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Selective release of integral proteins from human erythrocyte membranes by hydrostatic pressure

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The overt effect of pressure on biological membranes is mediated predominantly through lipid condensation and disintegration of cytoskeletal polymers. These may lead to selective shedding of integral proteins, which could then be isolated by conventional means. In this study we have used the well characterised human erythrocyte membrane in order to establish the technical requirements for future use of pressure, as an alternative to detergents, in isolation of membrane proteins. Pressure of varying magnitude (300–1640 bar) and duration (5–60 min) was applied on human erythrocyte ghost membranes in suspension at different temperatures (4, 24 and 37°C) and in the presence of various solutes. After ultracentrifugation protein and lipids remaining in the supernatant were quantified and analysed. It is indicated that selective integral membrane proteins can be shed off under defined conditions and presumably remain in solution by the support of strongly associated phospholipids and specific solutes. On the basis of our findings a series of technical recommendations for the isolation of specific membrane proteins is outlined.

Introduction

Effects induced by hydrostatic pressure (P) on a thermodynamic system relate to the energetic quantity $P\Delta V$, where ΔV is the corresponding change in specific volume of the complying elements. Non-compressible compartments remain essentially unaffected by pressure [1]. When a biological membrane is subjected to hydrostatic pressure below approx. 2000 bar (1 bar = 1.013 atm) the fluid lipid domains, which are highly compressible, condense and thus become more viscous and more ordered. In addition, assemblies of proteins dissociate down to monomers followed by decrease in specific volume due to the filling of intermolecular vacuous spaces with water [2]. The microscopic events involved in the reduction of specific volume include strengthening of hydrogen bonds and weakening of ionic and aliphatic-hydrophobic interactions [1]. At this range of pressure the effects on water structure and density or on tertiary structure of monomeric proteins is small and for all practical considerations negligible [1,2].

While under pressure, membrane proteins are likely to undergo a significant rearrangement due to the decrease in both lipid fluidity [3] and electrostatic interactions [1], in addition to the elimination of diffusion barriers upon dissociation of the underlying cytoskeletal assemblies and dissociation of physical bonding between integral and cytoskeletal proteins [2,4]. At this new equilibrium position integral membrane proteins are expected to be more exposed by a magnitude which corresponds to the ambient pressure and at a defined threshold-pressure specific proteins are expected

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to be shed off the membrane into the supernatant [5]. This rationale has been previously employed for the shedding and isolation of blood group antigens from human erythrocytes [5] and immunogenic proteins from tumour cells [6]. Future use of this method as a tool for isolation of integral membrane proteins entails, however, a systematic study on a well characterised system. We have therefore undertaken to investigate the ambient factors involved in the release by pressure of integral proteins from the human erythrocyte membrane, as presented in this communication.

Materials and Methods

Membranes. Erythrocytes were isolated from freshly drawn human peripheral blood and washed three times with Tris-HCl buffered saline (pH 8.0). Erythrocyte ghost membranes were then prepared essentially as described [7], except that 5 mM Tris-HCl buffer (pH 8.0) was used for haemolysis. Most experiments were carried out with membrane samples of 1 mg protein/ml.

Hydrostatic pressure. For pressure application on samples of small volume (less than 2 ml) the membrane suspension was placed in a plastic capped tube and the sample buffer was added to the top. After sealing, a short 22 G needle was inserted through the cap to act as a vent for pressure equilibration. Air bubbles were avoided. The tube was placed in the cavity of a 45 ml pressure bomb (French Press cell, Aminco) and covered with the sample buffer. The bomb was then sealed without air space and subjected to hydrostatic pressure with a French Press (Aminco). For samples of larger volumes the membrane suspension was filled directly into the cavity of the pressure bomb. Hydrostatic pressure was gradually applied at a rate of 250 bar/min maintained for up to 60 min at the desired pressure and thereafter slowly released. The sample was ultracentrifuged ($150000 \times g$, 90 min, 4°C) and the supernatant collected.

Chemical analyses. Proteins were assayed according to Bradford [8]. For lipid determination samples were first dried by lyophilisation, treated with isopropanol and one hour later supplemented with equal volume of chloroform [9]. After two hours of incubation at room temperature the organic solvent was separated by centrifugation, partially evaporated under nitrogen and then used for phospholipid [10] and cholesterol [11] determination.

SDS-polyacrylamide gel electrophoresis. This was performed as described [12] using a 7.5 to 15% acrylamide gradient for the general protein pattern stained by Coomassie blue and a 12.5 to 20% acrylamide gradient for the glycoprotein pattern stained by periodic acid-arsenite-sulfite [7].

Modulation of lipid fluidity. Membrane rigidification was carried out by incorporation of cholesteryl hemisuccinate (Sigma) as previously described [13]. Briefly, erythrocytes ($2 \cdot 10^7$/ml) were incubated in Tris-HCl buffered saline (pH 8.0), containing 3.5% polyvinyl pyrrolidone (M_r 40000 Sigma), 1% bovine serum albumin and 100 μg/ml cholesteryl hemisuccinate at room temperature for 8 h. Treated erythrocytes were then washed three times with Tris-HCl buffered saline and processed for membrane preparation as described above. Membrane fluidisation was achieved by addition of benzyl alcohol (up to 1%) into the membrane suspension [14–16]. Change of membrane fluidity was monitored by the conventional fluorescence polarisation method with 1,6-diphenyl-1,3,5-hexatriene as a probe [17].

Results

When hydrostatic pressure is applied on an assembly of biological molecules, dissociation into smaller units takes place at the lower scale of the pressure (i.e. below 2000 bar) [1,2] while at a higher pressure protein denaturation may take place. This study was therefore limited to pressures below 1700 bar. In addition, conventional laboratory presses are limited to such pressure levels.

In preliminary experiments, where pressure was applied on membranes suspended in Tris buffer alone, a substantial amount of the shed proteins precipitated after ultracentrifugation, presumably due to aggregation. Different solutes, known to support solubility of hydrophobic proteins [18], were therefore tested for increase in yield of shed proteins remaining in solution after ultracentrifugation. The tested solutes were sucrose (2–10% w/v), dextran (M_r 150000; 2% w/v), polyvinyl

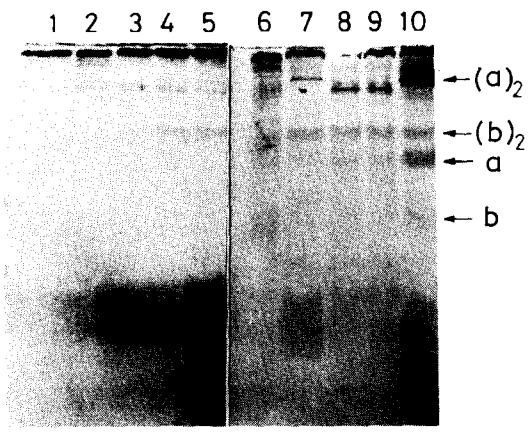

Fig. 1. Pattern of glycoproteins (50 μg/lane) after SDS-polyacrylamide gradient (12.5–20%) gel electrophoresis. Lanes 1–5: ultracentrifuged supernatants from pressurised (60 min, 24°C, 2% sucrose) human erythrocyte membranes. Lane 1, 328 bar; lane 2, 656 bar; lane 3, 985 bar; lane 4, 1313 bar; lane 5, 1640 bar. Lanes 6–9: Supernatants from pressurised (1640 bar, 24°C, 45 min) human erythrocyte membranes. Lane 6, no sucrose; lane 7, 2% sucrose; lane 8, 3% sucrose; lane 9, 4% sucrose. Lane 10, non-pressurised human erythrocyte membranes. Notations used: dimer of glycophorin a = $(a)_2$; dimer of glycophorin b = $(b)_2$; glycophorin a = a; glycophorin b = b.

pyrrolidone (M_r 40000, 2% w/v) with or without 0.34 M NaCl [18]. Of these sucrose and dextran without added NaCl were found to increase the yield of integral membrane proteins in the final supernatant, as evaluated by SDS-acrylamide gel electrophoresis (data not shown). The optimal concentration of sucrose was found to be 2–4%. At this range of sucrose concentration variations in the yield of particular integral proteins were observed (Fig. 1). Higher sucrose concentrations (up to 10%) caused a progressive decrease in the yield of integral membrane proteins in the supernatant.

Human erythrocyte ghost membranes (about 1 mg protein/ml) in 5 mM Tris-HCl buffer (pH 8.0) containing 2% sucrose (w/v) were subjected to increasing levels of hydrostatic pressure for 60 min at 24°C. Proteins, glycoproteins and lipids were analysed in the supernatant after ultracentrifugation as described in Materials and Methods. The results for protein and phospholipid release are shown in Fig. 2. Cholesterol was undetectable. The presence of integral membrane proteins in the ultracentrifuged supernatant was evaluated by SDS-polyacrylamide gel electrophoresis using either periodic acid-arsenite-sulfite or Coomassie

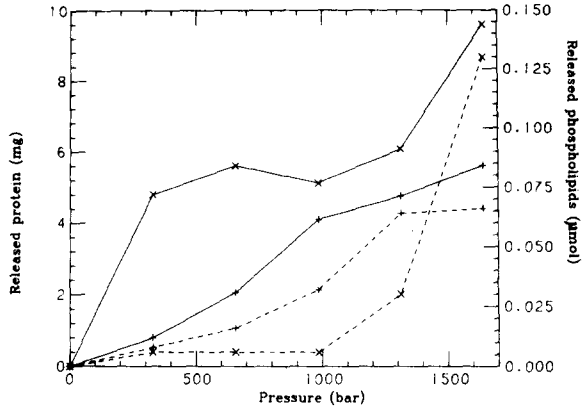

Fig. 2. Pressure profiles of protein (solid lines) and phospholipid (dashed lines) release from human erythrocyte membranes (20 mg in 20 ml Tris-HCl buffer (pH 8.0)) upon application of 1640 bar at 24°C for 60 min in the presence of 2% sucrose (+) or for 15 min in the presence of 2% sucrose and 1% benzyl alcohol (×).

blue staining. Protein release started at pressures of about 300 to 400 bar, but substantial amounts of phospholipids and integral proteins (monomer of glycophorin a and dimer of glycophorin b) were released only above about 1000 bar (Fig. 1), each integral protein at its own threshold level. Band 3 protein and the dimer of glycophorin a were not present in the supernatant. The identity of the protein, which constitutes the strong band below the dimer of glycophorin a (Fig. 1, lanes 8 and 9) is unknown, but could be attributed to a mixed dimer of glycophorin a and b.

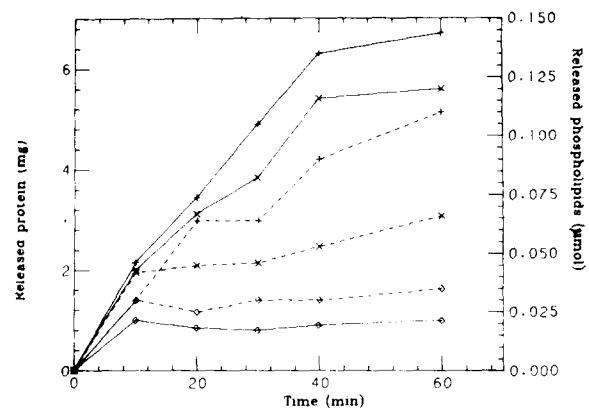

Fig. 3. Time profiles of protein (solid lines) and phospholipid (dashed lines) release from human erythrocyte membranes (20 mg in 20 ml Tris-HCl buffer, pH 8.0 containing 2% sucrose) upon application of 1640 bar at 4°C (◇), 24°C (×) and 37°C (+).

TABLE I

PROTEIN AND PHOSPHOLIPIDS RELEASED BY HYDROSTATIC PRESSURE FROM HUMAN ERYTHROCYTE MEMBRANES UNDER DIFFERENT CONDITIONS

	Protein (mg)	Phospholipid (μmol)	Protein/phospholipid (mg/μmol)
Erythrocyte membranes	20.0	$\approx 1.8 \cdot 10^{4}$	$\approx 10^{3}$
No sucrose;			
1640 bar, 45 min, 24°C	3.9	0.03	130
2% sucrose;			
1640 bar, 60 min, 24°C	5.6	0.06	84
2% sucrose; 1% benzyl alcohol;			
1640 bar, 15 min, 24°C	9.6	0.10	72
2% sucrose;			
1640 bar, 45 min, 24°C; membranes pretreated with cholesteryl hemisuccinate	4.16	0.06	69

The rate of protein release upon application of 1640 bar was determined at 4, 24 and 36°C. The time profiles and the concomittant values for phospholipid release are shown in Fig. 3. Increase in temperature was found to increase the yield of protein and phospholipid release, most significantly between 4 and 24°C. At 4°C hardly any material was recovered in the ultracentrifuged supernatant, while at 24 and 36°C plateaus were reached around 60 min pressurisation.

Erythrocyte membranes were rigidified or fluidised by incorporation of cholesteryl hemisuccinate or benzyl alcohol, respectively (see Materials and Methods), prior to pressure application in the presence of 2% sucrose (see above). When compared to untreated membranes the overall yield of integral and peripheral proteins shed off the cholesteryl hemisuccinate-treated membranes at 1640 bar for up to 45 min at 24°C was reduced (see Table I and Figs. 4 and 5), yet the dimer of glycophorin a, which was absent in the supernatant of the untreated pressurised membranes, became apparent (Fig. 6). Intact erythrocyte as well as ghost membranes can be readily fluidised by benzyl alcohol (see Fig. 7). At concentrations of up to 1.5% (v/v) benzyl alcohol does not haemolyse intact erythrocytes (Fig. 7). In the presence of 1% benzyl alcohol (v/v) and 2% sucrose the yield of peripheral and integral proteins were markedly increased at a pressure of 1640 bar (24°C), in particular that of the dimer of glycophorin a (Fig. 6). In addition, the rate of protein and phospholipid release was considerably faster, reaching a plateau after about 15 min (Figs. 2, 4, 5). The release of the dimer of glycophorin b started at pressures as low as 330 bar, whereas a pressure of at least 655 bar was required to release glycophorin a and and its dimer into the supernatant (Fig. 6). The anion transport protein (Band 3 protein) was not released at any of the applied

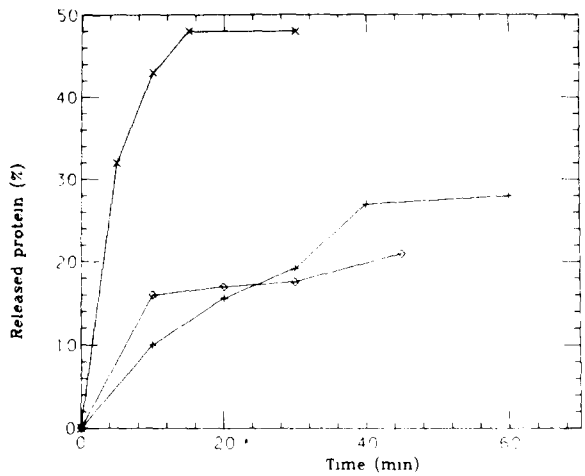

Fig. 4. Time profiles of protein release from human erythrocyte membranes in the presence of 2% sucrose (+), 2% sucrose and 1% benzyl alcohol (×) or from cholesteryl hemisuccinate-pretreated membranes in the presence of 2% sucrose (◇) upon application of 1640 bar at 24°C.

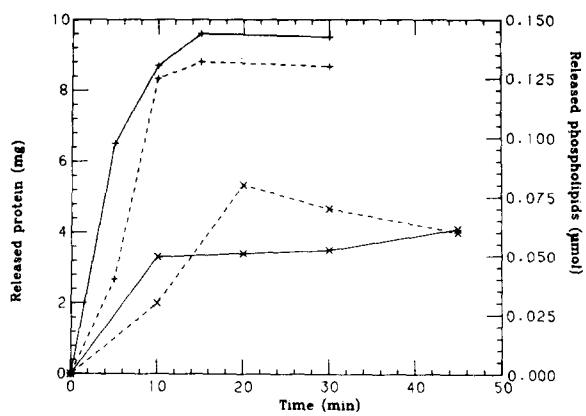

Fig. 5. Time profiles of protein (solid lines) and phospholipids (dashed lines) release from human erythrocyte membranes (20 mg in 20 ml Tris-HCl buffer (pH 8.0)) in the presence of 2% sucrose and 1% benzyl alcohol (+) or from cholesteryl hemisuccinate-pretreated membranes in the presence of 2% sucrose (×) upon application of 1640 bar at 24°C.

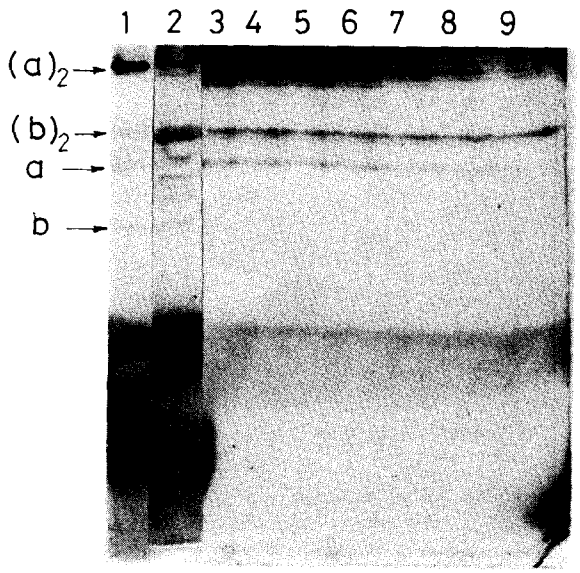

Fig. 6. Pattern of glycoproteins (50 μg/lane) after SDS-polyacrylamide gradient (12.5–20%) gel electrophoresis. Lane 1, non-pressurised human erythrocyte membranes; lane 2, ultracentrifuged supernatant of pressurised (1640 bar, 45 min, 24°C, 2% sucrose) membranes pretreated with cholesteryl hemisuccinate; lanes 3–9, ultracentrifuged supernatants of membranes pressurised in the presence of 2% sucrose and 1% benzyl alcohol. Lane 3, 1640 bar, 15 min; lane 4, 1640 bar, 10 min; lane 5, 1640 bar, 5 min; lane 6, 1313 bar, 15 min; lane 7, 985 bar, 15 min; lane 8, 656 bar, 15 min; lane 9, 328 bar, 15 min. Notation used: dimer of glycophorin a = $(a)_2$; dimer of glycophorin b = $(b)_2$; glycophorin a = a; glycophorin b = b.

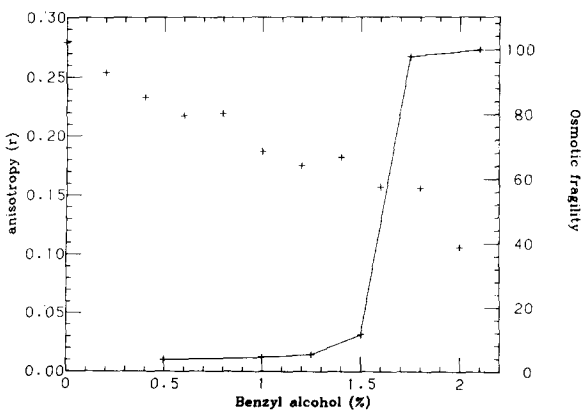

Fig. 7. Dependence of osmotic fragility (+ ——— +) of intact human red blood cells and fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene-labelled human erythrocyte membranes on the percent of benzyl alcohol in phosphate-buffered saline.

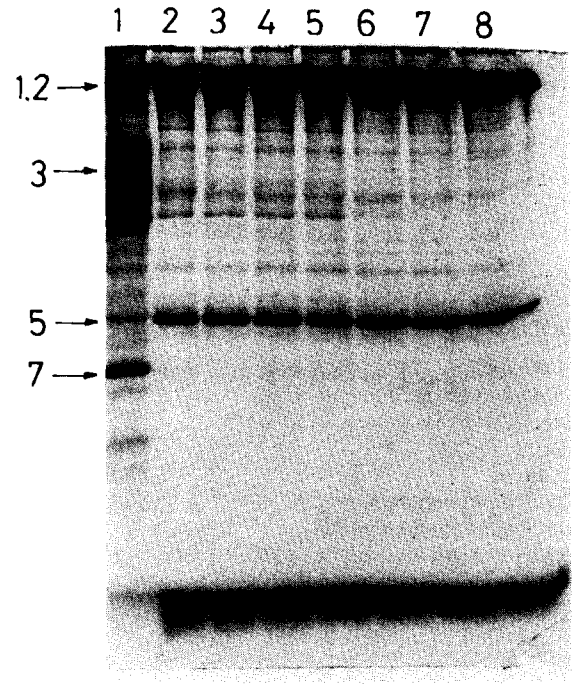

Fig. 8. Pattern of proteins (50 μg/lane) stained with Coomassie blue after SDS-polyacrylamide gradient (7.5–15%) gel electrophoresis. Lane 1, non-pressurised human erythrocyte membranes. Lanes 2–8 are equivalent to lanes 3–9 in Fig. 6. 1, 2 = spectrin (240000 and 220000); 3 = anion-channel protein (95000); 5 = actin (43000); 7 = band 7 protein (29000).

pressures (Fig. 8). Under these conditions peripheral proteins were shed in substantial amounts already below 300 bar and almost quantitatively at 1645 bar (data not shown). It is noteworthy, that in the presence of 2% sucrose 0.25, 0.5, 0.75 and 1% benzyl alcohol were equally effective, whereas in the absence of sucrose 1% benzyl alcohol was required to obtain the same results (data not shown). At 4°C the presence of 1% benzyl alcohol increased the release of peripheral membrane proteins, while almost no integral proteins and phospholipids were shed off at this temperature.

Discussion

Virtually all conventional methods currently used for the isolation of integral membrane proteins employ detergents for solubilisation. However, due to the replacement of the lipid annulus by detergent molecules, the solubilised protein is

likely to irreversibly loose its activity. An alternative approach is based on passive shedding of membrane proteins by hyperrigidification of the lipid bilayer. This can be achieved by chemical (e.g. increase in cholesterol) or physical (e.g. hydrostatic pressure) means or their combination [5].

The effect of pressure, which is solely induced by decrease in volume, is expressed as the condensation of the complying elements. The microscopic events, which are involved in the effect of pressure, are primarily weakening of aliphatic-hydrophobic and ionic bonds and strengthening of hydrogen bonds [1]. Therefore, when hydrostatic pressure of up to approx. 2000 bar is imposed on a biological membrane, the free volume of the lipid bilayer is reversibly reduced while proteins and water, which are considerably less compressible [1,2], are only slightly affected. The increase in density or microviscosity of the membrane lipid layer by pressure may induce lateral and vertical displacements of diffusible membrane proteins directed towards a new structural equilibrium. It is therefore expected that under such conditions some of the membrane proteins are shed off to the external medium. In addition, protein-protein interactions in the cytoskeletal network, as well as between the cytoskeleton and integral membrane proteins, is largely reduced under pressure [4], which facilitates the shedding process.

The membrane of human erythrocytes is well characterised and can therefore serve as an adequate system for studying the various technical parameters affecting the solubilisation of membrane proteins. With this as a model system, we have optimalised the magnitude of hydrostatic pressure, its duration, the effects of temperature as well as inter- and intramembrane additives for shedding of integral membrane proteins. Application of hydrostatic pressure above approx. 1000 bar was found to release integral membrane proteins into the supernatant. The amount of the released protein increased with the magnitude of pressure reaching maximum level in about 60 min at 25 or 36°C. Interestingly, phospholipids were found to be concomitantly released at a similar rate observed for integral proteins. It is plausible, that these phospholipids constitute the lipid annulus of the proteins. Their relative amount, however, was in the range of 1 μmol phospholipids per 100 mg protein (Table I), which is presumably by far too small to retain the proteins in a monomeric form in the aqueous environment. This metastable state might explain the tendency of the proteins in the supernatant to precipitate during ultracentrifugation. The use of solutes served therefore two simultaneous tasks, loosening the membrane boundaries and supporting the solubility of the released membrane proteins. The combination of sucrose and benzyl alcohol (2–4% and 0.25–1%, respectively) was found optimal. Under these conditions release of protein started well below 1000 bar and levelled off after about 15 min of pressurisation. The predominant action of the solutes may therefore be interpreted as to prevent protein aggregation and to a lesser extend to modulate lipid fluidity. However, it may be postulated, that in other membranes, where shed proteins are more densely coated with annulus phospholipids, solubilised proteins will be less susceptible to aggregation and therefore the amount of added solutes could be reduced accordingly.

As might be expected, virtually all peripheral proteins in the human erythrocyte membrane (spectrin, actin and bands 4.1, 4.2, 5 and 6) are readily released by hydrostatic pressure (Fig. 8). Among the integral proteins of the human erythrocyte membrane the correspondence to hydrostatic pressure varied: Glycophorin a and glycophorin b were released in substantial quantities, while the anion channel protein (Band 3 protein) was virtually unaffected. Other minor bands of integral protein characteristics also appear in the pressurised membrane supernatant (Figs. 1 and 6).

Conventional techniques for isolation of integral proteins, where the use of detergent is a prerequisite, have a series of drawbacks including loss of activity, due to the substitution of annulus lipids by detergent molecules, and technical difficulties in removing the detergent in later stages. The pressure technique, which is described in this study, is carried out under much milder conditions and seems to surpass the above mentioned difficulties. For convenience, we include some recommendations for the application of this method on other membranes:

(1) First trials should be carried out at room temperature and approx. 1600 bar for about 30 min.

(2) In cases of low yield, the addition of sucrose (2–4%, w/v) and benzyl alcohol (0.25–1.00%, w/v) prior to application of pressure, is recommended.

(3) In case of a particular integral protein which resists shedding hydrostatic pressure should be applied on cholesteryl hemisuccinate rigidified membranes and in the presence of sucrose and benzyl alcohol.

(4) Once the above conditions are established lower pressures could be applied for evaluation of the pressure threshold required for shedding of the desired protein. Fractionation by pressure according to this threshold value can increase the relative yield of the protein.

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