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ROLE OF DEHYDRATION IN CHANGING THE PERMEABILITY OF ERYTHROCYTE PLASMA MEMBRANES BY FREEZE-THAWING

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The effects of hypertonic solutions and slow freezing to different temperatures in the range of 0 to −80°C on the permeability of plasma membranes of reconstituted erythrocytes were investigated. It has been found that even increased concentrations of NaCl or KCl (1.2 to 4.2 M) do not change appreciably release of K^+, [^{14}C]sucrose and haemoglobin (by 8 to 20%), while leakage of markers into the supernatant fluid is essentially completed on freezing to −25°C. If the content of mobile water in the sample of reconstituted erythrocytes is lower than 1 to 2%, the permeability of plasma membranes for [^{14}C]sucrose and haemoglobin changes after freezing to −19 to −22°C. These findings suggest that the existence of water-dependent structural changes in the membranes can be responsible for a primary mechanism of altering the permeability at low temperatures.

Introduction

The existing evidence suggests that biological membranes are very sensitive targets of cryoinjury [1]. Membrane damage on freeze-thawing can be related to such physico-chemical factors as mechanical pressure of ice, low temperatures, salt concentrations, pH changes, etc. A possible role of the individual factors in the mechanism of freezing injury to the membrane has been reflected in hypotheses on mechanical damage caused by growing ice crystals [2] and salts [3,4], in the two-factor hypothesis [5], and hypothesis on thermal shock [6]. Relatively little is known concerning freezing of water that plays an important role in maintaining the structure-functional state of biological membranes and, particularly, their barrier function [7–9]. Upon freezing biological systems cell plasma membranes appear under conditions of minimum amount, or even complete absence, of mobile water in the surrounding medium resulting from water 'freeze-out' [10]. We have found no experimental evidence in the available literature on the role of dehydration in the mechanism of changing the permeability of membranes by low temperatures. This study was undertaken to investigate the relationship between amount of mobile water and permeability of plasma membranes after freezing at different temperatures.

Mateials and Methods

[^{14}C]Sucrose of specific activity 340 mCi was purchased from Institute for Research, Production and Use of Radioactive Isotopes, Prague, Czechoslovakia. Reagents chemically pure were KCl, NaCl, $MgCl_2$, Na_2HPO_4, and NaH_2PO_4. Tris was a product of Sigma Chemicals. Human albumin (M_r 67000) was a product of Serva. Human haemoglobin was obtained as described [5].

Preparation of reconstituted erythrocytes

Human donor blood stored for 1 to 3 days, free

of leucocytes and plasma [12], was washed 3- to 4-times with a cold isotonic solution containing 150 mM NaCl and 10 mM Tris-HCl (pH 7.4) and centrifuged at 2000 to 3000 rev./min for 10 min. Then the pellet was lysed in a cold hypotonic solution containing 10 mM Tris-HCl (pH 7.4) and 4 mM $MgCl_2$ in the ratio of 1 : 5. After 5 min incubation at 2 to 4°C erythrocyte ghosts were reconstituted by addition of 3 M KCl to a final concentration of 120 mM, kept at 2 to 4°C for 10 min, and subjected to additional incubation at 37°C for 40 min [13]. When [^{14}C]sucrose was used as permeability marker, it was added to the lysing solution in the amount of 1 μCi per ml. To remove the extracellular haemoglobin and [^{14}C]sucrose, the reconstituted erythrocytes were washed with a reconstitution medium containing 150 mM KCl, 4 mM $MgCl_2$, and 10 mM Tris-HCl buffer (pH 7.4) by centrifugation at 10000 to 12000 $\times g$ for 5 min in the cold. The ratio of reconstituted erythrocytes to reconstitution solution was 1 : 10. Washing was repeated util disappearance of haemoglobin and radioactivity in the supernatant [14].

Preparation of white (haemoglobin-free) ghosts

White ghosts were obtained from human donor blood as described [12]. The procedure was slightly modified, the pH of 5 mM sodium phosphate buffer being 7.4 instead of 8.0.

Native and reconstituted erythrocytes were treated with hypertonic salt solutions. The cells were placed in hypertonic KCl or NaCl solutions for 15 min, then mixed with 50 vol. of isotonic solution containing 150 mM NaCl or KCl, 4 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) followed by centrifugation at 10000 to 12000 $\times g$ for 5 min. Haemoglobin and [^{14}C]sucrose were determined in the supernatant. To measure the content of K^+ in the pellet, the reconstituted erythrocytes were additionally washed 3 to 4 times with a solution containing 90 mM $MgCl_2$ and 30 mM Tris-HCl (pH 7.4) by centrifugation at 10000 to 12000 $\times g$ for 5 min. Slow freezing was performed in a cryostat at a rate of 1 to 2 K/min, the latter being maintained by supplying nitrogen vapor to the cooling chamber. After thawing, the reconstituted erythrocytes were washed with the reconstitution solution, and the haemoglobin and [^{14}C]sucrose contents were determined in the pellet.

Analytical procedures

Haemoglobin was determined spectrophotometrically by absorption at 420 nM [15]. The content of K^+ was analysed by flame photometry [15]. The radioactivity was measured with a SL-40 scintillation counter (France), acid-insoluble products in the samples being sedimented with 5% trichloroacetic acid. ZHS-7 scintillation liquid (100 g of naphthalene and 5 g of PPO (2,5-diphenyloxazole) per liter of dioxane) was used as scintillator. The haematocrit was determined using an Adams-Readacrit centrifuge.

The amount of liquid phase was registered using a pulse NMR technique by falloff in the amplitude of proton free induction with the 90°C pulse width and 90 MHz resonance frequency. The signal-to-noise ratio was increased using a 100-fold accumulation of signals. Freeze-thawing was performed directly in the sensor of Bruker NMR spectrometer by means of cooling the samples with nitrogen vapor. The temperature was controlled to ± 1 K. 0.4 ml samples were kept at each temperature for at least 15 min. The percentage of mobile water was determined as a ratio of NMR signal amplitudes after and before freezing.

Results and Discussion

Fig. 1 shows that complete freezing of mobile water upon freezing the reconstitution medium occurs at -18°C, i.e., markedly lower than the eutectic temperature of pure KCl solutions (-11.2°C) [16]. During thawing the mobile water was observed only at -13°C, as a result a hysteresis loop was formed in the freeze-thaw cycle, thus indicating a possibility of supercooling a portion of mobile water at the freezing stage [17].

Reconstituted erythrocyte plasma membranes per se are known to be capable of retaining a portion of mobile water on freezing [17,18]. So, it was of interest to investigate the dynamics of mobile water freezing not only in the reconstitution solution, but also in the suspension of plasma membranes and in the suspension of reconstituted erythrocytes. Our experiments indicated that the process of water freezing out and thawing was essentially the same in the suspension of plasma membranes as in the reconstitution medium. The difference was that at the temperature of complete water freezing out, namely -17 to -19°C, some

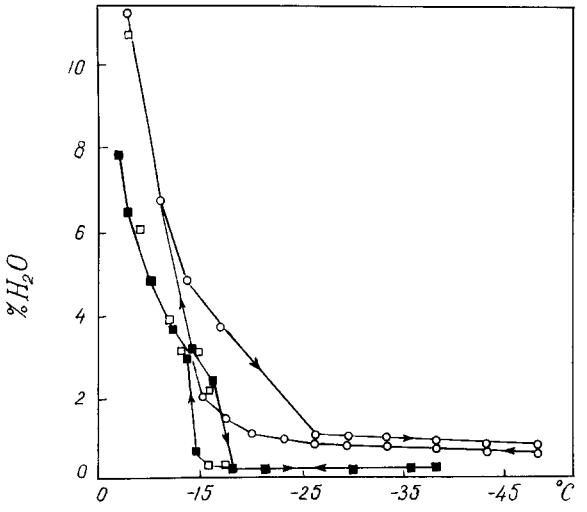

Fig. 1. Freezing and thawing of water in reconstitution solution, suspension of white ghosts, and suspension of reconstituted erythrocytes. □———□, Reconstitution (cell-free) solution; ■———■, suspension of white ghosts in reconstitution solution; ○———○, suspension of reconstituted erythrocytes. An arrow designates the course of freeze-thawing. The haematocrit of white and red ghosts is 60 to 65%.

water remained fluid in the suspension of white ghosts (no more than 0.1–0.2% of the initial water content in the sample) and was observed to the extent of −35°C. This water seems to be membrane-bound.

In the suspension of reconstitued erythrocytes freezing of mobile water was shifted to lower temperatures and could be observed even at −40°C, the lowest temperature used in our experiments. From the results in Fig. 1 it is found that the intensive freezing of mobile water in the suspension of reconstituted erythrocytes stops at −25°C, and the remaining water freezes very slowly in spite of further lowering the temperature. It seems likely that this portion of mobile water is bound to haemoglobin and can be retained until the temperature has fallen down to −70°C [17].

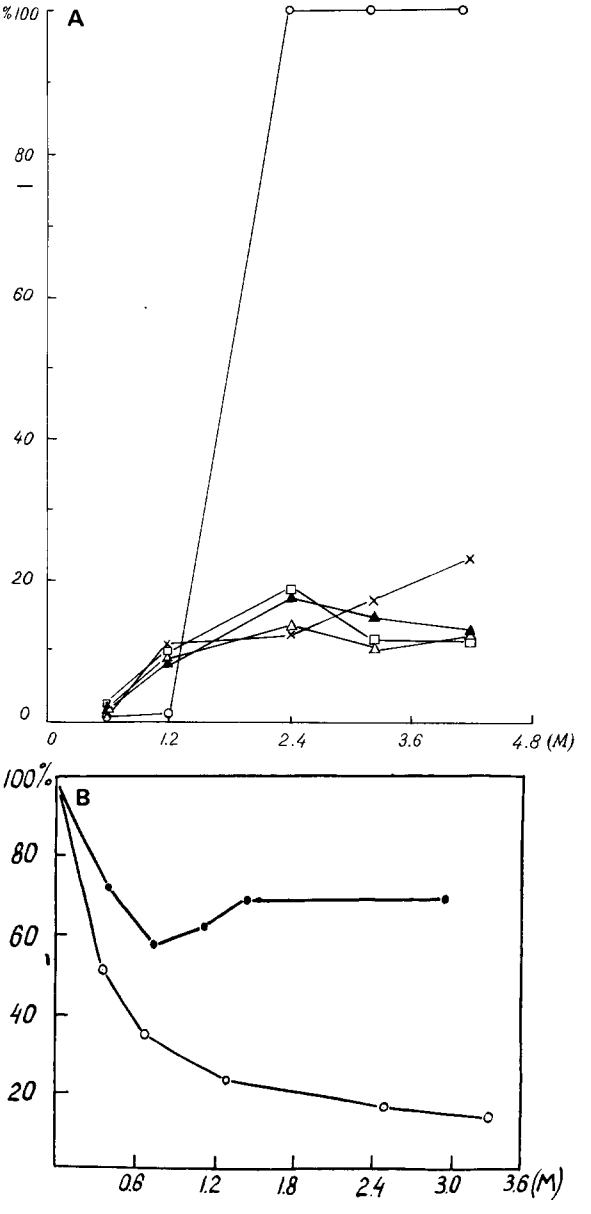

Fig. 2. (A) Release of haemoglobin, [^{14}C]sucrose and K^+ from native and reconstituted erythrocytes into the supernatant fluid when treated by hypertonic salt solutions and placed into isotonic solutions. Reconstituted erythrocytes suspended in KCl solutions: (a) △———△, release of haemoglobin; (b) ×———×, release of [^{14}C]sucrose. Reconstituted and native erythrocytes suspended in NaCl solutions: (c) ▲———▲, release of haemoglobin from reconstituted erythrocytes; (d) □———□, release of K^+ from reconstituted erythrocytes; (e) ○———○, release of haemoglobin from native erythrocytes. The content of haemoglobin, [^{14}C]sucrose, and K^+ in the control sample is assumed to be 100%. (B) Effect of hypertonic NaCl solutions on the haematocrit of native and reconstituted erythrocytes: (f) ●———●, native erythrocytes; (g) ○———○, reconstituted erythrocytes. NaCl solutions were prepared in 10 mM HCl buffer (pH 7.4). The haematocrit of native and reconstituted erythrocytes in isotonic NaCl solution (0.15 M) is assumed to be 100%. An arrow designates appearance of haemoglobin in the supernatant of native erythrocytes.

A pecularity of mobile water freezing and thawing in the sample of reconstituted erythrocytes is the occurrence of more extensive hysteresis loop, as compared to that in previous samples. This is, probably, due to the capability of intracellular haemoglobin of increasing the amount of supercooled water in the system [17] at a given temperature.

According to the two-factor hypothesis of freezing injury which is generally accepted in cryobiology, cell death on slow freezing is caused by increased salt concentration produced by water freezing [5]. As a result, phase transitions of the lipid complexes and their lateral separations [19], as well as denaturation of the lipid-protein complexes [3] and osmotic tension of plasma membranes [4], produce defects leading, ultimately, to cell lysis through hypertonic or colloid osmotic swelling at the thawing stage.

However, our experiments indicate that erythrocyte plasma membranes per se are sufficiently resistant to the action of increased salt concentration. Fig. 2A e shows that the treatment of native erythrocytes with 2.4 M NaCl and subsequent transfer into isotonic solution results in a nearly complete release of haemoglobin into the supernatant. This is in a good agreement with the data [4] which suggest that increase in the intracellular salt concentration causes posthypertonic haemolysis due to changed permeability of plasma membranes in salt solutions of concentration higher than 0.6 M. In contrast to native erythrocytes, in reconstitued erythrocytes treated with KCl or NaCl solutions even of very high concentrations, including a 4.2 M concentration, the intracellular contents of haemoglobin (Fig. 2A a, c), smaller molecules of [^{14}C]sucrose (Fig. 2A b), and K^+ (Fig. 2A d) are reduced only by 10 to 20% of the control. At the same time slow freezing to $-25°C$ and subsequent thawing liberated the markers into the supernatant fluid essentially completely (Fig. 3).

According to the hypothesis [4], permeability changes of the plasma membrane in hypertonic solutions are due to the osmotic stress which occur when cells reach their minimum volume and are not able to response to increased salt concentration in the extracellular medium by shrinkage. Our results support the data [4] on shrinkage of native erythrocytes in solutions of only up to 0.6 M concentration of NaCl (Fig. 2B f), and further increasing the salt concentration induces swelling that is believed to be associated with changed permeability of plasma membranes for cations [4]. It should be noted that $12.7 \pm 3.96\%$ haemoglobin of the initial sample releases into the supernatant of 3.0 M NaCl solution, thus indicating haemolysis of erythrocytes in a hypertonic solution, but to a lesser extent than in an isotonic medium. Probably, it is haemolysis of some cells that terminates the increase of haematocrit in 3.0 M NaCl solution (Fig. 2B f).

In contrast to native erythrocytes, reconstituted cells shrunk up to 3.3 M NaCl concentration in the extracellular medium, the highest concentration studied in these experiments (Fig. 2B g). There was no heamoglobin in the supernatant of the samples. So, the resistance of reconstituted erythrocytes to the effects of high salt concentrations could be due to their ability to behave as passive osmometers in the range of extracellular salt concentrations up to the eutectic concentra-

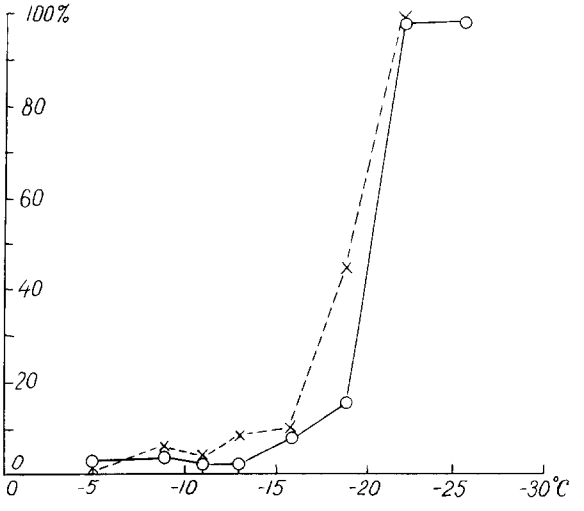

Fig. 3. Release of [^{14}C]sucrose and haemoglobin into the supernatant fluuid after thawing the reconstituted erythrocytes subjected to slow freezing to different temperatures in the range of 0 to $-25°C$. ×---×, Release of [^{14}C]sucrose; O——O, release of haemoglobin. The content of [^{14}C]sucrose and haemoglobin in the supernatant of reconstituted erythrocytes of control sample is assumed to be 100%. The content of [^{14}C]sucrose is approx. 106092 pulses per min. The content of haemoglobin in the control sample is 76.3 mg.

tion, thus preventing an excess of the tension and damage of plasma membranes. This could be accounted for a lower concentration and organization (other than in native erythrocytes) of haemoglobin whose colloid-osmotic state has a great influence in the phenomenon of shrinkage [20].

This evidence would suggest that increased salt concentration does not seem to play an important role in the primary mechnism of permeability changes in plasma membranes caused by low temperatures, as previously believed [3].

This hypothesis is strengthened by the experiments undertaken to investigate the temperature range of permeability changes in plasma membranes for [^{14}C]sucrose and haemoglobin on slow freezing. As is seen form Fig. 3, the injury occurs in the range of -19 to -22°C, i.e. significantly lower than the eutectic temperature of the reconstitution solution. After exposure of reconstituted

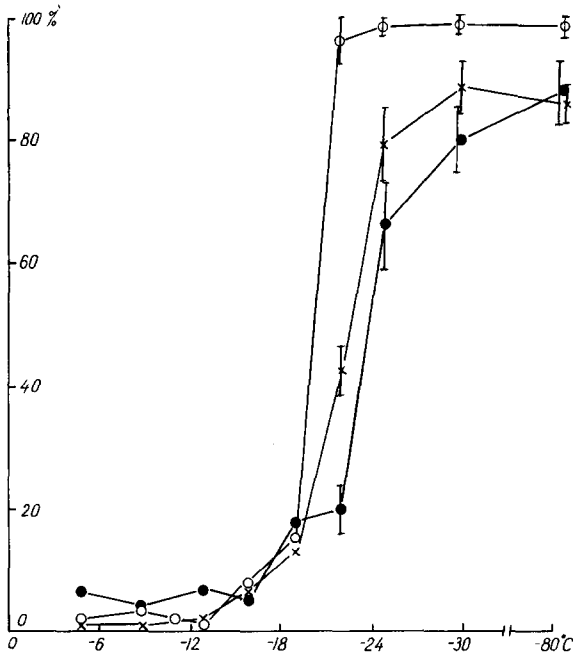

Fig. 4. Release of haemoglobin into the supernatant fluid after thawing the reconstituted erythrocytes subjected to slow freezing to different temperatures in the range of 0 to -80°C in the presence of extracellular protein. O——O, Control; ×——×, freezing with 24.2 mg/ml of albumin; ●——●, freezing with 29.6 mg/ml of haemoglobin. The content of haemoglobin in the supernatant of reconstituted erythrocytes of control sample is assumed to be 100%.

erythrocytes to higher temperatures, namely 0 to -16°C (when KCl and other components of the reconstitution solution are concentrated up to the eutectic concentration), no release of markers into the supernatant was observed.

Hence, these results can be interpreted as indicating that the dehydration is primarily due to the permeability changes in plasma membranes caused by slow freezing and subsequent thawing rather than to increased salt concentration. The hypothesis can be supported indirectly by shifts of the temperature at which reconstituted erythrocytes are damaged to lower values, between -22 and -25°C, in the presence of extracellular protein (Fig. 4), the latter being able, as mentioned above, to delay complete freezing of mobile water due to partial supercooling and binding.

Fig. 4 shows that the addition of albumin of haemoglobin to the freezing medium shifts the temperature at which reconstituted erythrocytes are damaged to lower values. Moreover, in the presence of extracellular protein the amount of retained haemoglobin increases by 9 to 11% even in samples frozen to -80°C, so the corresponding portion of cells seems to be intact.

The data provide evidence for the relationship between dehydration and permeability changes in plasma membranes, thus allowing a hypothesis to be advanced for freezing injury which proposes alterations of the plasma membrane as a result of dehydration.

According to the hypothesis, the primary mechanism of cryoinjury to the permeability of plasma membranes lies in the structural water-dependent alterations of membrane components (e.g. lipids, proteins, etc.) whose nature remains to be established. Damage to the permeability of cells due to dehydration occurs at lower temperatures than could be accounted for by solution effects, however the dehydration mechanism is believed to the primary with respect to plasma membranes.

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