Effect of Preparative Procedures on Ghostcells from Bovine Erythrocytes

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Several methods for the preparation of "white ghosts" or "resealed ghosts" were described in the recent literature. This article compares three methods to prepare white, resealed ghosts from bovine erythrocytes based on the principle of hypotonic lysis. The methods described differ by the removal of hemoglobin from the empty cells. The main difference between the standard centrifugation, the gelfiltration and the hollow-fibre diafiltration is the mechanical stress on the leaky membranes after swelling in hypotonic media. Mean cellular volumes, rates of potassium-efflux and the access of impermeable dyes to cytoplasmatic proteins are criteria to differentiate between ghostcell-populations.

Introduction

For a number of physical studies on membrane phenomena of erythrocytes the deeply coloured hemoglobin has to be removed. The resultant "empty" particles surrounded by the membrane envelope are called "ghosts". The loading and subsequent separation of the loaded red blood cells from extracellularly administered substances is also important. In recent years efforts were made to prepare ghostcells from erythrocytes without damaging the membrane. Cells were obtained under conditions which guarantee a population of tightly resealed ghosts [1, 2]. The principal method described by several authors is based on hypotonic lysis followed by several washing-steps to remove the red blood cell dye in hypotonic media. Alternative methods for the preparation of ghosts in isotonic media [3, 4] or alternative possibilities for the removal of hemoglobin [5] are described. Recent investigations on membrane resealing and on properties of the ghost cells [6, 7] point out, that earlier assumptions on the resealing time of erythrocyte membranes in hypotonic media should be revised, *i.e.* the ghost-membranes begin to reseal even in hypotonic media. For flux- or permeation-experiments not only the intactness of the cell membranes is essential but also the volume to surface ratio should be known in order to determine permeability-coefficients. In addition to studies on erythrocyte membranes versatile methods should be available for the removal of extracellular material if erythrocytes are used as transport vehicles for encapsulated drugs.

Methods and Results

In our laboratory the water-exchange and the rate of pH-equilibration across membranes were measured on populations of ghostcells from human and bovine erythrocytes [8, 9]. The ghost cells were prepared by three methods:

i. standard centrifugation method (*e.g.* [10, 11]); lysis in hypotonic buffer (25 mosm) and several washing steps at low temperature with a maximal rca of $14{,}000 \times g$ followed by the restoring of physiological ionic strength and incubation at 37 °C;

ii. a gelfiltration method; cell membranes and hemoglobin are separated by gelchromatography; the combined membrane fractions are treated as described above;

iii. a hollow-fibre diafiltration method where hemoglobin is removed with the filtrate while cells (cell membranes) circulate through bundles of hollow-fibres.

The following buffers were used for the preparation procedures: potassium phosphate 5 mM, pH 7.4 (hypotonic, buffer A), potassium phosphate 5 mM, 150 mM NaCl, pH 7.4 (isotonic, buffer B). Fresh bovine blood (anticoagulated with Na-citrate) was

Abbreviations: A, membrane surface area; EITC, eosinisothiocyanate; P, permeability coefficient; SDS, sodiumdodecylsulfate; V, cell volume.

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obtained from the local slaughter-house. After washing in isotonic buffer (buffer B) by low speed centrifugation periods the red blood cells were lysed in buffer A (final osmolarity 25 mosM). All preparation steps were performed in the cold-room at 4 °C. In the routine centrifugation method hemoglobin was removed by 5 successive centrifugation steps in hypotonic buffer at 14,000 × g. After restoring of physiological salt concentrations resealing was completed for all samples by an incubation period of 1 h at 37 °C.

The gelfiltration method was performed using Sephacryl S-1000 columns (Pharmacia) with a total volume, V_t, of 150–300 ml. No lysis of intact erythrocytes was detectable when they were passed through the column in isotonic phosphate-buffered saline. Fig. 1A shows the separation of cellmembranes from hemoglobin (preparative scale; low resolution). Flow rates of 0.2–1.0 ml/min were useful for a baseline-separation of the hemoglobin from the membrane-fractions. In Fig. 1B eluation-profiles of intact erythrocytes in isotonic saline and NaCl (for the determination of the entrapped solvent volume) are superimposed on a chromatogram for the fractionation of erythrocyte-ghost from hemoglobin. The void-volume V_0 at about 95–100 ml contains the membrane- and erythrocyte-peak, while V_1 (solvent volume) at 190–200 ml includes the hemoglobin- and NaCl-fractions. Ghost-cells were measured by turbidity, hemoglobin by absorption spectroscopy and NaCl by conductivity, respectively.

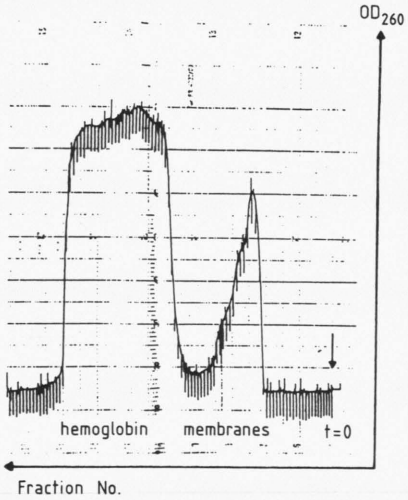

Fig. 1A: Separation-profile of bovine erythrocytes membranes from hemoglobin on a Sephacryl S-1000 column. The experiment was performed at 4 °C with a flow rate of 0.8 ml/min using a column with a V_1 of 200 ml (column length 60 cm). At $t=0$ maximal 5% of V_t (305 ml) lysed erythrocytes was layered on the gel-bed. Total time shown in Fig. 1A is 500 min.

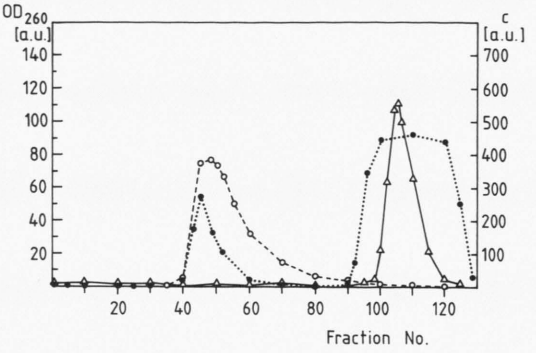

Fig. 1B. Determination of void-volume V_0 and bed volume V_1 of a Sephacryl S-1000 column using intact erythrocytes (in isotonic saline) (○) and NaCl (△). For direct comparison data-points of a preparation of erythrocyte ghosts are also shown (●). Temperature 4 °C, flow rate 0.8 ml/min, fraction-volume 2 ml.

The diafiltration through hollow-fibre walls was performed in a closed circuit apparatus allowing to perform lysis, hemoglobin-removal and concentrating of the ghosts in the same system. Shell and tube type modules consisting of bundles of capillary membranes with surface areas of about 0.5 m^2 from Enka AG, Wuppertal, were used. The modules were equipped with microporous polypropylene hollow fibres (average pore size 0.2 μm) with inner diameters of 330 μm and a wall thickness of 150 μm (ACCUREL^R, Enka AG). Fig. 2A exhibits schematically the construction of a diafiltration apparatus. In Fig. 2B the time-profile of the hemoglobin-elution and of the saltconcentration in the filtrate are shown. Compared with the time to prepare white erythrocyte ghosts by gelfiltration (about 4 h), a remarkable acceleration by a factor of at least 2 was reached with the diafiltration method. The possibility to increase the concentration of salts in the external medium before the hemoglobin is completely removed from the filtrate-cycle can lead to a further shortening of the time which the cells spend in the hypotonic medium. This is an advantage of the diafiltration over the centrifugation method.

After identical periods of resealing in media containing high K^+-concentrations (1 h at 37 °C) physical measurements were performed to characterize

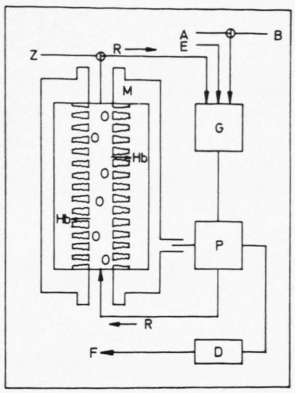

Fig. 2A. Principle of a diafiltration apparatus showing the hollow-fibre module M, inlets for hypotonic and isotonic buffer A and B, respectively, the detection-system D (OD or conductivity), inlet for cell-suspension E, compensation vessel G, the 4-channel peristaltic pump P, outlet Z and retentate- R, and filtrate- F-circuit. The pump P drives retentate- and filtrate-flux separately using tubes with different internal diameter to adjust flow-velocities.

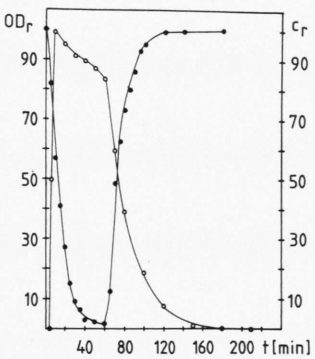

Fig. 2B. Time-course of hemoglobin-absorption (○) or saltconcentration (conductivity measurements) (●) at the detection-system D. OD_r and C_r refer to the relative hemoglobin absorption and electrolyte conductivity in percentage of the maximal values (at $t=0$), respectively. The apparatus was loaded with erythrocytes in isotonic saline in the retentate (R) and isotonic saline in the filtrate circle (F). At $t=0$ the filtrate is replaced by the hypotonic buffer A. In separate experiments for the determination of the NaCl-residence-time the salt-concentration was changed to isotonic buffer at 60 min.

the ghost populations. The measurements include the determination of K^+-efflux rates (K^+-sensitive electrodes or flame-photometry), the determination of mean cellular volumes (coulter counter) and, after labeling the membrane proteins with the amino-specific fluorophore eosinisothiocyanate (EITC), the determination of the ratio of accessible protein in the band 1, 2-region to stained integral band 3-protein. EITC was added to the resealed ghost. In intact red blood cells EITC is non-penetrating and only extracellular amino-residues are labeled.

The results presented in Table I reveal that ghost cells prepared by the centrifugation method have a 7-fold higher permeability for K^+-ions than those prepared by the gelfiltration-method. Diafiltration and gelfiltration lead to ghost-cells with 2- or 1−2-fold increased K^+-permeability compared with intact erythrocytes, respectively. Potassium exchange-rates, k, were determined from K^+-efflux curves shown in Fig. 3 by an exponential fit of the experimental data. Permeability-coefficients, P_K, were then calculated according to $P_k = k \cdot V/A$, with the volume to surface ratio V/A.

The mean cellular ghostvolumes V_m, determined by the coulter-counter-technique in isotonic saline after restoring of physiological salt-concentrations and incubation at 37 °C, show an increase in the order hollow-fibre < centrifugation < gelfiltration with only a slight difference of about 2−3 μm^3 for the alternative methods. The striking decrease of the V_m-values from intact erythrocytes to ghostcells (bovine erythrocytes) is in accord with recently published data on human erythrocyte ghosts showing a reduction of V_m by the factor of about 2.5 after 2 h in hypotonic media [7].

The membrane proteins were stained by covalently binding the amino-specific eosin-derivative, EITC, to the accessible NH_2-residues. The fluorophore was

Table I. K^+-permeability coefficients, P_K, cellular volumes, V_m, and ratio, $R(3/1, 2)$, of the membrane proteins stained by EITC for the three ghost preparations compared with values for intact bovine erythrocytes.

Method	P_K [cm/s][a]	V_m [μm^3][b]	$R(3/1, 2)$[c]
centrifugation	6.4×10^{-9}	14	0.5 − 1
hollow-fibre	2.0×10^{-9}	12	1 − 2
gelfiltration	1.0×10^{-9}	16	3 − 10
erythrocytes	0.9×10^{-9}	49	>100

[a] Permeability-coefficient for K^+ determined from K^+-efflux measurements according to $P_K = k \cdot V/A$ with $A = 90$ μm^2 (bovine erythrocytes and V-values listed in the second column.

[b] Mean ghostcell volume determined using the coulter-counter technique.

[c] Ratio of EITC-labeled band 3-region to band 1, 2-region from quantitative scans of the fluorescence intensity of the SDS-electrophoresis gels.

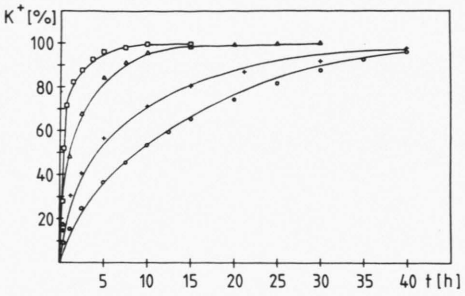

Fig. 3. Efflux of K^+-ions from resealed bovine erythrocyte ghosts prepared by the three different methods: gelfiltration (+), hollow-fibre diafiltration (△) and centrifugation (□). For comparison data from intact bovine erythrocytes (○) are included. The cells were loaded with high K^+-concentrations (K^+ replacing Na^+ in buffer B) and washed into a K^+-free buffer B. Extracellular K^+ was continuously monitored with a K^+-sensitive electrode in stirred solutions at 20 °C at times t (K^+_t) and after lysis ($K^+\infty$). K^+ [%] is defined according to K^+ [%] = $K^+_t/K^+\infty \cdot 100$.

added to the resealed ghosts. After short incubation periods the ghosts were separated from the unreacted EITC. Micrographs by phase contrast and eosin-fluorescence do not reveal major differences of the ghost populations prepared by the three methods. The membranes were further solubilized and SDS-gelelectrophoreses were performed. The gels were quantitatively scanned for the eosin-fluorescence on the stage of a fluorescence microscope. The fluorescent bands were compared with the bands after coomassie-blue staining. Controls (intact erythrocytes) exclusively showed the staining of band 3 even after prolonged incubation times with EITC. The appearance of stained band 1, 2-protein might be caused by the presence of leaky membranes or membrane fragments in the respective preparation. From *R*-values presented in Table I it is obvious that only the gelfiltration method is suited to preserve the membrane integrity and does not lead to a high degree of band 1, 2-staining. The centrifugation and the hollow-fibre diafiltration seem to produce more membrane-fragmentation during the preparation period. Circulation of intact erythrocytes in isotonic saline by the peristaltic pump used for the diafiltration experiments did not lead to a detectable lysis and band 1, 2-staining.

Discussion and Conclusion

Ghosts from bovine erythrocytes were prepared by three methods of hemoglobin removal. The ghosts obtained were characterized by three physical parameters: potassium permeability, volume and accessibility of amino-groups of membrane proteins to extracellular dye molecules. The comparative study underlines the influence of mechanical stress during the preparation procedure on ghostcells from bovine erythrocytes. It is assumed that the surface area, A, does not change as consequence of the three procedures.

The passive potassium-efflux shows a sequential increase of K^+-permeability from gelfiltration to hollow-fibre diafiltration and to centrifugation. In terms of the potassium permeation intact erythrocytes and ghosts prepared by the gelfiltration are comparable. The volume, V, and therefore the V/A-ratio changes by a factor of 2–3 within the preparation procedures. Thus for permeability measurements the volume has to be determined in parallel with the flux-rates.

The main factor concerning the integrity of membranes from ghostcells seems to be mechanical (shear) stress during the preparation period. Compared with the gelfiltration method the centrifugation and hollow-fibre diafiltration lead to a larger amount of cytoplasmic proteins or membrane fragments accessible to the extracellularly applied dye EITC.

In our hands gelfiltration is well suited to prepare almost intact erythrocyte ghosts on a laboratory scale for physicochemical measurements. Otherwise the necessity to handle large volumes in short preparation times could best be solved by the hollow-fibre diafiltration. If the intracellular hemoglobin has not to be removed completely, the time-period in hypotonic buffer can be shortened. This results in a smaller reduction of the ghost-cell volume as was recently verified in flow-dialysis experiments aimed to load red blood cells with drugs [12]. The standard centrifugation procedure has to be carefully inquired from case to case.

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[1] G. Schwoch and H. Passow, Molec. Cellular Biochem. **2,** 197–218 (1973).
[2] H. Bodemann and H. Passow, J. Membrane Biol. **8,** 1–26 (1972).
[3] S. Jausel-Huesken and B. Deuticke, J. Membrane Biol. **63,** 61–70 (1981).
[4] F. Schneeweiss, U. Zimmermann, and M. Saalemuddin, Biochim. Biophys. Acta **466,** 373–378 (1977).
[5] A. Faure, J. Delrieu, and M. Caron, Lectins: Biol., Biochem., Clin. Biochem. **3,** 661–665 (1983).
[6] B. Lee, K. McKenna, and J. Bramhall, Biochim. Biophys. Acta **815,** 128–134 (1985).
[7] G. B. Nash and H. J. Meiselman, Biochim. Biophys. Acta **815,** 477–485 (1985).
[8] H. Pitterich and R. Lawaczeck, Biochim. Biophys. Acta **821,** 233–242 (1985).
[9] H. Pitterich and R. Lawaczeck, J. Membrane Biol., submitted for publication (1987).
[10] J. T. Dodge, C. Mitchell, and D. J. Hanahan, Arch. Biochem. Biophys. **100,** 119–130.
[11] D. J. Hanahan and J. E. Ekholm, Methods Enzymol. **31A,** 168–172 (1974).
[12] C. Ropars, M. Chaissagne, M. C. Villereal, G. Avenard, C. Hurel, and C. Nicolau, Bibliotheca haemat. **51,** 82–91 (1985).