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CHARACTERISTICS OF THE PERMEABILITY BARRIER OF HUMAN ERYTHROCYTE GHOSTS TO NON-ELECTROLYTES

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SUMMARY

The non-specific diffusion barrier of practically hemoglobin-free human erythrocyte ghosts prepared in hypotonic phosphate buffer was studied by measuring the permeabilities to water-soluble non-electrolytes. In contrast to pink ghost preparations, these white ghosts are completely permeable to mannitol or sucrose, indicating that their diffusion barrier is completely lost. Such leaky ghosts can be resealed effectively by incubation at 37 °C in 0.1 isoosmolar balanced salt solution for 2 h. Temperature, Ca^{2+}, osmolarity and ionic strength are all factors which appear to control the resealing process. Such resealed ghosts, however, never exhibit complete restoration of the diffusion barrier and the permeabilities to non-electrolytes are 10- to 50-fold higher than those of the native membranes. Unlike the native membranes, the permeabilities of the resealed ghosts are little affected by changes in temperature, the molecular size or lipid solubility of the permeants. It is concluded that the resealed white ghost membranes represent an altered form of the native membrane structure, having water-filled discontinuities through which the water soluble non-electrolytes diffuse freely. Possible structural aspects of such discontinuity and its genesis are discussed.

INTRODUCTION

Great interest has been shown in recent years in the structure and function of biological membranes[1]. Human erythrocyte ghosts practically free of cytoplasmic content as prepared by the method of Dodge *et al.*[2], or modifications thereof, represents one of the most widely used preparations in such studies. In contrast to extensive studies[3–6] on the structure, composition, and specialized functions such as enzymatic, immunologic and specialized transport activities of this preparation, only limited attention has been directed to understanding the nature of its non-specific permeability barrier, a fundamental aspect of the biological membrane function[7]. We previously noted that the ghost preparation is readily permeable to mannitol, indicating virtually a complete loss of the permeability barrier characteristic to the native membrane[6]. This would point to the possibility that this white ghost preparation may represent a disarranged form of the molecule structure of the native membrane. The present study is an attempt to delineate the nature and extent of this alteration in molecular structure, by characterizing its non-specific

permeability to water-soluble non-electrolytes. It was shown in this study that the lost permeability barrier of this preparation can be restored under certain conditions but only to a limited extent. The resealed ghost reveals permeability characteristics entirely different from those of the native cell membrane. Based on these observations, the nature of molecular structure of the ghost membrane is discussed.

EXPERIMENTAL PROCEDURE

Fresh human blood drawn from healthy donors was used. The packed erythrocytes free from plasma, white cells and platelets were obtained by repeated washing in isotonic NaCl–Tris buffer (pH. 7.4). The washed cells were resuspended in 20 vol. of a specified hypotonic solution for 20 min at room temperature and recovered by centrifugation at 4 °C. This procedure was repeated for a specified number of times. The hypotonic solutions used for this hemolysing and washing procedure were 1/15 isotonic phosphate buffer (1/15 phosphate buffer) of that originally described by Dodge *et al.*[2], 1/10 isotonic balanced salt solution (1/10 balanced salt solution) containing 0.38 mM of Ca^{2+} and 0.25 mM of Mg^{2+}, 1/10 isotonic NaCl solution, 1/10 isotonic D-glucose solution and distilled water. All the solutions were at pH 7.4 and buffered, except the phosphate solution, with Tris–HCl (0.5 mM). A detailed description of these hypotonic solutions was given elsewhere[6].

Hemoglobin content was assayed as described by Dodge *et al.*[2]. The total protein was measured by the method of Lowry *et al.*[8]. Non-hemoglobin content was calculated as the difference between the total protein and hemoglobin content.

Both net and unidirectional fluxes were measured by a tracer technique which has been detailed elsewhere[6]. All flux measurements were done with ghosts suspended in 1/10 balanced salt solution, pH. 7.4, at a specified permeant concentration and temperature. Each temperature was maintained constant within ± 0.1 °C using water baths equipped with immersion bath coolers (Model PCB-2, Neslab Instruments, Portsmouth). The measurement of radioactivity and sampling technique were also described previously[6]. [U-^{14}C]Maltotriose, α-methyl-D-gluco-[U-^{14}C]-pyranoside, [1-^{14}C]lactose were obtained from Amersham/Searle, Arlington Heights, Ill. D-[U-^{14}C]Mannitol, [U-^{14}C]sucrose were from International Chemical and Nuclear Co., Irvine, Calif. D-[U-^{14}C]Glyceraldehyde and ^{3}H-labeled water were obtained from New England Nuclear Corp., Waltham, Mass., the former was purified on silica-gel G thin layer with a solvent system of *n*-butanol–acetone–water (4:1:5, v/v/v).

Partition coefficients of these solutes in chloroform–water bilayer system were measured at 24 °C, by a tracer technique as detailed elsewhere[9]. Both static equilibration (for 72 h) and equilibration by agitation followed by centrifugation were adopted with essentially identical results.

RESULTS

Ghosts were prepared by washing a given number of times with 20 vol. of specified hemolytic hypotonic solution. The 3-h time course of isotopic mannitol flux was followed using each of these preparations. For the ghosts prepared in 1/10 balanced salt solution (Fig. 1) the preparation washed once equilibrates with

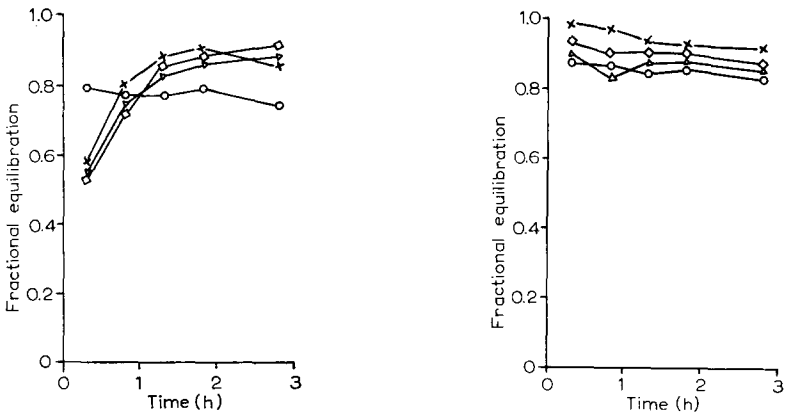

Fig. 1. Time courses of D-mannitol equilibration (influx) by the ghosts prepared in 1/10 balanced salt solution by applying different numbers of washes. The fluxes were measured in 1/10 balanced salt solution, pH 7.4, at 23 °C, using tracer amounts of [^{14}C]mannitol (0.1 Ci/ml). The permeant concentration was uniformly 5 mM. The fractional equilibrations to the water space of the ghosts (ordinates) were plotted against the time of arresting of the flux by separation of the ghost pellet from the medium after centrifugation (abscissa). Symbols are for the ghosts washed one time (○), two times (△), three times (□), and four times (×).

Fig. 2. Time courses of mannitol equilibration by the ghosts prepared in 1/15 phosphate buffer by applying different numbers of washes. The experimental conditions, data presentation and symbols are the same as Fig. 1.

the solute within less than 20 min, indicating almost a complete leakage. Each of the ghost preparations prepared by 2, 3 and 4 washes equilibrated with the solute only after at least 2 h. The intact cells were practically impermeable to this polyol, with a half-equilibration time of 12–26 h at room temperature. The permeability barrier abolished with hemolysis is apparently restored to some extent only after two or more washes. All of the ghost preparations made in the hypotonic phosphate buffer (Fig. 2), on the other hand, were found to have nearly a complete permeability to mannitol regardless of how often washing was carried out, indicating a failure to restore the permeability barrier with this medium*. All other ghost preparations including those made in 15 mM NaCl, 30 mM glucose and distilled water followed essentially the same type of the sequence in recovery in the permeabilitybarrier as that of the ghosts made in 1/10 balanced salt solution. The complete breakdown of the permeability barrier, however, does not appear to be unique to preparation in phosphate buffer: Ghosts made in 1/10 isotonic NaCl solution in the presence of 4 mM of EDTA (disodium salt) were also completely permeable to mannitol.

The 1/10 balanced salt solution ghosts prepared by washing 4 times did not lose their permeability barrier by additional washes with the hypotonic phosphate

* We have previously reported[6] that the phosphate ghosts stored overnight in the cold are completely permeable, whereas the same ghosts before the cold storage appear to retain some of their permeability barrier to mannitol. It should be stated here that the nonstored phosphate ghosts used in this previous report were preequilibrated at 37 °C prior to the flux measurements, a procedure which was found to reseal the leaky ghosts as described later in this present report.

buffer, indicating the importance of an early exposure to the buffer for this loss. Among the leaky phosphate ghosts, only those washed once or twice, but not those washed three times or more, greatly restored the permeability barrier, indicating that during the ghost preparation with the phosphate buffer some critical changes take place in the membrane structure at the third or fourth wash. The hemoglobin content of the ghosts washed four times ranges from 0.018 to 0.031% of the cellular contents. Further washing changed neither the hemoglobin content nor the permeability characteristics appreciably.

These almost hemoglobin-free, leaky ghosts prepared by washing four times in phosphate buffer can be made tight to some extent under certain conditions. The permeability barrier to mannitol was restored by suspension in 1/10 balanced salt solution and incubation at 37 °C for 2 or 3 h. Various resuspension media were examined for their effect on the restoration at 37 °C. 1/15 phosphate buffer or 1/10 balanced salt solution with 4 mM EDTA did not permit recovery of the barrier, suggesting the importance of Ca^{2+} and Mg^{2+} for this restoration. 1/15 phosphate buffer supplemented with these divalent cations, however, was also without effect. On the other hand, isotonic phosphate buffer was as effective as isotonic balanced salt solution, indicating that the presence of divalent cations is not the sole factor in inducing this restoration. With these suspension media, however, it was not clear whether high osmolarity or high ionic strength is responsible for the restoration. Resuspension in media of high osmotic content but low ionic strength, such as 1/15 phosphate buffer supplemented with 300 mM of glucose or mannitol, severely disintegrated the ghosts during the incubation so that meaningful results could not be obtained. None of these suspension media were effective at 24 °C for up to 3 h, indicating that the incubation temperature of 37 °C is absolutely critical for the restoration phenomenon. The leaky ghosts washed 4 times in 20 mM NaCl solution in the presence of 4 mM EDTA failed to restore the barrier even with incubation in 1/10 balanced salt solution at 37 °C for up to 3 h.

The phosphate ghosts resealed in 1/10 balanced salt solution were studied for further characterization of their permeability barrier to non-electrolytes. Fig. 3 represents a typical example of the time course of isotopic mannitol equilibration by the resealed white ghosts at four different temperatures. There was a surprisingly low temperature dependency of the mannitol permeability of these ghosts. This is in contrast to the normally high temperature dependency of the permeability of the native cell membrane[10] as exemplified in Fig. 4. The half-equilibration times for the ghosts range from 30 to 45 min. These are at least an order of magnitude smaller compared with those for intact cells, indicating that restoration of the permeability-barrier was incomplete.

Table I compares results of similar experiments but using different non-electrolytes. These non-electrolytes were shown to exhibit little affinity to the glucose carrier system and utilize a non-specific permeation mechanism (unpublished date). As evident in this comparison, the permeability changes little with a change up to 5-fold range in molecular size of the permeants. There appears to be no clear dependency of the permeability on the hydrophobicity of the permeants: For example, the permeabilities of α-methyl-D-glucoside and glucuronic acid differ less than 16% while their chloroform–water partition coefficients differ almost 100-fold.

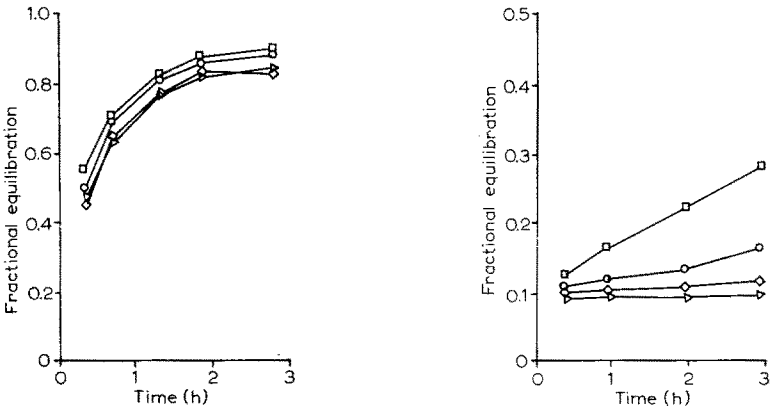

Fig. 3. Time courses of D-mannitol equilibration by the resealed ghosts at different temperatures. The ghosts were prepared by washing four times in 1/15 phosphate buffer then resealed by incubating in 1/10 balanced salt solution for 37 °C for 2 h. After an overnight storage at 4 °C, the resealed ghosts were pre-equilibrated for 30 min at a specified temperature for the flux measurement. All the ghosts were pre-equilibrated with D-mannitol at the concentration of 5 mM during the resealing step. The experimental conditions and the data presentations were otherwise the same as those of Fig. 1. Symbols are for the flux measurements at 4 °C (△), 15 °C (◇), 23 °C (○), and 37 °C (□).

Fig. 4. Time courses of D-mannitol equilibration by washed erythrocytes at different temperatures. The erythrocytes free from plasma and other blood cells resuspended in isotonic balanced salt solution were used. The symbols and the conditions of the flux measurements and the data presentations are the same as those of Fig. 3.

TABLE I

PERMEABILITIES AND THEIR ACTIVATION ENERGIES OF NON-ELECTROLYTES ACROSS HUMAN ERYTHROCYTE GHOST MEMBRANES

The ghosts were prepared in 1/15 phosphate buffer by washing four times, as detailed in the text. The relative permeabilities are expressed in terms of the inverse of the half-equilibration time at the solute concentration of 1 mM and shown as the mean ±S.D., with the number of determinations in parentheses. The activation energies were calculated from the Arrhenius plot of the data similar to that shown in Fig. 3. Each value represents a single determination. The chloroform–water partition coefficients, Q, as the mean ± S.D. of three measurements at 24 °C for each permeant, were also shown in this table.

Permeants	$t_{\frac{1}{2}}^{-1}(min^{-1})$ *at 24 °C*	E_{act} $(kcal \cdot mole^{-1})$	$Q_{chloroform/water} \times 10^{-4}$
Glycelaldehyde	0.031 ± 0.003 (3)	4.5	2.09 ± 0.61
Mannitol	0.025 ± 0.004 (8)	4.8, 4.6, 4.3	0.30 ± 0.04
Glucose 1-phosphate	0.028 ± 0.003 (4)	4.6	0.13 ± 0.01
α-Methyl-D-glucose	0.026 ± 0.005 (8)	5.0, 4.8, 4.6	1.94 ± 0.06
Glucuronic acid	0.030 ± 0.006 (4)	4.7	0.02 ± 0.01
Sucrose	0.027 ± 0.003 (8)	4.8	0.03 ± 0.01
Maltotriose	0.024 ± 0.003 (4)	4.6, 4.8	0.85 ± 0.16

These two permeants differ little in molecular size, thus there would be little possibility of appreciable size effect offsetting the effect of the lipid solubility. A uniformly low activation energy was also noted which was little affected by the size of these permeants.

DISCUSSION

It has been generally established that the non-specific permeation of water-soluble non-electrolytes across living cell membranes is essentially a molecular diffusion across a lipophilic polymerlike lattice of high viscosity[10]. This process is known to exhibit an extreme sensitivity to changes in temperature and in certain molecular parameters of the permeants particularly the size and the hydrophobicity. Thus, the reported values of activation energies for the non-specific permeation of non-electrolytes across various cell membranes are uniformly greater than 10 kcal·mole^{-1}, and increase steeply as the size of the permeants increases[6,10]. The size-dependency on the permeability itself is even more striking as it appeared in a careful analysis of Lieb and Stein[10], where the permeability was shown to decrease as an exponential function of the molecular weight of permeants with negative values of the exponents being 2.9 or greater. A direct linear dependency of these permeabilities on the lipid-solubility of permeants has been repeatedly documented[6,10]. On the other hand, the results of the present study clearly demonstrate that the non-specific permeation of non-electrolytes across the membrane of the ghost preparations is strikingly insensitive to the changes in each of these parameters. The observed values for the activation energy are low, ranging from 4.3 to 5.0 kcal·mole^{-1}, and almost independent of the molecular weight, or lipid–water partition coefficient. These facts indicate that the permeation mechanisms of the ghost membrane is different from that of the native cell membrane. On the other hand, the permeation characteristics observed with the ghosts in this study are similar to the characteristics of molecular diffusion in bulk water. Reported activation energies for diffusion in water of various water-soluble non-electrolytes range between 4.5 and 5.0 kcal·mole^{-1} (ref. 11) and are almost independent of the molecular weight. Data on the effect of molecular size on diffusion in water have shown that for solutes whose molecular weight is less than 1000, the diffusion coefficient decrease as an exponential function of the molecular weight, with the negative values of the exponent being only 0.5 (ref. 10). There has been no direct dependency observed between diffusion in water and the hydrophobicity of diffusant. These comparisons suggest that the non–electrolytes cross the ghost membrane predominantly by free diffusion through water-filled channels, which may have been created by the isolation procedure.

The exact molecular nature or the genesis of the aqueous channels postulated here in the resealed ghosts is not clear. In the present study, however, several factors have been shown to be important in the resealing process. First, the importance of Ca^{2+} is suggested by the fact that the inclusion of EDTA at 4 mM concentration during the preparation hinders the ghosts from being resealed even in the usual resealing medium. The fact that the 1/10 balanced salt solution, but neither the same 1/10 balanced salt solution *plus* EDTA, nor the 1/15 phosphate buffer, can induce resealing may also support this notion. In the human erythrocyte membrane, about 79% of the membrane calcium is known to be extractable from the protein, most of the remaining being associated with the phospholipids[12]. Although its exact role is not known, the ions, by interacting with the protein or the lipid, or both may permit restoration of the damaged lipid bilayer into more integral configuration. Secondly, the fact that the isotonic phosphate buffer and

isotonic NaCl solution as well were as effective in the resealing as 1/10 balanced salt solution, suggests importance of an effect of an osmotic shrinkage or of ionic strength. Thirdly, the most desisive parameter in the resealing was temperature. In no case was any appreciable resealing observed if the incubation was carried out at temperature of 22 °C or lower. This temperature effect may best be understood if the resealing process involves some rearrangement of lipid structure and if some degree of fluidity of the lipid structure is required for this repair process. Although the exact value of the transition temperature of the lipid structures in native membrane is not available, it has been shown that a phase transition at about 37 °C has been reported with *in vitro* bilayer structure made up of membrane lipid extracts[13].

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