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# THE INFLUENCE OF LIPID COMPOSITION ON THE BARRIER PROPERTIES OF BAND 3-CONTAINING LIPID VESICLES

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**Band 3 protein has been incorporated into lipid vesicles consisting of 94:6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine or total erythrocyte lipids by means of a Triton X-100 Bio-Beads method, with an additional sonication step prior to the removal of the detergent. This method results, for both types of band 3 lipid vesicles, in rather homogeneous vesicles with comparable protein content and vesicle trap. Freeze-fracture electron microscopy revealed that band 3-egg phosphatidylcholine-bovine heart phosphatidylserine vesicles have considerably more intramembrane particles as compared to the band 3-erythrocyte lipid vesicles. The dimensions of the nonspecific permeation pathways present in the band 3-lipid vesicles were measured using an influx assay procedure for nonelectrolytes of different size, in which the vesicles were sampled and subsequently freed from nonenclosed labeled permeant by means of gel-filtration. The band 3-egg phosphatidylcholine-bovine heart phosphatidylserine vesicles have nonspecific permeation pathways (pores), with diameters of up to 60 Å. In contrast, the band 3-total erythrocyte lipid vesicles are more homogeneous and show much smaller nonspecific permeation pathways, having a diameter of about 12 Å. These results suggest that the nonspecific permeability of the band 3-lipid vesicles is strongly lipid-dependent. Increase in specific anion permeability expected as a consequence of the presence of band 3 in the erythrocyte lipid vesicles was found to be very limited. However, stereospecific, phloretin-inhibitable D-glucose permeability could clearly be demonstrated in these vesicles. The difference of the nonspecific permeability of the band 3-egg phosphatidylcholine-bovine heart phosphatidylserine vesicles and band 3-erythrocyte lipid vesicles, is discussed in the light of the presence of defects at the lipid/protein interface and protein aggregation, which may induce formation of pores.**

## Introduction

The anion transporter of the human erythrocyte membrane, band 3 (nomenclature according to Fairbanks et al., Ref. 1) is an attractive candidate for studying protein-lipid interactions in model systems such as recombinant vesicles. This is due to, firstly, the abundant availability of this protein, since this protein represents about 25% (by weight) of the erythrocyte membrane proteins, and secondly, the relative ease with which this protein can be purified by means of a selective extraction procedure with Triton X-100 [2]. Moreover, several reconstitution procedures for band 3 into lipid vesicles are described. The methods are based on the removal of the detergent Triton X-100 from a band 3/lipid/detergent suspension by means of

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Abbreviations: DIDS, 2,2′-diisothiocyanostilbene-4,4′-disulfonic acid; DNDS, 2,2′-dinitrostilbene-4,4′-disulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

toluene-phosphatidylcholine extraction [3], adsorption to Bio-Beads [2,4–6] or after replacement of Triton X-100 by *N*-octylglycoside, by dialysis [7]. It has been reported that by the use of the Triton X-100-Bio-Bead method, band 3 was successfully reconstituted into lipid vesicles [2,4–6]. This suggestion was supported by the findings that band 3-lipid vesicles show an enhanced $SO_4^{2-}$ permeability compared to protein-free vesicles [4] which could be inhibited by competitive anions [2,8] or high concentrations of specific inhibitors of the native anion transporter, like DIDS [2] and DNDS [4,5].

However, recently, the suggestion that band 3 was functionally reconstituted into egg phosphatidylcholine-bovine heart phosphatidylserine vesicles was weakened by the observation that these band 3-lipid vesicles suffered from an increased nonspecific pemeability which partially contributes to the enhanced $SO_4^{2-}$ permeability [8]. In addition, an enhanced $SO_4^{2-}$-DIDS-inhibitable permeability was observed with egg phosphatidylcholine-bovine heart phosphatidylserine vesicles in which glycophorin, the major sialoglycoprotein of the human erythrocyte membrane which has no known transport function, was incorporated [8].

The nonspecific permeability increase, caused by the incorporation of the integral proteins, band 3 or glycophorin, and other integral proteins [8–14], is probably caused by packing defects in the membrane barrier. These packing defects are probably present in protein aggregates or located at the protein/lipid interface [9,15].

Recent investigations indicate that the packing defects, caused by incorporation of glycophorin in dioleoylphosphatidylcholine bilayers have a diameter of 15–18 Å [15]. In addition, this increased bilayer permeability is strongly dependent on the lipid composition of the bilayer, since glycophorin incorporated in a bilayer composed of total erythrocyte lipids results in vesicles which are hardly permeable for $K^+$ [9].

The increased nonspecific permeability of the band 3-lipid vesicles hampers the study of the band 3-related specific anion transport. Therefore, we have investigated in this study, firstly, the extent of the nonspecific permeability and seondly, in order to try to minimize the nonspecific permeability, the band 3 protein was incorporated in a lipid bilayer composed of the lipid extract of the native erythrocyte membrane, followed by a study of the permeability characteristics of the resulting vesicles.

## Materials

Chemicals and radiochemicals used were from the following sources: $Na_2^{35}SO_4$ (25–40 Ci/mg), D-[1-$^3$H]glucose (7.0 Ci/nmol), [$^3$H]Dextran (461 mCi/g) from Amersham; [1,2-$^3$H]poly(ethylene glycol) 4000 (1.6 mCi/mg), [1,2-$^3$H]poly(ethylene glycol) 900 (4.5 mCi/g), (6)-[$^3$H]raffinose (7.8 Ci/nmol), L-[1(*n*)-$^3$H]glucose (10.7 Ci/nmol) from New England Nuclear. Stractan (arabino galactan) from Sigma. Triton X-100 was purchased from Rohm and Haas and was purified according to the method of Ashani and Catravas [16]. DIDS was obtained from Pierce Chemicals, SM-2 Bio-Beads from Bio-Rad (pretreated according to Holloway [17]). Sephacryl S-1000, superfine, Sepharose 4B and Sephadex G-75 from Pharmacia Fine Chemicals. Phloretin was obtained from Roth. Egg phosphatidylcholine was purified from egg yolk according to standard procedures. Phosphatidylserine was purified from bovine heart as will be descirbed elsewhere. The phospholipids were pure, as they showed one spot on high-performance thin-layer chromatography.

Erythrocyte lipids were extracted using the method of Reed et al. [18] from human erythrocyte ghost, prepared by hypotonic lysis of human erythrocytes according to Parpart [19]. The erythrocyte lipids showed their characteristc composition as revealed by two-dimensional thin-layer chromatography [20]. All other chemicals were at least of analytical grade.

## Methods

*Purification and reconstitution of band 3 protein.* The band 3 protein was purified from human erythrocyte membranes, by means of a selective extraction procedure, with increasing concentrations of Triton X-100 as has been described in detail previously [2,5,8]. The incorporation of the band 3 protein was performed as outlined in Ref. 8 with one modification. The final 0.5% (g/v)

Triton X-100/10 mM $Na_2SO_4$/0.2 mM EDTA/0.2 mM $NaN_3$/0.2 mM dithiothreitol 10 mM sodium-Hepes (pH 7.0) extract, which contains band 3, was, after raising the dithioerythritol concentration to 6.5 mM, mixed with a film of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine or total erythrocyte lipids in a 20 : 1 (w/w) lipid-to-protein ratio. Prior to the removal of Triton X-100 by means of the SM-2 Bio-Beads treatment (0.3 g/ml, for 18 h at 4°C), the band 3/Triton X-100/lipid suspension was sonicated in a Bransonic 12 bath sonicator at maximum power under a $N_2$ atmosphere at 0°C, until the suspension was clear. For egg phosphatidylcholine-phosphatidylserine/band 3/Triton X-100, this took about 30 min, while for the erythrocyte lipid/band 3/Triton X-100 suspension, about 60 min were needed. This reconstitution procedure results in more homogeneous vesicles, with respect to vesicle size and protein content, as compared to the reconstitution procedure without the additional sonication step (see below and Ref. 8).

*Characterization of band 3-lipid vesicles.* Protein/lipid ratios were determined using the method of Peterson [21] for protein and Rouser et al. [22] for phosphorus content. The total amount of lipid in the band 3-erythrocyte lipid vesicles was calculated from the phosphorus content, assuming that the cholesterol content of the Reed extract (45 mol% of total lipid) is comparable with that of the vesicles.

Determination of the vesicle trap was performed by enclosure of [$^3$H]Dextran during the reconstitution. For this purpose, [$^3$H]Dextran (20 $\mu$Ci/ml) was added to the band 3/Triton X-100/lipid suspension, which also contains 0.1% (g/v) Dextran, prior to the removal of the detergent. The vesicles (30 $\mu$mol lipid) were separated from nonenclosed [$^3$H]Dextran by elution over a Sepharose 4B column (50 ml wet bed volume) using 10 mM $Na_2SO_4$/0.2 mM EDTA/0.2 mM $NaN_3$/0.2 mM dithiothreitol/0.1% (g/v) Dextran/10 mM sodium-Hepes (pH 7.0) buffer and an elution velocity of 1 ml/min. The column was preequilibrated with the elution buffer.

The vesicle heterogeneity was investigated by Stractan density gradient centrifugation as outlined previously in detail [8] and elution over Sephacryl S-1000 superfine (50 ml wet bed volume in 10 mM $Na_2SO_4$/0.2 mM EDTA/0.2 mM $NaN_3$/0.2 mM dithiothreitol/10 mM sodium-Hepes (pH 7.0)) at 4°C, using an elution velocity of 3 ml/h. This column material has been previously used for size analysis of lipid vesicles of 30–250 nm diameter [23]. In addition, freeze-fracture electron microscopy was used to investigate the morphology of the vesicles [24]. 25% (g/v) glycerol was added to prevent freeze damage.

The protein composition of the band 3-lipid vesicles was determined by gel electrophoresis according to Fairbanks et al. [1]. After staining with Coomassie brilliant blue, gels of band 3-egg phosphatidylcholine-phosphatidylserine vesicles, as well as band 3-erythrocyte lipid vesicles, showed the same gel electrophoresis pattern as the 0.5% Triton X-100 extract; about 85% of the total protein was located in the band 3 region, while 15% was located in the band 4 region. Probably, due to strong interaction with phospholipids, the band 4 region was rather vague. Therefore, we cannot exclude the presence of band 4.2 as well as band 4.5 in these vesicles.

*Protein-free vesicles.* These vesicles were prepared as outlined previously in detail [8] with a few modifications. Firstly, no [7(n)-$^3$H]cholesterol was used in the preparation of the mixed film. Secondly, prior to the removal of the detergent, a similar sonication procedure as described for the band-3-containing vesicles was performed.

*Flux measurements.* The permeability of the vesicles for solutes of different sizes was measured using an influx assay procedure as previously described in detail [8,15].

Briefly, prior to the flux measurements (carried out at 30°C), the vesicles were equilibrated for 24 h at 4°C with 10 mM $Na_2SO_4$/0.2 mM EDTA/0.2 mM $NaN_3$/10 mM sodium-Hepes (pH 7.0) buffer, which contains in addition, 1 mM solute with a molecular weight of less than 900 or 0.25% (g/v) solute with a molecular weight of not less than 900.

The influx of these solutes into the vesicles was monitored by mixing the vesicles with a buffer containing radioactively labelled solutes, followed by a separation of the vesicles from nonenclosed labelled solutes, at appropriate time intervals using ice-cold Sephadex G-75 columns (1.5 ml wet bed

volume) for solutes with a molecular weight of less than 900 [8]. For influx assays performed with [$^3$H]poly(ethylene glycol) 900 and [$^3$H]poly(ethylene glycol) 4000, ice-cold Sephadex G-150 columns (6 ml wet bed volume) were used [15]. In the case of [$^3$H]Dextran, a Sepharose 4B column was used (50 ml wet bed volume) with an elution velocity of 1 ml/min.

Influx curves were plotted as $(1 - dpm_t)/dpm_\infty$ versus time, in which $dpm_t$ corresponds with the vesicle trap at that particular time-point and $dpm_\infty$ corresponds with the vesicle trap as determined with entrapment of the large nonpermeable molecule [$^3$H]Dextran (see below). Alternatively, vesicle trap was determined by a subsequent incubation of the remainder of the vesicle suspension for 18 h at 37 °C [4,8]. If the influx curve showed more than one phase, half-time values of the influx and error therein were estimated form the straight part of the flux curve crossing the 0.500 ($dpm_t/dpm_\infty$) line. In the case of an influx curve with one phase, the half-time value was derived from the direction coefficient using the measurement points indicated in the text. The error in the half-time value was calculated using the least-squares method.

## Results

*Characterization of band 3-lipid vesicles*

Incorporation of band 3, using the Triton X-100-Bio-Beads method described by Wolosin [2], into 94 : 6 molar ratio phosphatidylcholine-phosphatidylserine vesicles, results in a rather heterogeneous vesicle mixture, with respect to protein content and vesicle size [8]. This vesicle heterogeneity greatly complicates the interpretation of a possible lipid dependency of specific as well as nonspecific leakages [8]. Therefore, the band 3-lipid vesicles were prepared, using a slightly different reconstitution method. This method is, in principal, the common Bio-Beads-Triton X-100 reconstitution method, with an additional sonication step, prior to the removal of the detergent. In the case of reconstitution of band 3 with 94 : 6 (molar ratio) phosphatidylcholine-phosphatidylserine, this method results in vesicles which showed, after Stractan density gradient centrifugation, only two bands located at 7.5 and 8.0% Stractan [8]. In comparison, the reconstitution procedure without sonication results in vesicles which are more heterogeneous, as shown by Stractan gradient centrifugation (more bands are present) and freeze-fracture electronmicroscopy [8].

In a typical experiment, equal amounts of phosphorus were present in both regions of the Stractan gradient. Vesicles located in 7.5% Stractan had a 18 : 1 (w/w) phospholipid/protein ratio, while the vesicles located in 8.0% Stractan had a 10 : 1 (w/w) phospholipid/protein ratio. The average lipid-to-protein (w/w) ratio of independently reconstituted vesicles varied between 14 : 1 and 18 : 1 (w/w) lipid/protein, which corresponds with a 1500 : 1 to 2450 : 1 molar lipid/protein ratio, assuming that the molecular weight of band 3 is 88 000–105 000 [25] and the average molecular weight of egg phosphatidylcholine-bovine heart phosphatidylserine is 775.

The initial lipid-to-protein (w/w) ratio of the Triton X-100 extract, being 20 : 1, is somewhat higher than the lipid/protein ratio of the vesicles. This difference is probably caused by a different adsorption of lipid and protein to the Beads.

Band 3-erythrocyte lipid vesicles, prepared with this reconstitution method, show after Stractan density gradient centrifugation only one vesicle band, located at 9.5% Stractan. Using the same method, protein-free erythrocyte lipid vesicles are located at 8.0% Stractan. The higher density of the vesicles prepared from the band-3-containing Triton X-100 extract, indicate the presence of protein in the bilayer, which is confirmed further by chemical analyses which revealed a 16 : 1 to 18 : 1 (w/w) lipid/protein ratio. These values correspond with 2300 : 1 to 3150 : 1 molar ratio/band 3, assuming an average molecular weight for erythrocyte lipids of 600.

Measurement of the vesicle trap by enclosure of [$^3$H]Dextran (see Methods) indicate a trap of 2.7–3.3 l/mol lipid for 94 : 6 molar ratio phosphatidylcholine- or phosphatidylserine-band 3 vesicles and about 3.6 l/mol lipid for erythrocyte lipid-band 3 vesicles.

The interpretation of the results of this vesicle trap determination procedure is, however, complicated by the finding that 20% of the [$^3$H]Dextran is adsorbed to the Bio-Beads during the formation of the vesicles. This results in an under-

estimation of the vesicle trap of, at the most, 20%. In addition, it can be expected that during the removal of Triton X-100, structures are formed which enclose [$^3$H]Dextran but still contain a considerable amount of Triton X-100. Upon further removal of the detergent, the membrane surface area will decrease, resulting in shrinking of the vesicles. This will lead to concentration of the impermeable Dextran [26]. As a consequence, the vesicle trap will be overestimated. For these reasons, vesicle traps of band 3-phosphatidylcholine-phosphatidylserine vesicles and band 3-erythrocyte lipid vesicles are considered to have a comparable value of 3 l/mol lipid. As a consequence, all influx rates of the studied permeants are related to this trap value.

Size analysis of the band 3-phosphatidylcholine-phosphatidylserine and band 3-erythrocyte lipid vesicles by means of elution over Sephacryl S-1000 superfine [23] (see Methods) and freeze-fracture electron microscopy revealed in both cases vesicles with a diameter which ranges from 500 to about 2500 Å. The band 3-phosphatidylcholine-phosphatidylserine vesicles show a considerable amount of intramembrane particles on their fracture faces with diameters up to 100 Å (Fig. 1A), In sharp contrast, no such particle could be detected in the fracture faces of band 3-erythrocyte lipid vesicles (Fig. 1B). This may indicate a higher aggregation state of band 3 in the band 3-phosphatidylcholine-phosphatidylserine vesicles as compared to the band 3-erythrocyte lipid vesicles.

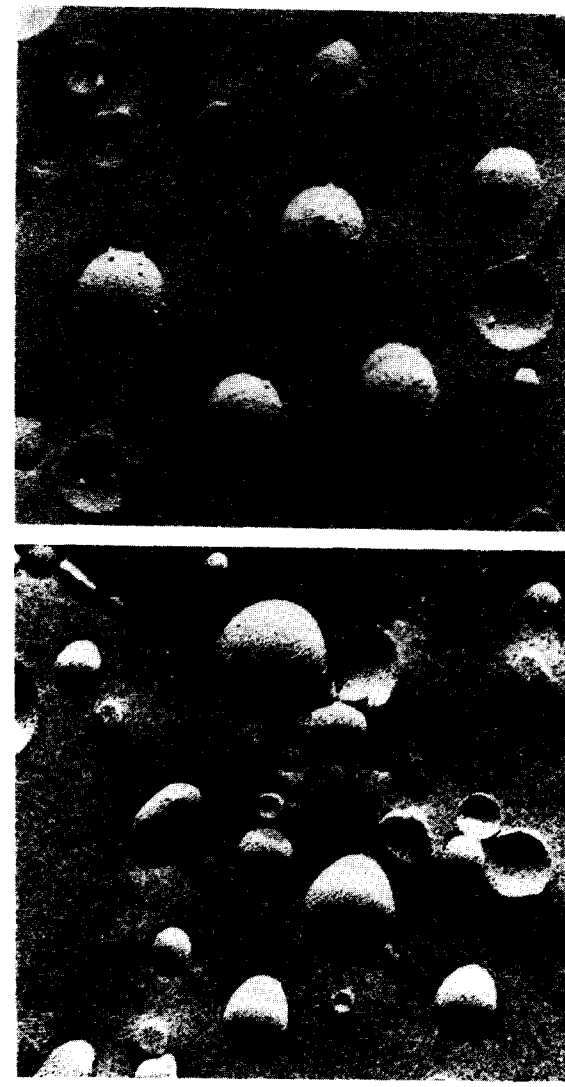

Fig. 1. Freeze-fracture electron-microscopy pictures of band 3-lipid vesicles. (A) Band 3-phosphatidylcholine-phosphatidylserine vesicles. (B) Band 3-erythrocyte lipid vesicles. Magnification ×100000.

*Nonspecific permeability characteristics of band 3-phosphatidylcholine-phosphatidylserine vesicles*

In a former study, it was shown that band 3-94 : 6 (molar ratio) phosphatidylcholine-phosphatidylserine vesicles have, compared to pure lipid vesicles, an increased nonspecific permeability for L-glucose [8]. In order to investigate whether the increased nonspecific permeability of the band 3-phosphatidylcholine-phosphatidylserine vesicles is of the extent reported for glycophorin-phosphatidylcholine vesicles [15] (pore diameter 15–18 Å), we studied the permeability of the band 3-lipid vesicles for nonelectrolytes of different size. In Fig. 2, such an experiment is depicted. It is shown that the band 3-phosphatidylcholine-phosphatidylserine vesicles have a relatively high permeability at 30 °C for L-glucose (half-time of influx of about 120 min) and for the much larger molecule poly(ethylene glycol) 4000 (half-time of influx 224 ± 24 min as derived from the first six measurement points). However, unexpectedly, poly(ethylene glycol) 900, a molecule with a size intermediate between those of L-glucose and poly(ethylene glycol) 4000, shows an influx rate which appears to be much faster than L-glucose (half-time

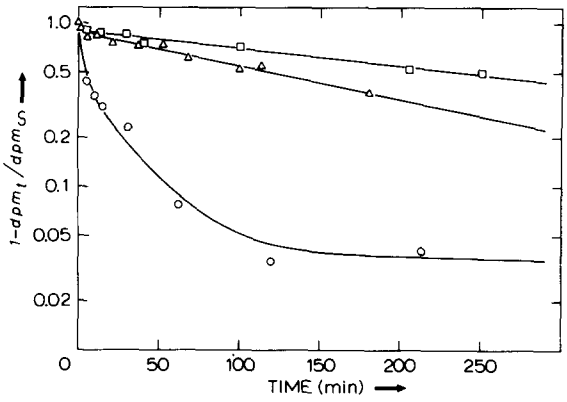


Fig. 2. Nonspecific permeability characteristics of band 3-phosphatidylcholine-phosphatidylserine vesicles. △———△, Influx curve of L-glucose; ○———○, influx curve of poly(ethylene glycol) 900; □———□, influx curve of poly(ethylene glycol) 4000. All influx assays were performed at 30 °C, as outlined in Methods. All influx curves were related to a trap value of 3 $\mu$l/$\mu$mol phospholipid, obtained as an average value by enclosure of [$^3$H]Dextran (see Methods).

of influx about 5 min). Before attempting to rationalize this finding, it is important to realize that the amount of labelled solute in the vesicles is determined by two processes. Firstly, the influx of the solute during the incubation and secondly, the efflux of the solute during the rapid separation at 0 °C of the vesicles from the medium. In the case of an extremely permeable molecule, this could result in an apparent low permeability. To get an insight into these possibilities, the equilibrium isotope distribution obtained after 18 h incubation of the vesicles at 37 °C was determined (Table I, left column). As can be noticed, the apparent vesicle trap increased upon increasing the molecular weight of the permeant to 900. The relatively low vesicle-trap values, obtained for L-glucose or raffinose, indicate a loss of trapped, labelled small solutes during the elution over the ice-cold Sephadex columns. The influx curve for L-glucose (Fig. 2) thus reflects only a part of the vesicle population. The remaining part of the vesicles apparently has an extremely high permeability which cannot be detected with this influx assay.

In contrast, the high trap value obtained for poly(ethylene glycol) 900 (Table I) indicates that the separation of the vesicles during the influx assay from the medium, is not accompanied by a loss of a considerable amount of enclosed [$^3$H] poly(ethylene glycol) 900. As a consequence, the influx of poly(ethylene glycol) 900 into the total vesicle population, including the vesicles which are very permeable to L-glucose, can be followed. This will result in an influx curve for poly(ethylene glycol) 900 with an apparent shorter half-time of influx and higher end trap value, than for L-glucose. In view of the uncertainties in the trap determinations, as discussed above, the high trap value obtained for poly(ethylene glycol) 900 of 4.2 $\mu$l/$\mu$mol phospholipid can be considered to be comparable with the trap value of 3 $\mu$l/$\mu$mol as obtained by enclosure of [$^3$H]Dextran.

The vesicle trap obtained after prolonged incubation with poly(ethylene glycol) 4000 and Dextran shows about 50–60% of the trap value as determined by enclosure of [$^3$H]Dextran. This re-

TABLE I

BAND 3-LIPID VESICLES TRAP AS DETERMINED WITH PERMEANTS OF DIFFERENT SIZES AFTER PROLONGED INCUBATION AFTER THE INFLUX ASSAY AT ELEVATED TEMPERATURE

Vesicle traps were determined after a subsequent incubation of the remainder of the vesicle suspension with the permeant in question for 18 h at 37 °C. The presented trap values (l/mol) are the average of at least three independent experiments, S.D. 10%. n.d., not determined.

| Permeant | Band 3-egg phosphatidylcholine-bovine heart phosphatidylserine vesicles | Band 3-erythrocyte lipid vesicles |
|---|---|---|
| L-Glucose | 1.4 | 4.6 |
| Raffinose | 3.0 | 4.7 |
| Poly(ethylene glycol) 900 | 4.2 | 2.3 |
| Poly(ethylene glycol) 4000 | 2.1 | 0.6 |
| Dextran | 2.6 | n.d. |

sult suggests that these large molecules are only able to equilibrate in 18 h at 37 °C with a part of the vesicle population which is leaky for L-glucose. Apparently, these large molecules cannot equilibrate with that part of the total vesicle population which does not suffer from a loss of trapped L-glucose.

In conclusion, only in the case of poly(ethylene glycol) 900, a molecule of a size which is in between that of L-glucose and poly(ethylene glycol) 4000, the permeability of the entire vesicle population can be followed.

Taking the trap values, obtained after prolonged incubation at elevated temperature as reference, it can be stated that the relatively less permeable vesicle population is permeable for molecules of $M_r$ of less than 900 which corresponds with a pore diameter of 15–18 Å [15]. Since, even the very big molecule Dextran ($M_r$ 80 700) is able to equilibrate with the rest of these vesicles, these vesicles must have pore diameters of more than 60 Å [15]. In agreement with this, in a former study of Cabantchik et al. [4] in which the sulfate permeability of band 3-94 : 6 molar ratio phosphatidylcholine-phosphatidylserine vesicles was studied, it was suggested that only about 20% of the vesicles are tightly sealed. The existence of two subpopulations of band 3-phosphatidylcholine-phosphatidylserine vesicles, having different permeation pathway diameters might be a reflection of the two vesicle bands which show up after Stractan density gradient centrifugation (see Results). One vesicle population is not permeable towards Dextran, while the other vesicle population is permeable to Dextran and Stractan, which has the same molecular weight. Therefore, it may be expected that the population permeable for Stractan will show up in a higher density region of the stractan gradient.

*Nonspecific permeability characteristics of band 3-erythrocyte lipid vesicles*

The band 3-phosphatidylcholine-phosphatidylserine vesicles suffer from a greatly enhanced nonspecific permeability. Before an insight into a possible lipid dependency of the transport functions of band 3 can be obtained, it is therefore necessary to study first the lipid dependency of the nonspecific permeability.

Since glycophorin-erythrocyte lipid vesicles show a much smaller nonspecific permeability as compared to glycophorin-phosphatidylcholine vesicles [9], it might be expected that the nonspecific permeability of the band 3-erythrocyte lipid vesicles could also be decreased. In Fig. 3, influx curves for L-glucose, poly(ethylene glycol) 900 and poly(ethylene glycol) 4000 are depicted. In contrast to the band 3-phosphatidylcholine-phosphatidylserine vesicles, the influx rate relates to the size of the studied permeant. The smallest molecule, L-glucose, has a half-time of influx of about 150 min, the largest molecule, poly(ethylene glycol) 4000, 1792 ± 340 min (as calculated using all the measurement points) and the molecule with intermediate size, poly(ethylene glycol) 900 has an intermediate half-time of influx of about 310 min.

As expected, the vesicle trap, determined after prolonged incubation of the vesicles, decreases with increasing molecular weight of the permeant (Table I, right column). This indicates that the bigger molecules are not able to equilibrate with the available vesicular volume in 18 h at 37 °C. The vesicle trap as determined by prolonged incubation with the smallest molecule, L-glucose, has a value comparable with the vesicle trap value determined by the enclosure of [$^3$H]Dextran. From this, it can be conluded that during elution of the vesicles, in contrast to band 3-phosphatidylcho-

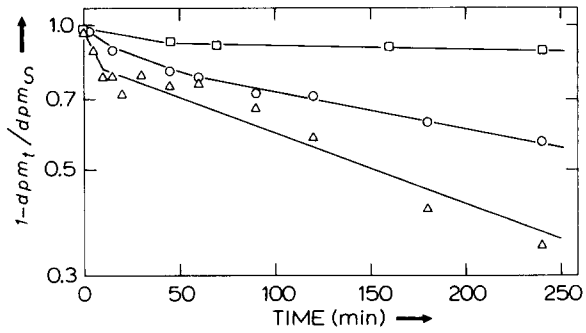


Fig. 3. Nonspecific permeability characteristics of band 3-erythocyte lipid vesicles. △———△, Influx curve of L-glucose; ○———○, influx curve of poly(ethylene glycol) 900; □———□, influx curve of poly(ethylene glycol) 4000. All influx assays were performed at 30 °C as outlined in Methods. All influx curves were related to a trap value of 3 μl/μmol phospholipid, obtained as an average value by enclosure of [$^3$H]Dextran (see Methods).

line-phosphatidylserine vesicles, no loss of enclosed permeants occurs. The permeability of the band 3-erythrocyte lipid vesicles for poly(ethylene glycol) 4000, as compared to the band 3-phosphatidylcholine-phosphatidylserine vesicles, is also lower (half-times of influx as respectively 1792 ± 340 min, derived from all measurement points, and 224 ± 24 min). Furthermore, it should be noted that although Stractan density gradient centrifugation reveals only one vesicle band, suggesting a homogeneous vesicle preparation, the influx curves seem to be composed of more than one phase.

In conclusion, from these results it can be suggested that the band 3-erythrocyte lipid vesicles show a much smaller nonspecific permeability (Fig. 3) as compared to band 3-phosphatidylcholine-phosphatidylserine vesicles. The decrease in nonspecific permeability upon incorporation of band 3 into its natural lipid environment, as compared to band 3-phosphatidylcholine-phosphatidylserine vesicles, is the same as the decrease of nonspecific permeability observed in glycophorin-lipid vesicles [9]. However, as compared to the protein-free erythrocyte lipid vesicles, the band 3 lipid vesicles still show an increased (nonspecific) permeability. Taking again the vesicle trap values of Table I as reference, it is clear that the band 3-erythrocyte lipid vesicles are only able to equilibrate with raffinose. This corresponds with a pore diameter of about 12 Å [15] which is much smaller than the pores present in the band 3-phosphatidylcholine-phosphatidylserine vesicles having a diameter of up to 60 Å.

*Specific permeability properties of erythrocyte lipid-band 3 vesicles*

Since the band 3-total erythrocyte lipid vesicles show a low nonspecific permeability, it is now worthwhile to study whether the anion transport system in these vesicles is functional. The common approach to investigate this in model systems, like lipid vesicles, is to study the magnitude of sulfate permeability of the vesicles and its sensitivity to anion transport-specific inhibitors like DIDS or DNDS [2–5,7,8]. However, in the band 3-erythrocyte lipid vesicles we could not demonstrate any inhibiting effect of 10 $\mu$M DIDS on the $SO_4^{2-}$ permeability, whereas similar concentrations of DIDS inhibited the $SO_4^{2-}$ transport of erythrocytes completely (data not shown).

Another approach to investigate whether there is some specific increase in anion permeability in the band 3-lipid vesicles is the comparison of the ratio of the sulfate permeability to L-glucose permeability of protein-free vesicles with the same ratio of the band 3-lipid vesicles [8]. Such an experiment is depicted in Fig. 4. The protein-free vesicles show an extremely low permeability for sulfate (half-time of influx 13 800 ± 1400 min, as derived from all the measurement points). The half-time of L-glucose is 1260 ± 260 min (as derived from all the measurement points). The band 3-erythrocyte lipid vesicles show an increased sulfate permeability with a half-time of about 200 min. The L-glucose permeability of these vesicles was also increased, as compared to protein-free vesicles, and had a half-time of about 160 min.

The other native transport function of band 3 [27,37,38] or band 4.5 [39–41], which are both present in the vesicles, is D-glucose transport. To check whether this transport function in the recombinant vesicles is intact, two approaches have been followed. Firstly, since the glucose carrier is highly stereospecific [28], the permeability of the band 3-lipid vesicles towards D-glucose is compared with that of L-glucose. Secondly, the sensitivity of the D-glucose, as well as the L-glucose permeability for an inhibitor of the native glucose transport system, phloretin, was tested.

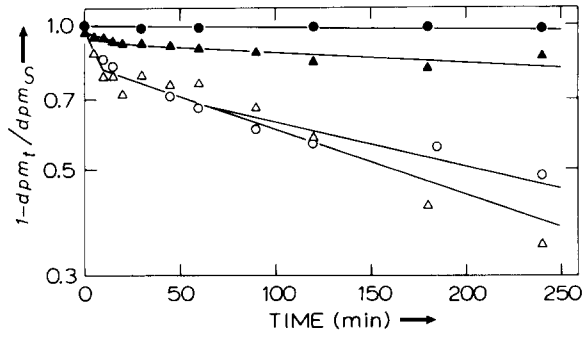


Fig. 4. Specificity of enhanced $SO_4^{2-}$ permeability of band 3-erythrocyte lipid vesicles, compared with protein-free-erythrocyte lipid vesicles. ●———●, $SO_4^{2-}$ permeability of protein-free vesicles; ▲———▲, L-glucose permeability of protein-free vesicles; ○———○, $SO_4^{2-}$ permeability of band 3-erythrocyte lipid vesicles; △———△, L-glucose permeability of band 3-erythrocyte lipid vesicles. All influx assays were performed at 30 °C, as outlined in Methods.

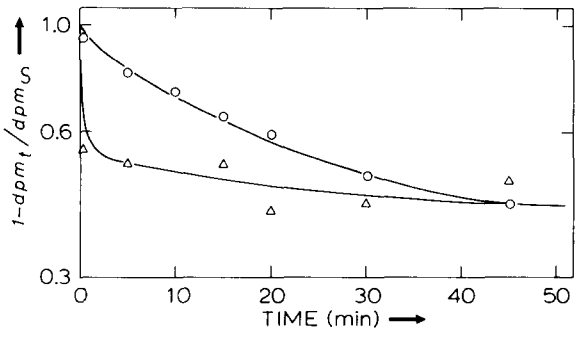


Fig. 5. Stereospecificity of glucose permeability of band 3-erythrocyte lipid vesicles. O——O, L-glucose permeability; Δ——Δ, D-glucose permeability. Influx assays were performed at 30 °C, as outlined in Methods.

The results of the first approach are depicted in Fig. 5. The influx curve of D-glucose is composed of two phases. The first phase has a half-time of less than 1 min, followed by a phase with a much lower influx rate. In contrast to the D-glucose influx, the L-glucose influx has a much slower influx rate, with a half-time value of about 28 min. Since the first phase of the D-glucose influx curve corresponds with the half of the available vesicular volume, it might be concluded that half of the vesicles contain the functional D-glucose carrier.

In contrast to the band 3-erythrocyte lipid vesicles, no difference in D- and L-glucose permeability of the band 3-phosphatidylcholine-phosphatidylserine vesicles could be observed (data not shown).

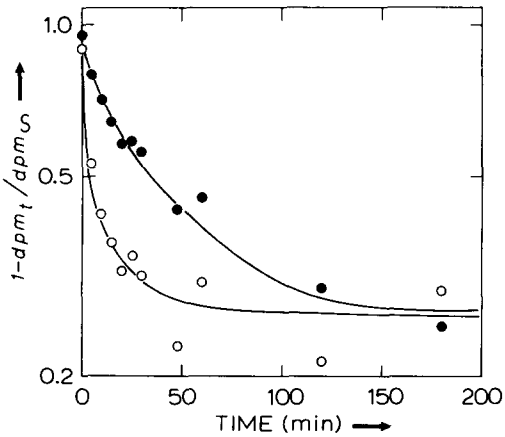


Fig. 6. Effect of 0.25 mM phloretin on the D-glucose flux at 10 °C into band 3-erythrocyte lipid vesicles. O——O, Influx curve without 0.25 mM phloretin; ●——●, influx curve with 0.25 mM phloretin. Influx assays were performed at 10 °C, as outlined in Methods.

The effect of 0.25 mM phloretin on the D-glucose influx rate into band 3-erythrocyte lipid was studied at 10 °C, since at 30 °C, the D-glucose influx rate was too fast to determine the kinetic parameters. At 10 °C, the half-time of D-glucose of influx without inhibitor is about 4 min, while the half-time of D-glucose influx with inhibitor is about 35 min (Fig. 6).

The large difference in D-glucose permeability as compared to L-glucose permeability, and the inhibiting effect of phloretin on the D-glucose permeability, strongly suggests that the glucose transport system in the band 3-erythrocyte lipid vesicles is still intact.

## Discussion

In this study, we describe the influence of lipid composition on the barrier properties of band 3-lipid vesicles.

By the introduction of a sonication step prior to the removal of the detergent, the homogeneity of the vesicles could be improved. However, size analysis by means of Sephacryl S-1000 chromatography and freeze-fracture electron microscopy reveal that in both types of band 3-lipid vesicles there is still a spread in vesicle diameter of 500 to 2500 Å. Since the average vesicle size, as measured with the enclosure of [$^3$H]Dextran, and the protein content of the band 3-phosphatidylcholine-phosphatidylserine and band 3-erythrocyte lipid vesicles ase similar, it is allowed to compare the permeability properties of both types of vesicles.

In order to characterize the nonspecific permeability of both types of vesicles, the influx rates of nonelectrolytes of different sizes were measured. In a former study, influx rates were related to a vesicle trap obtained after prolonged incubation after the influx assay at elevated temperature [8]. However, these trap determinations gave irreproduceable trap values. Therefore, to overcome this difficulty, we determined the vesicle trap by means of enclosure of a large molecule, for example, Dextran and related the influx rates to the trap value of 3 $\mu$l/$\mu$mol phospholipid found with this

method. Moreover, the present more homogeneous vesicles showed a much more reproducible trap volume value as obtained from the prolonged incubation (variation less than 10%, ten experiments).

It has been hypothesized that the increased nonspecific permeability, due to the incorporation of an integral membrane protein, is caused by packing defects at the lipid/protein interface and/or channels present in protein aggregates [9.15]. Freeze-fracture electron microscopy reveals that the band 3-erythrocyte lipid vesicles do not have any protein particle on their fracture faces, while in contrast, the band 3-phosphatidylcholine-phosphatidylserine vesicles show considerable amount of protein particles with diameters up to 100 Å. Therefore, it is tempting to speculate that the decrease nonspecific permeability of the band 3-erythrocyte lipid vesicles is caused by the low aggregation state of the band 3 protein in the erythrocyte lipid bilayer. This idea is supported by the finding that the aggregates of the tryptic hydrophobic segment of glycophorin increase the bilayer permeability relative to the situation in which monomers are present [29].

Another possible explanation for the decreased nonspecific permeability of band 3-erythrocyte lipid vesicles may be found in the geometry of the lipid/protein interface. In order to match the irregular protein surface in a membrane, a mixture of lipids, all with varying molecular shape, might be required as was previously suggested by Israelachvili [30]. As compared to the phosphatidylcholine-phosphatidylserine bilayer, a broad spectrum of differently shaped molecules are present in the erythrocyte-lipid bilayer. Therefore, it is likely that the band 3 molecules are immersed in the erythrocyte-lipid bilayer in such a way that packing defects do not exist at the lipid protein interface any longer. As a consequence, the nonspecific permeability is low.

Due to the high nonspecific permeability of the band 3-phosphatidylcholine-phosphatidylserine vesicles, the detection of specific permeability, related with the transport functions of band 3, is difficult [8]. An observed, increased $SO_4^{2-}$ permeability of these vesicles, as compared to protein-free vesicles, might therefore be partial nonspecific and partial related with the anion transporter [8].

Since the nonspecific permeability in the band 3-erythrocyte lipid vesicles is low, it can be expected that the permeability properties of these vesicles can be more easily understood. The anion-transport functions of band 3 can be evaluated by comparison of the sulfate/L-glucose ratio of the band 3-lipid vesicles and protein-free vesicles. The increase in sulfate permeability, relative to that of L-glucose permeability, caused by the incorporation of band 3 into the lipid bilayer is about 9-fold. This might suggest the presence of a functional anion-transport system in the band 3-erythrocyte lipid vesicles.

In order to evaluate the efficiency of transport, transport numbers ($TN$) (defined as the number of sulfate ions transported per band 3 protein molecule per minute at a standard sulfate concentration) can be calculated, using the equation:

$$TN = \frac{k_{app} \cdot C_{SO_4^{2-}} \cdot \bar{V}}{\bar{C}_{prot}}$$

in which $k_{app}$ is the rate coefficient of influx, $C_{SO_4^{2-}}$ the standardized $SO_4^{2-}$ concentration in the vesicles (10 mM, equivalent with $6 \cdot 10^{15}$ ions/$\mu$l), $\bar{V}$ the vesicle trap ($\mu$l buffer/$\mu$mol lipid) and $\bar{C}_{prot}$ the mean number of protein molecules per $\mu$mol phospholipid [6,31]. For band 3-erythrocyte lipid vesicles, these factors have the following value at 30 °C: $k_{app}$, 0.693/260 $min^{-1}$ (see Fig. 4); $C_{SO_4^{2-}}$, $6 \cdot 10^{15}$ ions/$\mu$l; $\bar{V}$, 3 $\mu$l/$\mu$mol lipid and $\bar{C}_{prot}$, $(1/2875) \cdot 10^{-6} \times 6 \cdot 10^{23}$ band 3 molecules/$\mu$mol phospholipid, giving a $TN$ number of about 0.3 [sulfate ions/band 3 · min], which is about 30-times too low as compared to $TN$ of the human erythrocyte of 8 h at 25 °C [32,6].

The low molecular efficiency of the anion transport in the reconstituted vesicles, suggests that most of the band 3 molecules are not properly reconstituted or that every band 3 molecule functions very poorly. This is supported by the observation that 10 $\mu$M DIDS does not inhibit the sulfate permeability of the vesicles. Although the presence of a functional anion-transport system in the band 3-erythrocyte lipid is suggested by the finding that the anion (sulfate) permeability is increased with respect to the L-glucose permeability, the lack of inhibiting effect of 10 $\mu$M DIDS on the system and the low transport numbers

makes clear that band 3, even in its native lipid environment, has not adopted its functional conformation. Since band 3 has interactions with almost all the other erythrocyte membrane proteins [33], these interactions might be responsible for the functional conformation. Therefore, the lack of anion-transport activity or DIDS sensitivity of band 3 might be caused by the absence of these protein-protein interactions in the recombinant vesicles. In this respect, it is worthwhile to note that, using the Triton X-100 extraction procedure, only 50% of the band 3 molecules are extracted [8]. In addition, it has been suggested that this may be a consequence of the fact that 15–40% of the band 3 molecules in the native membrane are strongly associated with the cytoskeleton via band 2.1 [42]. Therefore, if only the tightly bound band 3 fraction is representing the active anion channel, no transport activity of the loosely bound band 3 molecules which are present in the recombinant vesicles can be expected. In addition, since the erythrocyte lipid vesicles probably do not show lipid asymmetry as being present in the native erythrocyte membrane [45,46], it might be expected that the functioning of the band 3 protein is also governed by the existence of lipid asymmetry. The lack of transport activity of the band 3 molecule, might also be a consequence of irreversible structural change of the protein during the sonication procedure. However, this is unlikely, because the band 3-phosphatidylcholine/phosphatidylserine vesicles prepared by means of the reconstitution procedure with and without sonication, have similar permeability characteristics with respect to the sulfate/L-glucose permeability ratio and the protein-particle size as revealed by freeze-fracture electron microscopy [8].

The presence of the D-glucose carrier system in the band 3-erythrocyte lipid vesicles is unambiguous. This conclusion is drawn from the findings that, firstly, the band 3-erythrocyte lipid vesicles show a preferential uptake of D-glucose over L-glucose and secondly, phloretin, an inhibitor of the transport system in the erythrocyte membrane [28], shows an inhibiting effect on D-glucose influx.

These results are in agreement with previous studies, concerning the permeability characteristics of D-glucose carrier containing model systems [34,35]. Recently, it has been suggested that the native D-glucose transporter of the human erythrocyte membrane is an accompanying component of band 3 and that band 4.5 represents a partially active fragment of the native transporter [36]. This finding is in agreement with our results, which show an active D-glucose transport in vesicles, containing band 3 and probably band 4.5.

In conclusion, we have shown in this study that the nonspecific permeability of band 3-lipid vesicles is strongly lipid-dependent. Therefore, a fruitful investigation into the possible lipid dependency of a reconstituted transport protein should be accompanied by a thorough study of the lipid dependency of nonspecific transport processes induced by the presence of the protein in the bilayer.

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