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# Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. III. Permeability of spectrin-depleted inside-out membrane vesicles to hydrophilic nonelectrolytes. Formation of leaks by chemical or enzymatic modification of membrane proteins

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Spectrin-depleted inside-out vesicles (IOV's) prepared from human erythrocyte membranes were characterized in terms of size, ground permeability to hydrophilic nonelectrolytes and their sensitivity to modification by SH reagents, DIDS and trypsin. IOV's proved to have the same permeability of their lipid domain to erythritol as native erythrocytes, in contrast to resealed ghosts (Klonk, S. and Deuticke, B. (1992) Biochim. Biophys. Acta 1106, 126–136 (Part I in this series)), which have a residual leak. On the other hand, IOV's have a slightly elevated permeability for mannitol and sucrose, nonelectrolytes which are almost (mannitol) or fully (sucrose) impermeant in the native membrane. These increased fluxes, which have a high activation energy and can be stimulated by phloretin, are, however, also much smaller than the corresponding leak fluxes observed in resealed ghosts. In view of these differences, formation of IOV's can be concluded to go along with partial annealing of barrier defects persisting in the erythrocyte membrane after preparation of resealed ghosts. Oxidation of SH groups of the IOV membrane by diamide produces an enhancement of permeability for hydrophilic nonelectrolytes which is much less pronounced than that induced by a similar treatment of erythrocytes or ghosts (Klonk, S. and Deuticke, B. (1992) Biochim. Biophys. Acta 1106, 126–136 (Part I in this series)). Moreover, proteolytic treatment of the vesicle membrane, although leading to a marked digestion of integral membrane proteins, only induces a minor, saturating increase of permeability, much lower than that in trypsinized resealed ghosts (Klonk, S. and Deuticke, B. (1992) Biochim. Biophys. Acta 1106, 137–142 (Part II of this series)). Since absence of the cytoskeletal proteins, spectrin and actin, is the major difference between IOV's and resealed ghosts, these results may be taken as further evidence for a dependence of the barrier properties of the erythrocyte membrane bilayer domain on its interaction with cytoskeletal elements. In contrast, these barrier properties seem to be rather insensitive to perturbations of integral proteins.

## Introduction

Resealed inside-out vesicles are formed when unsealed erythrocyte ghosts are incubated in $Mg^{2+}$-free alkaline buffer of very low ionic strength [1]. In the course of this treatment the membrane loses a major fraction of its skeletal proteins (spectrin, actin), which provides for the change of spontaneous curvature required for extroversion of the cytoplasmic membrane surface. Such vesicles are interesting tools for the study of certain aspects of transport mechanisms based on the activity of integral proteins.

An important prerequisite for transport studies with inside-out vesicles is the restoration of the original barrier function of the membrane. For the purpose of ion (for references see Ref. 2) and glucose transport studies [3] vesicle membranes may in fact be assumed to be tight barriers. Detailed studies about the basal barrier properties, however, are not available to the best of our knowledge. This report presents a study of the permeability of resealed inside-out vesicles to hydrophilic nonelectrolytes, some of which are impermeant, others permeant in the original erythrocyte membrane.

Abbreviations: BANA, $N^{\alpha}$-benzoyl-L-arginine-4-nitroanilide hydrochloride; diamide, diazenedicarboxylic acid bis(dimethylamide); NEM, *N*-ethylmaleimide.

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Moreover, since inside-out vesicles are completely devoid of spectrin and actin they could be used to study the molecular basis of membrane leakiness induced by modification of membrane proteins with SH oxidizing agents and by limited proteolysis. In companion papers [4,5] we have examined the influence of such treatments on the barrier properties of the membrane of resealed ghosts and on the resealing after osmotic lysis.

## Materials and Methods

### *Materials*

Fresh human blood from healthy donors was obtained from the local blood bank. Standard chemicals and compounds used as test permeants were from Merck (Darmstadt), Fluka (Neu Ulm) or Sigma (Munich).

$^{14}$C-labelled test permeants (erythritol, mannitol, sucrose, inulin) were from Amersham-Buchler (Braunschweig). Trypsin (3.5 U/mg, substrate BANA) was from Merck (Darmstadt), phloretin from Carl Roth (Karlsruhe), diamide from Calbiochem (Frankfurt), cytochalasin B from Sigma (Munich) and NEM from Fluka (Neu Ulm).

### *Methods*

*1. Preparation of cells*

Fresh human blood anticoagulated with citrate and stored at 4°C in a conventional storage medium containing glucose (20 mM) and adenine (25 $\mu$M) was used for experiments within the following 10 days. Erythrocytes were isolated by centrifugation (5750 $\times g$, 5 min). After removing plasma and buffy coat, the erythrocytes were washed three times in an excess of isotonic NaCl and packed at 5750 $\times g$ for 5 min.

*2. Preparation of inside-out vesicles*

Sealed inside-out vesicles were prepared according to Steck and Kant [1] with some modifications. Washed cells were suspended in one volume of 51 mM sodium phosphate buffer (pH 7.7–8.0). 1 volume of this suspension was rapidly mixed with 8.5 vols. ice-cold lysis buffer (5.1 mM sodium phosphate, pH 7.7–8.0). The lysate was stirred for 15 min at 0°C. Subsequently the unsealed ghosts were pelleted by centrifugation (32000 $\times g$, 10 min, 0°C) and washed three times with ice-cold lysis buffer to remove all residual hemoglobin. To initiate vesiculation and to load the vesicles with test permeants 1 vol. of unsealed, white ghosts was mixed with 2 vols. 0.5 mM sodium phosphate buffer (pH 9.7) containing various $^{14}$C-labeled hydrophilic nonelectrolytes (erythritol, mannitol, sucrose, inulin, 100 nCi/ml) and 2 mM of the unlabeled nonelectrolyte (0.1 mM in case of inulin). The pH was adjusted to 8.2–8.4. After 1.5 hours incubation at 0°C followed by 0.5 h at 37°C the vesicles formed were pelleted by centrifugation (32000 $\times g$, 15 min, 0°C). After removing the supernatant, the pellet was homogenized by vigorous vortexing [6]. The vesicles were washed twice with 0.5 mM sodium phosphate buffer (pH 8) and immediately used for measurements of permeability.

*3. Characterisation of inside-out vesicles*

The sidedness of vesicle preparations was determined according to Steck and Kant [1] by measuring the activity of the exofacial marker enzyme acetylcholinesterase.

Protein contents was measured by the assay of Lowry [7], lipid phosphorus according to Bartlett [8]. Membrane SH groups were quantified with dithiodipyridine as described earlier [9]. When determination of lipid phosphorus was intended, we used Tris buffer for the preparation of vesicles [1]. The volume trapped between the packed vesicles was determined by isotope dilution of an impermeant probe ([$^{14}$C]inulin ) [10].

Electrophoresis was carried out according to Fairbanks et al. [11]. After Coomassie blue staining the gels were scanned at 546 nm using an ISCO gel scanner (Mode 1310).

*4. Measurements of permeability*

Tracer efflux was initiated by suspending 1 vol. tracer-loaded vesicles in 10 vols. tracer-free sodium phosphate buffer (0.5 mM, pH 8). At various times 300-$\mu$l samples were centrifuged (32000 $\times g$, 10 min, 0°C) and the radioactivities in the supernatants measured. Rate coefficients were obtained by linear regression analysis as described in a previous paper [4]. As a measure for the yield of tight vesicles after membrane modification, we determined the retention of test solute as described earlier [4].

Results derived from three or more experiments ($n \geq 3$) are given as mean values $\pm$ S.D., for $n < 3$ arithmetic means are presented.

*5. Modification of inside-out vesicles*

After 30 min incubation of vesicles at 37°C modifying reagents, dissolved in sodium phosphate buffer (0.5 mM, pH 8), were added to the vesicle suspension (desired concentrations see Results). After further incubation at 37°C the vesicles were washed at 0°C and used for efflux measurements as usual.

## Results

*Characteristics of inside-out vesicles*

Vesicles prepared by the simple procedure described in Methods were oriented inside-out to about

$65 \pm 6\%$ ($n = 30$) as indicated by the inaccessibility of acetylcholinesterase to acetylthiocholine in the absence of Triton X-100. The remaining 35% membrane material consist of unsealed vesicles, since resealing with right-side-out orientation is not possible in the absence of magnesium ions [1]. Further indication for this assumption is given by the complete tryptic cleavability of ankyrin, reported later in this paper. These unsealed vesicles do not disturb our efflux measurements since they are unable to trap test solutes.

The preparation of inside-out vesicles results in a decrease in membrane protein content (mg protein/$\mu$mol lipid phosphorus) from 1–1.1 in native cells [12] to 0.75 ($n = ?$). The decrease of protein contents is caused by the rapid and complete removal of extrinsic proteins, predominantly spectrin (bands 1 and 2) and actin (band 5) during vesicle preparation [13–15].

The vesicle diameters, obtained by scanning electron microscopy, are distributed symmetrically and range from 0.38 to 1.17 $\mu$m with a mean value of $1.05 \pm 0.32$ $\mu$m ($n = 75$) (for details see Ref. 16). Since the vesicles collapsed on the slide in the course of air drying, the observed diameter should be slightly larger than the real diameter. Based on geometric considerations a real mean diameter of the spheric vesicles of 0.7 $\mu$m could be calculated from the measured mean diameter of 1.05 of the collapsed spheres. Sze and Solomon [17] reported an average measured diameter of $0.93 \pm 0.23$ $\mu$m, which agrees with our measurements.

*Ground permeability of inside-out vesicles*

In order to characterize the barrier properties of inside-out vesicles, permeabilities for a number of nonelectrolytes were derived from efflux measurements. Moreover, we determined the activation energies and the sensitivity to phloretin. Test permeants were (Stokes-Einstein radii in nm [18]): erythritol (0.36), mannitol (0.42) and sucrose (0.52).

The time course of release of entrapped erythritol and mannitol from inside-out vesicles at different temperatures is shown in Fig. 1. In this semi-log presentation the data can be fitted by single exponentials. In the case of erythritol, cytochalasin B was added to the efflux medium (10 $\mu$M) to inhibit the glucose transporter, which is still active in vesicles [3,17]. Erythritol fluxes measured in presence of the inhibitor can be regarded as simple diffusion, reflecting the ground permeability [4].

In Table I rate coefficients are listed for the efflux of erythritol, mannitol and sucrose at different temperatures. At 0°C the vesicles are impermeable to sucrose and mannitol, while a measurable release of erythritol is observed. Upon elevation of the efflux temperature to 37°C a slow release of sucrose becomes measurable, mannitol efflux is well measurable, whereas the efflux

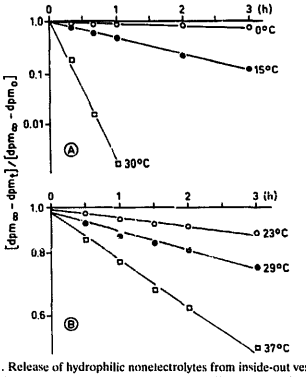


Fig. 1. Release of hydrophilic nonelectrolytes from inside-out vesicles at different temperatures. Time course of efflux measured as described in Methods. (Panel A) Erythritol (in presence of 10 $\mu$M cytochalasin B), (Panel B) mannitol. (Note different ordinate scales!)

of erythritol becomes too rapid for our method already at 30°C.

The activation energies, derived from Arrhenius diagrams (ln $k$ versus $1/T$ ($K^{-1}$)) are quite similar (about 100 kJ $mol^{-1}$) for erythritol ($T = 0°C–30°C$) and mannitol ($T = 15°C–37°C$ (Fig. 2). This value suggests diffusion through the lipid domain of the membrane [19].

In order to further substantiate that the permeation of the test permeants through the vesicle membrane involves the lipid domain we measured the release of test permeants in the presence of phloretin. Phloretin (3-(*p*-hydroxyphenyl)-2,4,6-trihydroxypropiophenone), known as an inhibitor of many carrier-mediated transports, also stimulates diffusion of nonelectrolytes through the lipid domain of membranes [19–22]. We found a progressive enhancement of the release of mannitol and sucrose at increasing levels of phloretin

TABLE I

*Ground permeability of inside-out vesicles at different temperatures*

Efflux rate coefficients ($k_0 \times 10^3$, $min^{-1}$) of various test solutes at several temperatures. Number of experiments in brackets. n.d., not determined.

| Test solute | $k_0 \times 10^3$ ($min^{-1}$), $T$ | | |
|---|---|---|---|
| | 0°C | 30°C | 37°C |
| Erythritol | $1.56 \pm 0.03$ (4) | 75.8 (2) | 201 * |
| Mannitol | 0.016 * | 1.5 (2) | $3.82 \pm 0.82$ (9) |
| Sucrose | n.d. | n.d. | $0.63 \pm 0.4$ (9) |

* Values derived from extrapolation of Arrhenius diagrams.

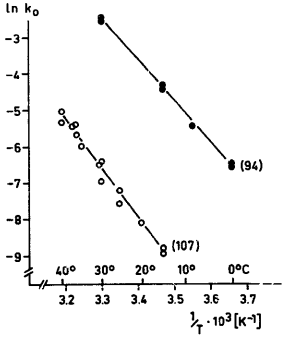


Fig. 2. Arrhenius diagram for the ground permeability of inside-out vesicles to erythritol (●) and mannitol (○). Fluxes were measured as described in Methods. Numbers in brackets are activation energies (kJ/mol), calculated from the slopes of the lines (regression analysis).

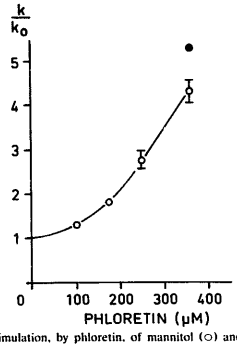


Fig. 3. Stimulation, by phloretin, of mannitol (○) and sucrose (●) efflux from inside-out vesicles. Changes described under the assumption of a multiplicative amplification of an existing pathway ($k_{phloretin}/k_0$). $k_{0,mannitol} = (3.8 \pm 0.8) \cdot 10^{-3}$ min$^{-1}$ ($n = 9$); $k_{0,sucrose} = (0.63 \pm 0.4) \cdot 10^{-3}$ min$^{-1}$ ($n = 9$). Fluxes measured at 37°C.

(Fig. 3). A stimulation of similar magnitude was previously reported for the influence of phloretin on the permeabilities of human red cells to 2,3-butanediol, propionamide and ethylene glycol [21,22].

*Modification with covalently reacting reagents*

Treatment of native erythrocyte or ghost membranes with diamide, a mild SH oxidizing reagent, induces crosslinking of spectrin by formation of intermonomeric disulfide bonds [4,23,24] and furthermore oxidizes about 45% of the membrane SH groups. This modification is paralleled by an enhancement of the permeability to hydrophilic nonelectrolytes [4,23]. A causal relationship between crosslinking of spectrin and enhancement of permeability has been claimed [23] but not firmly established.

Surprisingly it proved to be impossible to induce a comparable enhancement of permeability in inside-out vesicles with diamide (Table II), though the extent of SH group modification was the same in ghosts and inside-out vesicles (about 45%). We observed only a minor increase of permeability even at high levels of diamide.

In the unmodified vesicle membrane the passive diffusion of mannitol and erythritol is markedly temperature-dependent ($E_a$ about 100 kJ/mol, Fig. 4). In contrast, the diamide-induced permeabilities to these test solutes have a low temperature dependence ($E_a$

TABLE II

*Formation of membrane leaks by diamide in inside-out vesicles*

Leaks quantified by the rate coefficients ($k \times 10^3$, min$^{-1}$) of efflux of various test solutes. Concentration of diamide (45 min, pH 8, 37°C) and temperature of efflux are given in the table. Further experimental details as described in Methods. Number of experiments in brackets. Data of resealed ghosts are given for comparison.

| Test solutes | Diamide (mM) | $T_{efflux}$ (°C) | $k \times 10^3$ (min$^{-1}$) | |
|---|---|---|---|---|
| | | | inside-out vesicles | resealed ghosts |
| Erythritol | 0 | 0 | 1.56 ± 0.03 (4) | 1.1b ± 0.03 (12) |
| | 10 | 0 | 4.37 (2) | 21.0 (2) |
| Sucrose | 0 | 0 | < 0.1 (6) | 0.34 ± 0.1 (30) |
| | 5 | 0 | < 0.1 (2) | 1.15 ± 0.3 (5) |
| | 10 | 0 | < 0.1 (2) | 1.20 (1) |
| | 0 | 37 | 0.63 ± 0.4 (9) | 0.74 ± 0.1 (3) |
| | 5 | 37 | 0.65 (2) | 2.5 (2) |
| | 10 | 37 | 1.18 ± 0.1 (3) | n.d. |

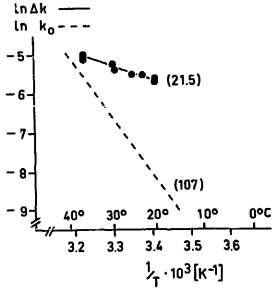


Fig. 4. Arrhenius diagram for diamide-induced (10 mM, 45 min, 37°C, pH 8) mannitol leak permeability in inside-out vesicles. The diagram for unmodified inside-out vesicles (from Fig. 2) is given for comparison. Fluxes were measured as described in Methods. Numbers in brackets are activation energies (kJ/mol) (see Fig. 2).

about 21 kJ/mol (Fig. 4). This corresponds to the activation energy of diffusion of nonelectrolytes in bulk water and identifies the small leak formed by diamide in vesicles as an aqueous pathway [23].

Similar results were obtained for NEM (Table III), which did not produce a detectable leak in resealed ghosts [4] and erythrocytes [23].

In a preceding paper [4] leak formation could be induced in ghosts by DIDS acting on the cytoplasmic side of the membrane after addition to open ghosts before resealing. As shown in Table III, treatment of inside-out vesicles with DIDS does not enhance permeability although the originally cytoplasmic surface of the membrane was exposed to the agent.

TABLE III

*Temperature dependence of nonelectrolyte fluxes in inside-out vesicles before and after covalent membrane modification by various agents*

Rate coefficients of mannitol efflux ($k \times 10^3$, $min^{-1}$). Modification carried out as described in Methods. $E_a$ values calculated from Arrhenius diagrams. Number of experiments in brackets. n.d., not determined.

| Reagent | $k \times 10^3$ ($min^{-1}$), T° | | | $E_a$ (kJ/mol) |
|---|---|---|---|---|
| | 0°C | 26°C | 30°C | |
| None | 0 | 0.3 (2) | 1.5 (2) | 107 |
| Diamide | | | | |
| 10 mM, 45 min | 1.35 ± 0.18 (4) | 3.3 (2) | 6.49 (2) | 21.5 |
| NEM | | | | |
| 10 mM, 45 min | 5.33 (2) | 6.8 (1) | 9.5 (2) | 10.0 |
| DIDS | | | | |
| 0.2–0.5 mM, 45 min | 0 (2) | 0 (2) | n.d. | – |

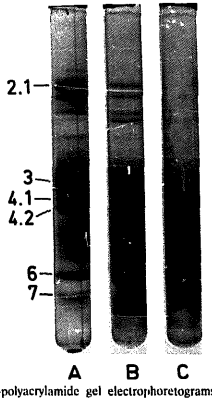


Fig. 5. SDS-polyacrylamide gel electrophoretograms of trypsin-digested inside-out vesicles (B, C). For comparisons gel of unmodified inside-out vesicles (A) is given. Trypsin concentrations 0.125 (B) and 0.5 (C) μg/ml. Nomenclature of peptide bonds in the SDS-PAGE gels (Coomassie staining) according to Ref. 32. Differences in intensitiy of band 6 and 7 result from different amounts of protein put on the gels.

*Influence of trypsination on inside-out vesicles*

Spectrin- and actin-free inside-out vesicles could also be used to study the origin of trypsin-induced leak formation in ghost membranes [5], since they allow to decide wether cleavage of spectrin or of intrinsic proteins is responsible for the enhancement of permeability.

Tryptic treatment of the outward directed surface of inside-out vesicles (Fig. 5, lane B and C) induced a complete degradation of all proteins in the range of 200 kDa, mostly ankyrin (band 2.1). This complete degradation of ankyrin is evidence for the absence, postulated above, of resealed right-side-out vesicles in our preparation. Moreover, band 3 (95 kDa) was degraded in the membrane of inside-out vesicles as evident from the decrease in staining intensity in the range of 95 kDa in parallel with the appearance of the membrane-bound tryptic 52 kDa residue of band 3 [25,26].

Trypsination of inside-out vesicles enhances the rate of sucrose exit. Normalized to the ground permeability, trypsin (0.5 μg/ml) induced a 15-fold enhancement of sucrose permeability at 37°C. This effect of trypsin saturates with concentration (Fig. 6) since increasing the trypsin concentration above 0.5 μg/ml did not further enhance permeability. The same was true for prolongation of the incubation time from 15 to 30 min

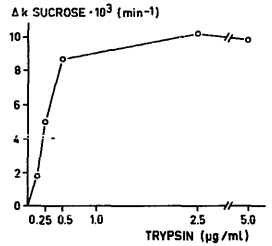


Fig. 6. Concentration-dependence of the effect of trypsin on sucrose permeability of inside-out vesicles. The effect is defined as the increase of rate coefficients ($\Delta k$) on top of the control ($k_0 = (0.63 \pm 0.4) \cdot 10^{-3}$ min$^{-1}$ ($n = 9$)). Efflux at 37°C. Fluxes were measured as descibed in Methods. Mean values from 3–5 experiments.

at high trypsin concentrations. Such saturation might be caused by self-digestion (autolysis [27]) of the trypsin. This interpretation was excluded by repeated addition of fresh, concentrated trypsin solution. Even then, no further enhancement of permeability could be observed (data not shown).

Experiments with inulin-loaded vesicles support the view that the trypsin-induced release of trapped solutes is not due to lysis of a fraction of vesicles, but to the formation of a leak pathway. After trypsin treatment even up to 5 μg/ml, which induced a saturating enhancement of sucrose permeability, inulin was not released (data not shown).

Further indication for the concept of a formation of defined leaks, rather than a destruction of a fraction of vesicles, is provided by the complete retention of trapped marker after the washing procedure (for methods, Ref. 4). The retention of test solute is defined here as the amount of the test solute retained by modified vesicles (or ghosts) relative to the total amount of test solute trapped in untreated vesicles (or ghosts) (for details, see Ref. 5). In case of ghosts, we found a diminished retention after trypsin modification [5] which was assigned to complete destruction of part of the ghosts during the modification period.

The activation energies for the trypsin-induced permeabilities of mannitol and sucrose inside-out vesicles are about 65–75 kJ/mol (data not shown).

## Discussion

Sealed inside-out vesicles are formed by endocytosis in fresh, well-washed ghosts following incubation in an alkaline buffer of low ionic strength and lacking divalent cations. Their high surface area to volume ratio provides the possibility to derive very low permeabilities from measurements of fluxes with rate coefficients of reasonable magnitude.

*Transport properties of inside-out vesicles*

In order to characterize the 'ground permeability' of inside-out vesicles, data obtained in this study are compared in Table IV with results available for ghosts and erythrocytes [4,23]. Results are expressed in permeabilities calculated according to Ref. 4.

In the case of erythritol, the permeabilities are essentially the same in all three systems. The high activation energy of 79–90 kJ/mol indicates diffusion through the lipid domain.

In the case of mannitol differences become evident between ghosts and vesicles (Table IV). Ghosts seem to have a defect in their membrane with the characteristics of an aqueous pore, as indicated by the low activation energy of the leak fluxes [4]. This effect seems to have vanished in the vesicles. The low permeability of vesicles to mannitol is only slightly higher than that of intact erythrocytes [28]. This 'leak' is most probably due to a permeation through the lipid domain, as indicated by the high activation energy and

TABLE IV

*Comparison of permeabilities and activation energies for different test solutes in intact erythrocytes, ghosts and inside-out vesicles*

Permeabilites calculated as described in the text. Activation energies were derived from Arrhenius diagrams. Data for erythrocytes and ghosts from Refs. 4 and 23. Efflux measurements at 15°C. Number of experiments in brackets. For ghosts and inside-out vesicles, volume ($V$) and surface area ($A$) were calculated from the radius determined by microscopy (see Ref. 4, and Results).

| Test solute | Membrane system | $V$ ($\mu m^3$) | $A$ ($\mu m^2$) | $k_0 \times 10^3$ (min$^{-1}$) | $P \times 10^{10}$ (cm/s) | $E_a$ (kJ/mol) |
|---|---|---|---|---|---|---|
| Erythritol | erythrocytes | 95 | 140 | 4.13 | 29.5 | 90 |
| | ghosts | 51 | 140 | 5.5 | 33.1 | 79 |
| | inside-out vesicles | 0.18 | 1.54 | 12.2 | 23.2 | 92 |
| Mannitol | erythrocytes | 95 | 140 | 0.02 * | 0.15 * | 108 * |
| | ghosts | 51 | 140 | 1.23 | 7.38 | 20.7 |
| | inside-out vesicles | 0.18 | 1.54 | 0.15 | 0.28 | 107 |

* Calculated from data of Ref. 28, assuming an activation energy of 108 kJ/mol.

the stimulation by phloretin, and is in line with the slight enhancement of the transbilayer mobility, in inside-out vesicles, of the phospholipid probe, lysophosphatidylcholine [29]. This residual enhancement of permeability might be due to bilayer perturbation following the aggregation of integral membrane proteins reported to occur in inside-out vesicles under some [13,30], though not all [31] conditions.

A decrease of membrane permeability clearly occurs between the ghost state and the vesicle state. The change of curvature during vesicle formation is probably not involved in this effect since the vesicles have still a mean radius of about 0.35 $\mu$m, not likely to impose constraints on the bilayer [32]. It is tempting to speculate that the removal of spectrin and actin in the course of vesiculation leads to a tighter sealing. In terms of the concept of a single, discrete hole in resealed ghosts [33], which is an alternative to our hypothesis of fluctuating defects in the ghost membrane bilayer, the normalisation of permeability accompanying vesicle formation could also result from the confinement of this discrete defect to only one of the large number of vesicles produced from one ghost. A distinction between these two concepts will have to await the final characterisation of the 'holes'.

In contrast to the vesicle data, which suggest a *decrease* of permeability following the *removal* of spectrin during preparation of inside-out vesicles, we have shown that an *enhancement* of permeability results from *tryptic cleavage* of membrane skeletal elements in resealed ghosts (see the preceding paper, Ref. 5). Removal of spectrin and actin by elution in $Mg^{2+}$-free medium and proteolytic cleavage of these proteins in situ are obviously not directly comparable in their consequences. In the former case, which leads to vesicle formation, the two skeletal proteins are removed while the anchor proteins (ankyrin and band 4.1) stick to the membrane [13–15]. In the latter case, which produces leaks in ghosts, skeletal and anchor proteins are cleaved, but it is presently unknown wether the skeletal elements are still linked to the membrane by the anchor proteins in spite of a scission in their peptide backbone, or are removed.

*Sensitivity of spectrin-depleted inside-out vesicles to diamide*

Crosslinking of spectrin by diamide has been claimed to be responsible for diamide-induced leakiness of erythrocyte or ghost membranes [4,23]. Vesicles lose essentially more than 95% of their spectrin during the vesiculation process. In parallel they become much less sensitive to diamide, although some leakiness can still be induced. Expressed in terms of permeabilities, 5 mM diamide (pH 8, 45 min, 37°C) enhances erythritol permeability (at 0°C) by $8.15 \cdot 10^{-10}$ cm/s in inside-out vesicles, to be compared with $118 \cdot 10^{-10}$ cm/s in ghosts. This small diamide-induced leak in vesicles seems, nevertheless, to be based on the formation of aqueous holes, as indicated by the low activation energy of leak fluxes. The lack of effect of diamide in vesicles devoid of spectrin and actin may be regarded as evidence for a major involvement of these proteins in the leak formation following diamide treatment in resealed ghosts and native erythrocytes. Alternatively, one would have to speculate that modification, by diamide, of proteins present in both systems induces different types of secondary changes in vesicles as compared to ghosts, leading to leak formation in the lipid bilayer of ghosts but not of vesicles.

The lack of effect of DIDS in the vesicle system probably points to an involvement of spectrin in the effect of DIDS in the ghost system. On the other hand, it should be considered, that in the ghost system DIDS was already present prior to and during the resealing, while in the vesicle system the modifier was added only after the formation of resealed vesicles. One could thus define DIDS as a modifier of resealing and not of the barrier function of the tight membrane.

*Sensitivity of inside-out vesicles to trypsin*

In the preceding paper [5], we reported an increase of leakiness to sucrose in parallel with tryptic digestion

TABLE V

*Comparison of trypsin-induced enhancement of permeability for different test solutes in ghosts and inside-out vesicles*

Permeabilities calculated as described in the text. Activation energies derived from Arrhenius diagrams (23–37°C). Note that different trypsin concentrations were used in the two preparations. $k$ values are given for 0°C, based on measurements (ghosts), respectively, calculated from Arrhenius plot (inside-out vesicles).

| Test solute | Membrane system | Trypsin (ng/ml) | $V$ ($\mu m^3$) | $A$ ($\mu m^2$) | $k \times 10^3$ ($min^{-1}$) | $P \times 10^{10}$ (cm/s) | $E_a$ (kJ/mol) |
|---|---|---|---|---|---|---|---|
| Mannitol | ghosts | 160 | 51 | 102 * | 1.46 | 11.8 | n.d. |
| | inside-out vesicles | 500 | 0.18 | 1.54 | 0.86 | 1.6 | ≈ 65 |
| Sucrose | ghosts | 160 | 51 | 102 * | 0.91 | 7.55 | 0 |
| | inside-out vesicles | 500 | 0.18 | 1.54 | 0.15 | 0.28 | ≈ 75 |

* For a discussion of the choice of this value, see the preceding paper [5].

of ghost membrane proteins. These studies had to be restricted to low trypsin concentrations, since the onset of vesiculation of ghosts, caused by digestion of spectrin, obviated quantitative efflux measurements after more extensive trypsination. Measuring fluxes after tryptic digestion posed no experimental problems in spectrin-depleted inside-out vesicles. We found no decrease in the retention of trapped test solute.

Tryptic treatment of inside-out vesicles induces drastic cleavage of membrane proteins. The induced leakiness, however, is considerably smaller than in resealed ghosts and also differs in size discrimination and activation energies (Table V).

Since spectrin is not present in these vesicles, its proteolytic cleavage can not be the reason for the leakiness. Moreover, trypsin does not cause vesiculation in this system, therefore leakiness can not be related to this phenomenon. By exclusion we have therefore to postulate that enzymatic cleavage of intrinsic proteins causes leakiness of inside-out vesicles. It appears, however, that a major proteolytic cleavage of these proteins, probably of their cytoplasmic domains, has to occur before the membrane begins to lose its barrier properties (see Fig. 5).

Generalizing, the hypothesis can be formulated that even major modifications of intrinsic membrane proteins, by chemical or enzymatic treatment will not impair the barrier function of the membrane to any extent comparable to that resulting from certain alterations of the membrane skeleton.

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