination of $t_{\text{max}}$ was made after each washing. $t_{\text{max}}$ increased linearly with the number of washings as shown in Figure 5. Young cells consistently reached a peak of crown production at shorter times than did old cells.

Localization of Crown Sites

The optimum concentration and fixation time in glutaraldehyde for unwashed red cells (ACD storage) was determined. A conventional smear on a glass slide was made of cells after such optimal fixation for crown production. After rapid air drying the slide was immersed vertically into distilled water for 20 seconds. Phase examination of the wet slide showed that crown formation was restricted to the rim of the cell, that is, parallel to the plane of the slide (Fig. 6). No crowns were seen attached to the biconcave region of the cell. Control experiments (Fig. 7) showed that the cells adhered to the surface of the glass throughout the fixation and subsequent hemolysis, with the flat side of the biconcave disks lying parallel to the plane of the slide. Thus the orientation of the cells relative to the glass was fixed at all times.

Discussion

The first question to be discussed is as to whether the single break hemolysis described here represents the mechanism of normal osmotic hemolysis, or whether, in fact, the development of hemoglobin crowns is the reaction to
Fig. 5.—The effect of multiple washes in isotonic saline on crown production is shown here for a population of young cells (Y) and old cells (O). The fixation time for maximum crown production (\( t_{\text{max}} \)) increases with repeated washings. The maximum number of crowns in the case of old cells is reached at longer fixation times than for young cells.

Hypotonicity of a red cell whose properties have been modified by contact with fixative. Several lines of evidence indicate that the latter explanation is the correct one. It has been established\(^7,10\) that ferritin with a diameter of 110 AU, if present in the hemolyzing solution, will enter a red cell during hypotonic hemolysis. Particles of colloidal gold larger than 250 AU are excluded. The exclusion of colloidal material larger than 250 AU would not be consonant with the existence of an opening as large as one micron. It is relatively easy to produce crowns by a simultaneous application of fixative and hypotonicity, or by a prior treatment with fixative followed by hypotonic shock. However, crowns have not been demonstrable with the reverse procedure; that is, osmotic shock followed by fixative. It seems clear that the mechanical properties of the membrane have been altered by the fixative.

It has been shown by Seeman\(^7\) that pores in the membrane about 200 AU in diameter open and close during hypotonic hemolysis. The ghost so produced is impermeable to hemoglobin and may regain its former selectivity to ion transport.\(^11-14\) Clearly the membrane, although breached at many sites, has repaired itself. The evidence for membrane repair in the case of the much larger openings produced by crown extrusion may be summarized as follows. Ghosts with large holes in a wet preparation would be easily detectable with phase microscopy, but they are never seen although many detached crowns are seen. In thin sections the appearance of a ghost with an attached crown and little or no loss in membrane continuity could be interpreted as a cut through the edge of a large opening. However, serial sections through such a
Fig. 6.—Red cells treated with 0.25 per cent glutaraldehyde were spread on a glass slide by a conventional smear technique. Osmotic shock of the spread cells produced crowns which emerged in a direction parallel to the slide, i.e., localized on the rim of the cell. Some detached crowns are seen. Densities are reversed in this figure and crowns appear black. Phase optics × 700.

Fig. 7.—Control red cell smears demonstrating adherence of cells to glass slide after 0.25 per cent glutaraldehyde-saline fixation for 30 seconds, air drying and hypotonic hemolysis. (A) Dry smear. × 400 (B) Same field with cover slip after hemolysis. × 400.

cell as well as the examination of hundreds of random cell sections tend to support the view that the ghosts are not left with a large opening, that is, membrane repair has occurred.

It has been known since the aldehydes were introduced15 as a tissue fixative for electron microscopy that membranes are not well preserved by glutaraldehyde fixation alone. However, membranes were apparent if the tissue were postfixed in osmium tetroxide. Thus, it appears that lipids are not stabilized by the fixation and may be extracted by the dehydration and embedding procedures. Since it is likely that the lipid is a most important membrane constituent insofar as repair is concerned,16-18 it therefore seems plausible that membrane repair could take place even after mild glutaraldehyde fixation. Glutaraldehyde is an efficient cross linking agent for proteins, reacting with amino and imino groups. Thus it might be expected that membrane protein as well as hemoglobin will be denatured and stabilized by glutaraldehyde. Polymerization with formation of aggregates of hemoglobin will be the result of thorough fixation. Short periods of fixation of highly concentrated hemoglobin with low concentrations of glutaraldehyde might result in poly-
mers of hemoglobin too large to diffuse through pores of the normal diameter (200 AU). With internal pressure increasing due to water entry and diffusion via the normal small pores not possible, a large break in the membrane would result. On the other hand, it is possible that the small pores never open, and the pressure must be relieved by a large break.

If the membrane were homogeneous over its area, then it would be logical that the break would occur at random anywhere on the surface of the cell. This is not the case—the crowns appear exclusively on the rim of the cell pointing to a difference in the visco-elastic properties. There is evidence that shape determining factors of the red cell reside in the membrane. Heterogeneity in chemical constitution was not demonstrable, however, until Murphy showed by autoradiography with tritiated cholesterol, that cholesterol was concentrated around the rim. It is possible of course that differences in protein composition also exist over the cell surface. Too little is known at this time to permit any useful correlation of visco-elastic properties with membrane composition.

The shift of $t_{\text{max}}$ to longer times as a result of multiple washes in isotonic saline reported here may reflect an increasing osmotic fragility. That a corollary exists between osmotic fragility and $t_{\text{max}}$ is shown by the fact that old cells must be exposed to fixative for longer times than young cells for maximum crown production (Fig. 5). It has been abundantly documented that old red cells are osmotically more fragile than young red cells. Another indication of a difference in visco-elastic behavior between young and old red cells is that stromalytic forms are abundant in young cells but almost non-existent in old cells.

It may be more than coincidence that the area of membrane disruption for crown formation is about the same in shape and size as the area of hemoglobin extrusion observed by Kochen and that inferred by Seeman from electron micrographs of thin sections. It has been shown here that the membrane break for crown formation occurs only on the rim of the cell. The site of the membrane pores reported by Seeman is unknown; it would be a significant finding if they were shown to be localized also to the rim of the cell.

**SUMMARY**

The mechanism of osmotic hemolysis of human red blood cells has been investigated after mild fixation in glutaraldehyde. A mass of precipitated hemoglobin (crown) is seen around a single membrane break which may be as large as 2μ in diameter. Ghosts with large holes are not seen and it is believed that membrane repair takes place.

Hemoglobin extrusion by this mechanism takes place only around the rim of the red cell. Both old and young red cells exhibit crown formation, but old cells require longer fixation than do young cells.

A correlation with previous work on mode of osmotic hemolysis of red cells is discussed.

**SUMMARIO IN INTERLINGUA**

Le mechanismo del hemolyse osmotic de erythrocytos human eseva investigate post leve fixation in glutaraldehyda. Un massa de hemoglobina precipitate es vidite circum un
sol fissura membranal que pote attinger un diametro de usque ad 2 μ. Chimera con grande foraturas non es vidite, e il pare que reparo membranal es in progresso.

Extrusion de hemoglobin per iste mechanismo occurre solo circum le margine del erythrocytos. Le formation coronari occurre in erythrocytos tanto vetule como etiam juvene, sed erythrocytos vetule require un plus longe fixation que cellulas juvene.

Es commentate relationes del presente constatationes con previe studios relative al modos de hemolyse osmotc in erythrocytos.

REFERENCES


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