Preparation of Human Erythrocyte Ghosts in Isotonic Solution: Haemoglobin Content and Polypeptide Composition

M. Saleemuddin *, U. Zimmermann, and F. Schneeweiß

Biophysical Chemistry, Nuclear Research Center Jülich

(Z. Naturforsch. **32 c**, 627–631 [1977]; received May 4, 1977)

Electrical Haemolysis, Ghost Cell Preparation, Protein Composition

Human red blood cell ghosts were prepared by dielectric breakdown of the cell membrane and subsequent electrical haemolysis in isotonic and isoionic solutions. Almost complete removal of haemoglobin (to about 0.1%) could be achieved by subjecting the erythrocytes suspended in isotonic solution to three consecutive electric field pulses of 16 kV/cm in the presence of 10 mM EDTA, followed by dilution with EDTA-free isoionic medium. The cup-shaped ghost cells were electrically homogeneous. The polypeptide composition of the electrically prepared ghost cells, as analyzed by polyacrylamide electrophoresis in 1% SDS, revealed a pattern similar to ghost cells prepared by osmotic haemolysis. However, the band 6, corresponding to glyceraldehyde-3-phosphate-dehydrogenase protomer, was absent from the electrically prepared ghost cells. Also the band 8, which is eluted at low ionic strength, appeared to be very prominent in the electrical preparations.

Introduction

The red blood cell membrane undoubtedly represents the best studied plasma membrane, and considerable information is available regarding the properties, function and structural arrangement of its several components. However, whilst the lipid composition of the human red blood cell membrane appears to be fairly well established [1, 2], discrepancies exist with respect to composition of the membrane polypeptides. The principle reason for this controversy seems to be the perturbation of the ionic strength during the generally used preparation procedure of ghost cell membranes. Various hypotonic preparation methods exhibit markedly differing protein compositions [3] and by small changes of the ionic strength of the external medium approx. 30% of membrane proteins can be readily eluted [4–6].

Using the dielectric breakdown technique described by Zimmermann *et al.* [7–10], erythrocyte ghosts, which are normally distributed, electrically homogeneous and fairly impermeable to sucrose, can be prepared in isotonic and isoionic solutions. The procedure does not involve the introduction of compounds which can possibly react with or elute membrane components, and therefore appears to offer the advantage over other recently introduced preparation methods using isoionic solutions [11, 12].

* Permanent address: Chemistry Department, Aligarh Muslim University, Aligarh/India.

Requests for reprints to Prof. Dr. U. Zimmermann, Institute of Biophysical Chemistry, Nuclear Research Center Jülich GmbH, P.O. Box 1913, *D-5170 Jülich 1*.

It was therefore envisaged that ghost membranes obtained by electrical haemolysis would more closely reflect the physiological composition of the intact red blood cell membrane. Polypeptide composition of the electrically prepared human red blood cell ghosts and the experimental conditions which permit the almost complete removal of haemoglobin from the ghost cells in isotonic solution are described in the present communication.

Materials and Methods

Fresh blood obtained from apparently healthy donors was used. The red blood cells were centrifuged and washed several times with solution I (138.6 mM NaCl, 12.3 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4). For electrical haemolysis the cells were suspended in solution II (105 mM KCl, 20 mM NaCl, 4 mM $MgCl_2$, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, and 10 mM glucose; the pH of the solution was 7.2). A suspension density of $2-3 \times 10^8$ cells/ml corresponding to a dilution ratio of 1 : 40 of packed cells to solution was used, unless otherwise stated. The cell suspension was subjected to an exponentially decaying electric field pulse of either 12 kV/cm or 16 kV/cm with a 40 μs decay time. The discharge chamber and high voltage circuit and theory is described elsewhere [9, 13]. It should be noted that the precooled suspension (at 0 °C) was filled between the two flat platinum electrodes and was layered by cell-free solution in order to expose all of the cells in the suspension to the same electric field [14]. Immediately after the field application the suspension was diluted to the desired ex-

tent with cold solution II. Haemolysis was complete in approx. 5 min. The cells were then centrifuged, washed, and reincubated in solution II; the suspension density was adjusted to $2-3\times10^8$ cells/ml (dilution ratio approx. 1:40). Resealing was performed for 10 min at 0 °C followed at least by 20 min at 37 °C. The size distributions of the resealed ghosts were measured in a hydrodynamically focusing Coulter Counter (AEG-Telefunken, Ulm, West Germany), applying increasing electric field strengths in the orifice (60 μm in diameter and length). The haemoglobin content of the ghost cells was determined by measuring the absorption of the Soret band at 415 nm after haemolysing the ghost cells in distilled water. Small concentrations of haemoglobin were estimated by the pyridine hemachromagen method described by Dodge *et al.* [1]. The solubilization of ghost proteins, analysis of the polypeptide pattern of the ghost cell membrane with polyacrylamide gel electrophoresis in 1% SDS and the subsequent staining of the proteins and glycoproteins, was performed as reported by Fairbanks *et al.* [15].

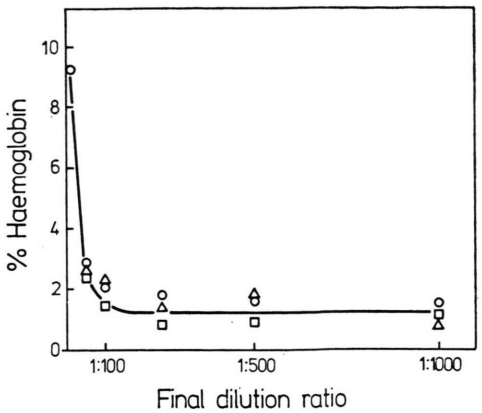

Fig. 1. Haemoglobin contents of the erythrocyte ghosts prepared by electrical haemolysis. Suspensions of the erythrocytes were subjected to the electric field pulse and adjusted to the final dilution ratios. ○—○, pulsed at a dilution ratio of 1 : 10 at 12 kV/cm; △—△, pulsed at a dilution ratio of 1 : 40 at 12 kV/cm; □—□, pulsed at a dilution ratio of 1 : 40 at 16 kV/cm. Omission of Mg^{2+} from the solution II (see text) produced no significant changes in the haemoglobin content of the ghosts.

Results and Discussion

Haemoglobin content of the ghost cells

Red blood cells were subjected to the electric field pulses (12 or 16 kV/cm; 40 μs duration) at two suspension densities (1 : 10 or 1 : 40 packed cells to solution). The pulsed suspension was diluted up to a dilution ratio of 1 : 1000 immediately after the field application. As indicated in Fig. 1, the haemoglobin content depends on the final suspension density (*i. e.* the suspension density at the time of haemolysis). However, it is independent of the suspension density at the time of field application. Within the limits of the accuracy of the measurements, the points of different runs coincide. The haemoglobin concentration decreases from about 10% at a dilution ratio of 1 : 10 to about 1% for a dilution ratio of 1 : 250. The haemoglobin content could not be lowered below this value by further dilution, nor was it possible to decrease the haemoglobin content by increasing both the field strength (to 16 kV/cm) and the number of field pulses injected into the suspension. Indeed, for ghost cells subjected to three consecutive pulses of 16 kV/cm the haemoglobin content does not seem to be significantly lower. These results suggest that in isotonic solutions a small amount of haemoglobin is strongly associated with the inner surface of the membrane. It is of interest to note that haemoglobin concentration of the ghosts can be readily lowered below 0.1% by osmotic haemolysis.

Almost complete removal of the haemoglobin can, however, be achieved by addition of EDTA during the application of the field pulse. EDTA induces a concentration dependent shift to very high values of the critical electric field strength required for electrical haemolysis. The "protective" effect of EDTA on electrical haemolysis can be used to generate several breakdown centers and/or enlarge the breakdown areas by increasing the number and strength of the electric field pulses injected into the solution without significant haemolysis [16]. Haemolysis occurs when the concentration of EDTA is lowered by dilution immediately after the field application. Under these experimental conditions the electrically induced permeability change in the membrane is much more pronounced and together with the chelating effect of EDTA haemoglobin can be removed more completely than in the experiments performed in the absence of EDTA. The almost complete release of haemoglobin from the cells pulsed in presence of EDTA may also be attributed to the release of haemoglobin in a synchronous manner from the cells upon dilution. Indeed it was possible to render the ghost cells essentially haemoglobin free ($\sim$0.1%) by subjecting the intact cells suspended in isotonic solution (dilu-

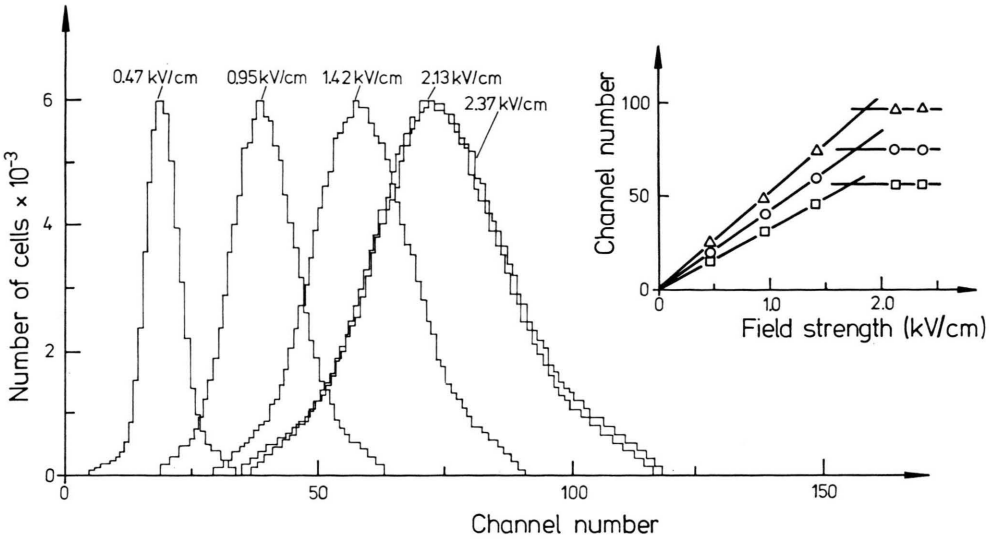

Fig. 2. Size distributions of human erythrocyte ghosts measured as a function of increasing electric field strengths in the orifice of the Coulter Counter. The ghost cells which were almost haemoglobin-free (approx. 0.1% haemoglobin) were prepared electrically from red blood cells suspended in isoionic solutions to which 10 mM EDTA was added. For experimental details, see text. The pulse heights (channel numbers) corresponding to different volumes of the size distributions (□ 91 μm^3; ○ 119 μm^3; △ 150 μm^3) plotted versus electric field strength (insert). The sharp discontinuity in the linear curves is induced by the dielectric breakdown of the cell membrane. Note that the sizes are normally distributed both in the subcritical and supercritical field range.

tion ratio 1 : 40) to three consecutive electric field pulses at 16 kV/cm in the presence of 10 mM EDTA, followed by dilution with the same solution containing no EDTA at 0 °C (final dilution ratio 1 : 1000). Best results were obtained when these lysed cells were centrifuged, washed at 0 °C, and resuspended in the solution II. To ensure complete resealing, it was necessary to extend the resealing time at 37 °C to 1 hour. Size distributions of ghost cells so obtained are shown in Fig. 2. At low electric field strength the sizes are almost normally distributed. The mean volume calculated from the size distribution determined in the low electric field range is about $110-120\ \mu m^3$. Preliminary measurements show, that the mean volume depends on whether or not Ca^{2+} is present in the resealing solution II. With increasing concentration (up to $1-2$ mM Ca^{2+}) the mean volume decreases. The data presented here refer to ghost cells resealed in solution II, which contains less than 10^{-5} M Ca^{2+}.

Above a critical external field strength in the orifice, an apparent under-estimation of the particle size occurs, due to the current flow through the cell interior. This is indicated in the inset of Fig. 2 by plotting the pulse heights (channel numbers) corresponding to different volumes as a function of increasing field strength in the orifice of the Coulter Counter. From the theory a linear relationship is expected [7]. However, a sharp discontinuity in the relationship is apparent indicating that dielectric breakdown has occurred. If the shift of the size distribution in response to dielectric breakdown is not accompanied with a skewness in the initially normally distributed size distribution, the conclusion can be drawn that the preparation is electrically homogeneous and consists of a single population [10]. Inhomogeneous populations which have either different shape factors or different membrane properties exhibit different breakdown voltages. This naturally produces a skewness in the distribution once breakdown of one of the populations has occurred [10].

As indicated in Fig. 2 skewness does not occur in the supercritical field range (*i. e.* at field strength beyond the critical value) for the haemoglobin free ghosts, which suggests that the ghost preparation is nearly electrically homogeneous. This conclusion is supported by phase contrast microscope observations. Under the phase contrast microscope the ghost cells appeared cup shaped and uniform. However, it should be pointed out that the phase contrast microscope is a much less sensitive tool to detect in-

homogeneities of the ghost cell population than the Coulter Counter. The nearly haemoglobin-free ghosts are quite fragile and within 1 hour of incubation at room temperature the ghost populations show skewness in the supercritical field strengths. On the other hand, the ghost cells containing more than 1% hoemoglobin are much more stable, which supports the suggestion that haemoglobin is essential for the stabilization of erythrocyte membrane.

Polypeptide composition

For the electrophoretical studies only ghost cell populations which were normally distributed and electrically homogeneous were used. Electrophoresis of electrically obtained ghost membrane proteins using polyacrylamide gels containing 1% SDS, yielded a pattern which was similar to that obtained for ghost cell membranes prepared by osmotic haemolysis. No significant differences could be observed in the glycoproteins of the electrically and osmotically prepared ghosts.

Typical electrophoretic runs of the protein composition of the ghost cell membranes prepared electrically at various degrees of dilution or subjected to EDTA treatment during the electrical haemolysis are presented in Fig. 3. There are two remarkable differences between the polypeptide composition of ghost cells prepared electrically and those subjected to hypotonic stress. Band 6 appears to be successively removed depending on the degree of dilution and on EDTA treatment, and band 8 is more prominent in all ghost cell preparations obtained by electrical haemolysis.

Band 6 is identified as the protomer of glyceraldehyde-3-phosphatedehydrogenase [16]. It has not been completely established as to whether or not this enzyme is associated with the red blood cell membrane *in vivo*. Association of the enzyme with specific sites of the inner membrane surface of the red blood cells has been demonstrated for membrane preparations in hypotonic solutions [18]. On the other hand, the elution of the enzyme by high ionic strength solutions and the absence of the enzyme in ghost cells prepared in isoionic solutions by glycol loading [12, 15] demonstrate that the presence of this enzyme in membrane fractions of osmotically prepared ghost cells is induced by the low ionic strength. This assumption is supported by experiments in which the resealed ghost cells prepared by the electrical haemolysis are subjected to hypotonic

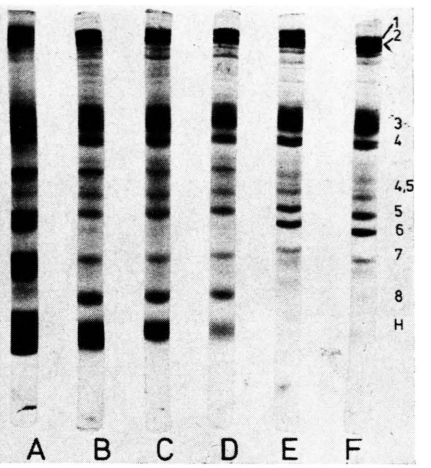

Fig. 3. Gel electrophoresis of the electrical and osmotic ghost preparations. Gels A–C, electrical ghosts prepared by application of a field pulse of 16 kV/cm to erythrocytes suspended at a dilution ratio of 1 : 10. The final dilution ratios were 1 : 10 (A), 1 : 100 (B), and 1 : 1000 (C). Gel D, ghost prepared by electrical haemolysis in the presence of 10 mM EDTA; Gel E, electrically prepared ghost (as Gel B) subjected to osmotic stress. After the application of the electric field pulse, the osmolarity of the suspension was lowered by dilution with water to 10 mOsm and the ghosts were washed with the 10 mOsm KCl solution; Gel F, osmotically prepared ghosts [15]. See text for experimental details. Because of the presence of varying amounts of haemoglobin and experimental conditions there are small variations in the amount of protein applied to the gels. The dark staining component at the Band 7 in Gel A is presumably the haemoglobin dimer. The protein bands are numbered according to Steck [20]. The fastest moving component is haemoglobin (H). The tendency of the band 4 to dissociate was more marked in osmotic preparations when the dye front was run to about 90 mm.

stress in the same solution in which the electric field induced haemolysis was performed; this solution contains the original enzyme present in the intact red blood cells. Electrophoretic runs of such ghost preparations (Fig. 3) indeed show band 6. This result shows that the sites which specifically bind glyceraldehyde-3-phosphatedehydrogenase in low ionic strength solutions are not affected by the high electric field strength pulse and also supports the suggestion that the enzyme is associated with the membrane only in low ionic strength solutions. It is also interesting to note that band 8 is removed from the electrically prepared ghost cells when they are subjected to hypotonic stress. The results reported here indicate that band 8 reflects a polypeptide normally present in the membrane of intact red blood cells. It should be pointed out that band 8 appears to be absent in ghost cell membrane pre-

parations obtained in isoionic solutions by glycol loading [12], presumably due to solubilization by glycol. However, these ghost cells, obtained by glycol loading seem to be impermeable to ATP which questions the role of the band 8 polypeptide in maintaining impermeability of the ghost cells to ATP [19].

From the results reported here we can conclude that significant differences in the polypeptide composition between ghost cells prepared electrically and those prepared by osmotic lysis exist, although, in general the polypeptide pattern from both preparations resemble each other very closely. The experiments do not rule out the possibility that the arrangement of the proteins within the membrane and the membrane integrity of both types of ghost cells may be quite different. This question can be only answered by ion transport measurerments or specific crosslinking experiments.

The work was supported by a grant to M. S. from the DAAD, Bonn Bad Godesberg, and from the SFB 160, DFG to U. Z.

[1] J. T. Dodge, D. Mitchell, and D. J. Hanahan, Arch. Biochem. Biophys. **100,** 119 [1963].
[2] R. I. Weed, D. F. Reed, and G. Berg, J. Clin. Invest. **42,** 481 [1963].
[3] F. H. Kirkpatrick and P. L. La Cella, Experientia **30,** 140 [1974].
[4] C. D. Mitchell and D. J. Hanahan, Biochemistry **5,** 51 [1966].
[5] S. A. Rosenberg and G. Guidotti, J. Biol. Chem. **244,** 5118 [1969].
[6] S. L. Marchesi and E. Steers, Jr., Science **159,** 203 [1968].
[7] U. Zimmermann, J. Schulz, and G. Pilwat, Biophys. J. **13,** 1005 [1973].
[8] U. Zimmermann, G. Pilwat, and F. Riemann, Z. Naturforsch. **29 c,** 304 [1974].
[9] U. Zimmermann, G. Pilwat, and F. Riemann, Biochim. Biophys. Acta **375,** 209 [1975].
[10] U. Zimmermann, G. Pilwat, and F. Riemann, Biochim. Biophys. Acta **436,** 460 [1976].
[11] P. S. Lin, D. F. H. Wallach, R. B. Mikkelson, and R. Schmidt-Ullrich, Biochim. Biophys. Acta **401,** 73 [1975].
[12] F. Billah, J. B. Finean, R. Coleman, and R. W. Mitchell, Biochim. Biophys. Acta **433,** 54 [1976].
[13] U. Zimmermann, G. Pilwat, F. Beckers, and F. Riemann, Bioelec. Bioenerg. **3,** 58 [1976].
[14] F. Schneeweiß, U. Zimmermann, and M. Saleemuddin, Biochim. Biophys. Acta **466,** 373 [1977].
[15] G. Fairbanks, T. L. Steck, and D. F. H. Wallach, Biochemistry **10,** 2606 [1971].
[16] U. Zimmermann, G. Pilwat, Chr. Holzapfel, and K. Rosenheck, J. Mem. Biol. **30,** 135 [1976].
[17] J. A. Kant and R. L. Steck, J. Biol. Chem. **248,** 8457 [1973].
[18] B. C. Shin and K. L. Carraway, J. Biol. Chem. **248,** 1436 [1973].
[19] T. A. Bramley, R. Coleman, and J. B. Finean, Biochim. Biophys. Acta **241,** 752 [1971].
[20] T. L. Steck, J. Cell. Biol. **62,** 1 [1974].