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THE ANION PERMEABILITY OF VESICLES RECONSTITUTED WITH INTRINSIC PROTEINS FROM THE HUMAN ERYTHROCYTE MEMBRANE

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Band 3 protein was reconstituted with lipid vesicles consisting of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine in a 2500 : 1 phospholipid : protein molar ratio by means of a Triton X-100/beads method. The SO_4^{2-} permeability of the resulting vesicles was measured using an influx assay procedure in which the vesicles were sampled and subsequently eluted over Sephadex columns at appropriate time intervals. The accuracy of the assay was greatly increased by using an internal standard in order to correct for vesicle recovery. In agreement with previous work, it could be demonstrated that incorporation of band 3 in the vesicles caused an increase in SO_4^{2-} permeability, which could be (partially) inhibited by high concentrations of DIDS or a competitive anion such as thiocyanate. However, the magnitude of the increased SO_4^{2-} permeability was highly variable, even when vesicles were reconstituted using band 3 isolated from one batch of ghosts. In addition, the SO_4^{2-} influx curves showed complex kinetics. These results are related to the existence of vesicle heterogeneity with respect to protein content and vesicle size as revealed by stractan density gradient centrifugation and freeze-fracture electron microscopy. Band 3 incorporation also increased the L-glucose permeability of the vesicles which could also be inhibited by DIDS. Glycophorin, which has no known transport function, reconstituted with lipid vesicles consisting of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine in a 400 : 1 phospholipid : protein molar ration increased the bilayer permeability towards SO_4^{2-} as well as towards L-glucose. Surprisingly, the SO_4^{2-} permeability in the vesicles could also be inhibited by DIDS and thiocyanate. It is concluded that the use of DIDS and a competitive anion, thiocyanate, in order to prove that band 3 is functionally reconstituted, is highly questionable. The increased SO_4^{2-} and L-glucose permeability of band 3-lipid as well as glycophorin-lipid vesicles and the inhibitory action of DIDS are discussed in the light of the presence of defects at the lipid/protein interface and protein aggregation, which may induce the formation of pores. Since the band 3-lipid vesicles are more permeable for SO_4^{2-} than for L-glucose, in contrast to the glycophorin-containing vesicles, it is suggested that some anion specificity of the increased bilayer permeability in the band 3-lipid vesicles is still preserved.

Introduction

In order to achieve and maintain a certain distribution of solutes over a semipermeable membrane, specific transport system are necessary in a membrane [1]. The most important system in the

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Abbreviations: DIDS, 2,2′-diisothiocyanostilbene-4,4′-disulfonic acid; DNDS, 2,2′-dinitrostilbene-4,4′-disulfonic acid; Hepes, : *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

human erythrocyte membrane is that involved in the exchange of bicarbonate for chloride. Tissue cells produce carbon dioxide as a result of their metabolic activity. The carbon dioxide diffuses freely through the erythrocyte membrane and is converted to bicarbonate by the action of carboanhydrase. The extra amount of bicarbonate disturbs the Donnan equilibrium of anions over the erythcrocyte membrane, which is compensated by a bicarbonate-chloride exchange. In the lung, the reverse process occurs.

During the past 10 years it has become apparent that a protein is responsible for this anion exchange. Treatment of erythrocytes with specific, radioactively labeled, impermeable inhibitors of the anion transport, showed that almost 100% of the label was incorporated in band 3 protein [2] (nomenclature according to Fairbanks [3]). For an excellent review of the properties of this transport protein, see Ref. 4.

One approach to gain insight in to the molecular details of a transport system and its dependency on lipid-protein interactions is to isolate and reconstitute it in simple model systems such as lipid vesicles. Recently, there have been several reports about reconstitution of band 3 in artificial bilayers [5–8]. In general, in these studies the protein was partially purified by selective Triton X-100 extraction of erythrocyte ghosts, this being in one case followed additionally by various chromatographic steps [7]. Reconstituted vesicles were prepared by mixing the protein, dissolved in Triton X-100-containing buffer, with phospholipids, this being followed by the removal of the detergent by means of treatment with Bio-Beads [5–8] or with a toluene/phosphatidylcholine extraction [7]. These band 3-lipid vesicles showed a significant permeability for sulfate, which could be partially inhibited by DIDS, a specific erythrocyte anion transport inhibitor.

Although the enhanced sulfate permeability in band 3-lipid vesicles and its sensitivity towards inhibitors and competitive anions seem to indicate that the anion transport system is indeed properly reconstituted, several basic properties of the increased anion transport still remain obscure. Until now it has not been clear whether the enhanced sulfate permeability in the band 3-lipid vesicles is partially caused by a nonspecific leakage due to packing defects in the bilayer caused by the presence of this integral membrane protein.

Reconstituted systems in general are more permeable than the native membrane. This has been documented for model membranes containing glycophorin and glycophorin fragments [9–11]. The presence of these polypeptides, which have no known transport function, greatly increases the bilayer permeability in a lipid-dependent fashion [12]. This poses two questions. Firstly, is the band 3-induced increase in permeability specific for anions such as sulfate? In other words, does the protein enhance the permeability of other molecules which normally cannot move across the erythrocyte membrane? Secondly, is the increase in sulfate permeability specific for band 3, or would other integral membrane proteins when incorporated in vesicles show a similar enhanced sulfate leak? Furthermore, it is obvious that the increased sulfate permeability of band 3-lipid vesicles can be critically evaluated only when the data are compared to similar measurements on protein-free vesicles. Therefore, to gain further insight into these various possibilities, we incorporated, respectively, band 3 by means of the Triton X-100 beads method and glycophorin by means of the method of MacDonald and MacDonald [13] in unilamellar vesicles and studied the effect of protein incorporation in the lipid bilayer on the permeability of the vesicles towards sulfate and L-glucose, comparing the results with similar measurements on protein-free vesicles.

Materials and Methods

Materials

$Na_2^{35}SO_4$ (25–40 Ci/mg, 0.9–1.5 TBq/mg) and [7(n)-^{3}H]cholesterol (5–15 Ci/mmol, 185–550 GBq/mmol) were purchased from Amersham International. L-[1(n)-^{3}H]Glucose (10.7 Ci/mmol, 396 GBq/mmol) was purchased from New England Nuclear. ^{3}H-labeled Triton X-100 (0.045 Ci/mmol, 1.67 GBq/mmol) was purchased from Rohm and Haas.

^{14}C-labeled phosphatidylcholine was purified from rat liver, previously injected with ^{14}C-labeled ethanolamine according to the procedure of Bjørnstad [14], and was a kind gift from Dr. R.A.

Demel. DIDS was obtained from Pierce Chemicals and its capacity for inhibiting anion exchange was tested on (a) $^{35}SO_4^{2-}$-efflux from erythrocytes according to the method of Cabantchik and Rothstein [15], and on (b) chloride-fluoride exchange over the erythrocyte membrane according to the method of Halestrap [16]. Egg phosphatidylcholine was purified from egg yolk according to standard procedures. Phosphatidylserine was obtained from bovine heart as will be described elsewhere. All phospholipids were pure, as they showed one spot on high-performance thin-layer chromatography using the appropriate solvent systems.

Glycophorin was purified according to the procedure as described by Taraschi et al. [17] and was a kind gift of Dr. A.T.M. Van der Steen. Stractan (*arabino*-galactan) was obtained from Sigma; SM-2 Bio-Beads from Bio-Rad, pretreated according to Holloway [18]. Triton X-100 was purchased from Rohm and Haas and was purified according to the method of Ashani and Catravas [19]. It was stored under nitrogen in the dark at 4°C. Wheat-germ lectin Sepharose 6MB and Sephadex G-75 were purchased from Pharmacia Fine Chemicals.

Band 3 protein purification

The band 3 protein was purified from the human erythrocyte membrane by means of selective extraction with Triton X-100 as previously described by Köhne et al. [8]. After the final extraction step with a 0.5% Triton X-100/24 mM NaCl/0.2 mM EDTA/0.2 mM dithiothreitol/0.2 mM NaN_3/10 mM Na-Hepes (pH 8.0) buffer, the suspension was centrifuged in a Beckman ultracentrifuge L5-65 (rotor 30, 20 min, 30000 rpm, 4°C) and the supernatant was immediately used for the reconstitution step. Typically, when ghosts of 4 mg protein/ml were used as starting material for the purification, the 0.5% Triton X-100 extract contained 0.1 mg protein/ml. The protein was mostly band 3 as shown by polyacrylamide gel electrophoresis [6,8], but still contained glycophorin, as could be detected by staining the gel with periodic acid-Schiff reagent [6,8].

Since 25% of ghost protein represents band 3 [4] and the absolute amount of band 3 present in the 0.5% Triton X-100 extract is 0.5 mg, the extraction percentage of band 3 is about 50%.

The band 3 : glycophorin molar ratio in the 0.5% Triton X-100 extract when analyzed by protein and sialic assays (see section, Analytical methods) appeared to be 2 : 1. When this number is compared with the band 3 : glycophorin ratio in the human erythrocyte ghosts, according to Ref. 20, of 2 : 1, it seems that the selective extraction step with 0.05% Triton X-100 of glycophorin in which glycophorin is extracted to 80% [6] does not increase the band 3 : glycophorin ratio. However, when this original ratio of band 3 : glycophorin in erythrocyte ghost is recalculated with the data available nowadays concerning the molecular weight of glycophorin (31500 [21]) and the sialic acid content per glycophorin (31–32 mol sialic acid/mol glycophorin [22]), the molar ratio appears to be 1 : 1. When we take into account that at the 0.05% Triton X-100 step, about 80% of the glycophorin [6] and no significant amount of band 3 is extracted and at the final 0.5% Triton X-100 extraction step the remaining glycophorin and about 50% of band 3 is extracted, the band 3 : glycophorin ratio will become 2 : 1, which is in agreement with our result.

The supernatant of the final extraction step was either immediately used for the reconstitution with phospholipids (see next section) or, in order to reduce the glycophorin content of the extract, was chromatographed on a wheat-germ lectin Sepharose 6MB column (5 × 1 cm) at 4°C [23]. The column material was preswollen and equilibrated in 0.5% Triton X-100/24 mM NaCl/0.2 mM EDTA/0.2 mM dithiothreitol/0.2 mM NaN_3/10 mM Na-Hepes (pH 8.0). The maximum amount of 0.5% Triton X-100 extract added was 10 ml. The elution velocity of the extract was 1 ml/min. Due to the specific binding of glycophorin to the covalently bound lectin, the band 3 : glycophorin ratio was increased from 2 : 1 to 8 : 1. This purified 0.5% Triton X-100 extract was treated and used for reconstitution with phospholipids in the same way as described for the original 0.5% Triton X-100 extract.

Reconstitution of band 3 with lipid vesicles

The incorporation of the band 3 protein into lipid vesicles was performed as outlined in Ref. 8 with a few modifications. Briefly, 2 vol. of the Triton X-100 extract were mixed at 0°C with 1 vol. of a buffer comprising 30 mM Na_2SO_4/0.2

mM EDTA/0.2 mM dithiothreitol/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). Then this mixture was added at 0°C to a film of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine containing a trace amount of [7(n)-^{3}H]cholesterol (phospholipid : cholesterol molar ratio, 10000 : 1) in a 2500 : 1 phospholipid : protein molar ratio. This film was prepared by thoroughly evaporating a chloroform solution of the lipids under a stream of nitrogen. After raising the dithiothreitol concentration to 6.5 mM, the lipid film was dissolved into the protein extract by means of agitation on a Vortex mixer. Subsequently, Triton X-100 was removed by treatment with SM2 beads according to Köhne et al. [8] or according to Gerritsen et al. [24] with identical results.

After this treatment, the beads were removed by means of filtration over glass wool. The vesicles formed upon the removal of Triton X-100 were harvested at 150000 × *g* for 90 min at 4°C using a Beckman ultracentrifuge L5-65, rotor 40 at 39500 rpm. The protein composition of the band 3-lipid vesicles as revealed by gel electrophoresis, according to Fairbanks [3] was about 95% band 3 and 5% band 4.2. No band 4.5 or band 7 could be detected. The sialic acid content of the vesicles, as measured with a sialic acid assay after extraction of the interfering, unsaturated phospholipids with chloroform/methanol [6] was 14 nmol sialic acid/mg protein for vesicles prepared from the purified 0.5% Triton X-100 extract by means of wheat-germ lectin chromatography and 32 nmol sialic acid/mg protein for vesicles prepared from the unpurified Triton X-100 extract.

If it is assumed that all the sialic acid is located on glycophorin, the band 3 : glycophorin molar ratio is, respectively with and without lectin chromatography, 25 : 1 and 11 : 1. However, since the precise sugar content of band 3 is not known, the contribution of band 3 to the amount of sialic acid cannot be fully evaluated. For this reason, the molar ratios have to be considered as the minimum band 3 : glycophorin ratio. After the ultracentrifugation step, the vesicle pellet was resuspended in a 10 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes buffer (pH 7.0), and this vesicle suspension was allowed to equilibrate for 24 h at 4°C. Without this equilibration procedure, the trap at isotopic equilibrium of the vesicles appeared to be much more variable (see next section).

Reconstitution of glycophorin-lipid vesicles

Glycophorin-containing vesicles were made according to the procedure of MacDonald and MacDonald [13]. Briefly, 1.3 mg of glycophorin was dissolved in 40 μl of an 1 mM Tris-HCl (pH 7.4) buffer. To this solution were added 3 ml methanol and 6 ml chloroform. After brief sonication at room temperature in a bath sonicator in order to assure complete dissolution of glycophorin, this solution was added to a film (dried under vacuum from chloroform) of 10 μmol lipids consisting of 94 : 6 (molar ratio) egg phosphatidylcholinebovine heart phosphatidylserine with a trace of [7(n)-^{3}H]cholesterol (phospholipid : cholesterol molar ratio, 10000 : 1). After dissolving the phospholipid film in the glycophorin-containing solution, the solvent was removed under vacuum using a rotary evaporator. The remaining mixed glycophorin-lipid film was resuspended in 1 ml of a 10 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0) buffer. The vesicles were centrifuged, first for 10 min at 10000 × *g* in order to remove large structures containing no or a small amount of protein; then the supernatant was centrifuged at 150000 × *g* for 20 min to collect the glycophorin-containing unilamellar vesicles, which have a 400 : 1 phospholipid : protein molar ration [25]. The vesicle pellet was, after suspension in the 10 mM sulfate buffer, treated the same way as outlined in the band 3 vesicle section.

Protein-free vesicles

Typically, these vesicles were prepared by mixing 6 ml 0.5% Triton X-100/24 mM NaCl/0.2 mM NaN_3 buffer/0.2 mM EDTA/10 mM Na-Hepes (pH 8.0) and 3 ml 30 mM Na_2SO_4/0.2 mM NaN_3/0.2 mM EDTA/10 mM Na-Hepes (pH 7.0) with 20 μmol 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine with a trace of [7(n)-^{3}H]cholesterol (phospholipid : cholesterol molar ratio 10000 : 1), previously dried under vacuum from chloroform, followed by the removal of Triton X-100 and harvesting as described in the section on band 3-lipid vesicle reconstitution.

Flux measurements

The anion $^{35}SO_4^{2-}$-influx assay under equilibrium conditions was essentially the same as described previously by Wolosin [6] with a few modifications. Before the assay was started, samples were drawn in order to determine the $^3H/P_i$ ratio. Then, typically 450 μl (approx. 2.5–5 μmol phospholipid) of the vesicle suspension was mixed with 50 μl of the same buffer with or without DIDS in the desired concentration. This mixture was allowed to equilibrate at the desired temperature for about 2 min. The influx assay was started by adding 500 μl assay medium buffer with $^{35}SO_4^{2-}$ (approx. $60 \cdot 10^6$ dpm/ml) at the same temperature. At appropriate time intervals, 50-μl samples were drawn and immediately applied to an ice-cold Sephadex G-75 column (1.5 ml wet bed volume), preswollen in the assay buffer. The Sephadex G-75 column were previously equilibrated with sonicated (94 : 6, molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine vesicles (150 μg phospholipid per column, eluted with 3 ml assay buffer) in order to avoid nonspecific adsorption of the vesicles by the column material.

After the vesicles were applied to the column the vesicles were immediately eluted with 800 μl of the ice-cold assay buffer (this usually took 1–5 min). In this way, the recovery of the vesicles was about 75%. Elution of $^{35}SO_4^{2-}$, in the absence of vesicles, was negligible. The eluate (850 μl) was collected directly in minivials (5 ml) and mixed with 2.35 ml Insta-Gel Packard scintillation liquid. The recovery of the vesicles was determined from the tritium radioactivity in the eluate. Radioactivity was counted in a Packard PRIAS Model PLD liquid scintillation counter. Initially, the $^{35}SO_4^{2-}$ influx under steady-state conditions will obey first-order kinetics and therefore a semilogarithmic plot of $1 - (dpm_t/dmp_\infty)$ versus time will yield a straight line [46]. dpm_∞, which represents the amount of enclosed $^{35}SO_4^{2-}$ at isotopic equilibrium, was obtained after the assay by a subsequent incubation of the remainder of the vesicle suspension for 18 h at 37°C [5]. From the slope of this curve, the half-time ($t_{1/2}$) of the $^{35}SO_4^{2-}$ influx can be obtained. The trap of the vesicles is derived from dpm_∞, the amount of $^{35}SO_4^{2-}$ and the amount of phosphorus per volume unit of the vesicle suspension and is called trap at isotopic equilibrium. In those cases where the influx curve showed the expected first-order kinetics, statistical analyses were applied by means of the least-squares method. When influx curves showed more phases, the error in the half-time was estimated from the last straight part of the flux curve crossing the $0.500(dpm_t/dpm_\infty)$ line. In the case of a $^{35}SO_4^{2-}$ influx curve with L-[1(n)-^{3}H]glucose, the influx assay procedure was similar, except that no [7(n)-^{3}H]cholesterol was incorporated into the vesicles. The recovery of vesicles from each column in those experiments was determined by a phosphorus assay on 200 μl of the eluent. The radioactivity was determined in 400 μl of the eluent by mixing it with 4.5 ml Insta-Gel and further as described for $^{35}SO_4^{2-}$ influx assay.

Gradient centrifugation

Gradient centrifugations were performed on discontinuous stractan gradients, made in 50 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). Stractan solutions from 9.5 to 7% with steps of 0.5% stractan and 0.5 ml volume were layered on top of each other. Samples of the vesicle suspension (0.5–4 ml) reconstituted in the same sulfate buffer were immediately drawn after the removal of the SM2 beads and layered on top of the gradients. After centrifugation (swing-out rotor L41, 35 000 rpm, 16 h at 4°C in a Beckman L65 ultracentrifuge) the vesicle bands which appeared in the gradient were collected. For electron microscopic purposes, the vesicles were concentrated by means of ultracentrifugation. Due to the high-density of the stractan suspension and the small size of the vesicles, the centrifugation (rotor 40, 40 000 rpm, 90 min at 4°C with a Beckman ultracentrifuge L65) had to be performed with a buffer of lesser density consisting of 75 mM LiCl/10 mM Tris-HCl (pH 7.0).

Analytical methods

Protein was measured by the method of Ross and Schatz [26] or the method of Peterson [27]. The protein content of vesicles from stractan gradient was measured by using the method of Peterson. Samples of the gradient were diluted to 1% stractan in order to avoid interference during the precipitation step of protein.

Phospholipids were determined as phosphorus

according to Rouser et al. [28] or Fiske and SubbaRow [29]. Sialic acid was measured according to Warren [30]. Freeze-fracture electron microscopy was performed as outlined previously [31]. 25% (v/v) glycerol was added to the sample to prevent freeze damage.

Results

Anion influx assay

The transport properties of the band 3-containing lipid vesicles were first tested using an SO_4^{2-}-influx assay. Due to its relatively slow permeability through the erythrocyte membrane, this anion is particularly attractive for kinetic studies using lipid vesicles [6]. In this influx assay after certain time periods, a separation between enclosed and free $^{35}SO_4^{2-}$ is accomplished using small ice-cold Sephadex column [6]. The vesicles were eluted with such a volume (800 μl) of ice-cold buffer to assure maximal (approx. 75%) vesicle yield with no significant contamination of free-labeled sulphate.

In our hands, it was found that the recovery of the vesicles was somewhat variable due to the difficulties in preparing the Sephadex columns and elution of the vesicles in a reproducible way. Therefore it was found to be essential to determine the vesicle recovery using an internal standard.

In Fig. 1 it is demonstrated that the construction of an influx curve without the use of the internal standard is hardly possible. Moreover, this figure illustrates the large scattering of vesicle recoveries which shows up when the vesicles are eluted with a low amount of buffer (680 μl). By correcting for the vesicle recovery and increasing the elution volume from 680 [6] to 800 μl this large scattering could be eliminated.

The half-time of the SO_4^{2-}-influx is about 4 min as related to the level reached at about 15 min. This value of the half-time of the influx curve is roughly in agreement with half-times of SO_4^{2-}-influx curves under similar conditions for band 3-lipid vesicles [6]. However, since the isotopic equilibrium level as determined after the subsequent incubation of the remainder of the vesicles with $^{35}SO_4^{2-}$ during 18 h at 37°C is much higher than the level reached at about 15 min, the half-time of 4 min is debatable (see further).

In order to compare the transport properties of different vesicles, it is essential that the $^{35}SO_4^{2-}$ influx is reproducible for each type of vesicle. Therefore, the influx assay itself needs to be accurate. This is the case, since influx assays performed on two parts of the same vesicle suspension result in the same influx curve (error about 10%; not shown). However, influx assays performed on vesicles prepared in exactly the same way, but reconstituted separately, show a large spread of

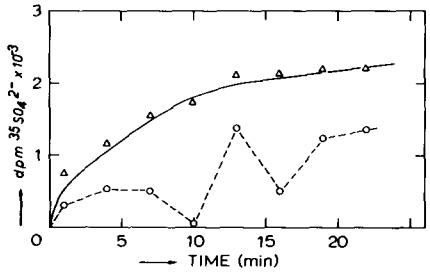

Fig. 1. $^{35}SO_4^{2-}$ influx into band 3-containing vesicles at 31°C. The vesicles were prepared in 10 mM Na_2SO_4 buffer as outlined in the Materials and Methods section. Assay was performed by eluting 50 μl vesicle suspension in $^{35}SO_4^{2-}$ medium with 680 μl ice-cold buffer over ice-cold Sephadex columns as outlined in the Materials and Methods section. dpm ^{35}S activity in the eluate of each Sephadex column is plotted versus time. O— — —O, Influx curve without correction for vesicle recovery; Δ———Δ, influx curve after correction for vesicle recovery using [7(n)-^{3}H]cholesterol as an internal standard.

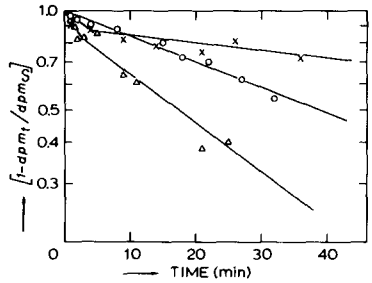

Fig. 2. Spread of $^{35}SO_4^{2-}$ influx into band 3-lipid vesicles, reconstituted in exactly the same way, but from different ghosts as starting material. Vesicles were made in an initial 2500:1 (molar ratio) phospholipid-protein in 10 mM Na_2SO_4 buffer as outlined in the Materials and Methods section. Influx rates at 12°C were related to traps at isotopic equilibrium. Δ———Δ, Related to a trap of 4.1 μl/μmol phospholipid; O———O, related to a trap of 1.7 μl/μmol phospholipid; ×———×, related to a trap of 2.4 μl/μmol phospholipid.

half-times, even if they are prepared from the same ghost preparation. This is demonstrated in Fig. 2, in which band 3-lipid vesicles prepared in 10 mM Na_2SO_4 buffer from different ghosts have half-times at 12°C of, respectively, 19 ± 1 min, 39 ± 2 min and 135 ± 35 min, all related to traps at isotopic equilibrium. However, it should be realised that, in principal, the influx rate should be related to the actual equilibrium position for the system in its actual state during the flux measurement instead of relating to the trap at isotopic equilibrium, obtained after the flux measurement. The equilibrium position of the anion, for the system in its actual state during the flux measurement is, however, impossible to determine. This difficulty inherent in this influx technique may contribute to an uncertainty in the flux rates.

The usefulness of this SO_4^{2-}-influx assay is further confirmed by its capacity of showing an inhibitory effect of DIDS and a competitive anion on the SO_4^{2-}-influx when samples of the same preparation of band 3-lipid vesicles are compared. In Fig. 3A, this inhibitory effect of 100 μM DIDS on band 3-lipid vesicles, when added to the outside, is demonstrated. Lower concentrations of DIDS (10 μM and 20 μM) did not cause a reproducible inhibition. The half-times were, respectively, 39 ± 2 min without inhibition and 105 ± 10 min with 100 μM DIDS, as related to their traps at isotopic equilibrium. Fig. 3B compares measurements on two different reconstitutions of band 3 from the same erythrocytes. The erythrocyte suspension was divided into two parts, one of which was treated with DIDS [15]. From both batches, band 3 was extracted and reconstituted into vesicles in the same way. The half-times of the SO_4^{2-} influx into the resulting vesicles were, respectively, 194 ± 33 min (from DIDS-treated erythrocytes) and 39 ± 2 min (from normal, untreated erythrocytes). A possible asymmetric incorporation of band 3 in the lipid bilayer, with respect to DIDS binding sites, can be derived from the percentage of inhibition caused by exofacially added DIDS

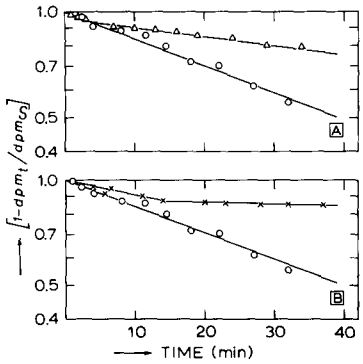

Fig. 3. Effect of DIDS, present on the outside (A) or on both sides (B) of the vesicles, on the SO_4^{2-} flux through lipid vesicles reconstituted with band 3. (A) Vesicles were made in 10 mM Na_2SO_4 buffer and assay with and without 100 μM DIDS was performed at 12°C as outlined in the Materials and Methods section. Influx rates were related to traps at isotopic equilibrium, which were, respectively, without inhibitor 1.7 μl/μmol phospholipid and with 100 μM DIDS 1.2 μl/μmol phospholipid. (B) Vesicles were made from normal or DIDS-pretreated erythrocytes. Erythrocytes were pretreated with 20 μM DIDS at 4°C during 20 min at pH 7.0, according to Ref. 15. The complete inhibition of SO_4^{2-} tracer flux was verified as described in Materials and Methods before proceeding to the preparation of ghosts. Vesicles were made in 10 mM Na_2SO_4 buffer and assayed at 12°C as outlined in the Materials and Methods section. Influx rates were related to the traps of isotopic equilibrium of, respectively, 1.7 μl/μmol phospholipid (vesicles derived from normal cells) and 2.9 μl/μmol phospholipid (vesicles derived from DIDS-treated cells). O——O, without inhibitor; Δ——Δ, 100 μM DIDS outside; ×——×, DIDS on both sides.

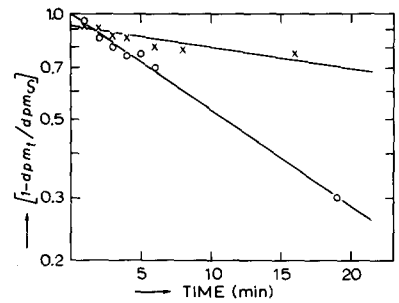

Fig. 4. Effect of thiocyanate on the SO_4^{2-} flux at 12°C into band 3-lipid vesicles. Vesicles were made in 10 mM Na_2SO_4/80 mM mannitol/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). 250 μl of the vesicle suspension were mixed with 250 μl of this buffer, while another 250 μl of the vesicle suspension were mixed with 250 μl of 10 mM Na_2SO_4/40 mM mannitol/20 mM KCNS/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). Both mixtures were allowed to equilibrate at 4°C for 24 h before the assay was started by adding $^{35}SO_4^{2-}$. Influx rates were related to traps at isotopic equilibrium and were, respectively, 0.33 μl/μmol phospholipid (without competitive anion) and 0.40 μl/μmol phospholipid (with competitive anion). O——O, Influx curve without 10 mM thiocyanate; ×——×, influx curve with 10 mM thiocyanate.

on the SO_4^{2-} influx into vesicles prepared from normal erythrocytes and the SO_4^{2-} influx into vesicles prepared from DIDS-treated erythrocytes [6]. However, since both vesicle preparations are independently reconstituted, a comparison of the permeability properties is very difficult in view of the variability of SO_4^{2-} fluxes (compare Fig. 2). Therefore, no conclusion can be drawn from these facts about a possible asymmetrical incorporation of band 3 in the lipid vesicles.

The inhibitory effect of a competitive anion thiocyanate, which inhibits the native band 3 transport system [42] on the SO_4^{2-} flux through band 3-lipid vesicles is demonstrated in Fig. 4. The SO_4^{2-} influx half-times were, respectively, 10 ± 0.4 min without and 50 ± 13 min with 10 mM thiocyanate as competitive anion.

The reason for the variability of the SO_4^{2-} flux in different reconstitutions of the same type can be: firstly, not all band 3 molecules which are incorporated in the bilayer have the same conformation, which is hard to detect by currently available analytical techniques; secondly, a possible heterogeneity of the vesicle population with respect to, for instance, vesicle size and protein content (see further); thirdly, uncertainties in the assay itself. A reason for such uncertainty could be the variation in trap at isotopic equilibrium, which in different reconstitutions of the same type ranged from 0.5 to 4.0 μl/μmol. This spread of traps (Fig. 2) could possibly be caused by the high temperature of 37°C at which the trap is determined. Incubation at 4°C for 24 h or 48 h, however, did not decrease the variability of traps. Furthermore, the trap at isotopic equilibrium of one vesicle population appeared to be dependent on the temperature at which the assay was performed. In a typical experiment when a vesicle preparation immediately after the centrifugation (see section on Gradient centrifugation) was assayed at 3, 15 and 30°C, the trap at isotopic equilibrium was found to be 1.0, 2.5 and 2.0 μl/μmol, respectively. By incubation of the vesicles for 1 h at 37°C or 24 h at 4°C prior to the particular assay temperature, the spread of traps at isotopic equilibrium of one vesicle population, assayed at different temperatures, could be reduced from 45% ($n = 10$) to 11% ($n = 18$).

For this reason, incubation at 4°C during 24 h prior to influx assay was always used. This preincubation could not, however, decrease the spread of traps of independently reconstituted vesicles. Therefore, in the following experiments, we related the influx to an arbitrary trap of 1.5 μl/μmol phospholipid, which is the average of numerous determinations of traps at isotopic equilibrium. The use of a fixed trap for different vesicle preparations is based on the assumptions that this trap is related to one type of vesicle and that the vesicles remain unaltered during the first part of the flux assay. However, the use of an arbitrary trap instead of the trap at isotopic equilibrium did not diminish the variability in influx rates as depicted in Fig. 2.

Specificity of enhanced sulphate permeability of band 3-lipid vesicles

In order to test whether the observed SO_4^{2-} permeability is specific, we also investigated the permeability properties of the band 3 lipid vesicles towards L-glucose, which has a very low permeability in the erythrocyte [32]. Therefore, the influx assay was performed with $^{35}SO_4^{2-}$ together with L-[1(n)-^{3}H]glucose and in order to test the role of the protein in the transport process, similar permeability experiments were carried out with protein-free vesicles.

As expected, the band 3-lipid vesicles showed, compared to protein-free vesicles, an enhanced SO_4^{2-} permeability at 3, 15 and 30°C, which could be inhibited at 15°C by 100 μM DIDS (Fig. 5A, C). Half-times of SO_4^{2-} fluxes into the band 3-lipid vesicles at 3 and 15°C, 15°C + 100 μM DIDS and 30°C were, respectively, 110 ± 20 min, 32 ± 2 min, 60 ± 5 min and 8 ± 2 min. For protein-free vesicles, half-times of SO_4^{2-} fluxes ar 3° and 15°C, 15°C + 100 μM DIDS and 30°C were, respectively, 370 ± 18 min, 2517 ± 157 min, 2517 ± 157 and 798 ± 42 min, which indicates that DIDS had no influence on the SO_4^{2-} flux into protein-free vesicles. All influx rates were related to a trap of 1.5 μl/μmol phospholipid.

At 3 and 15°C, the band 3-lipid vesicles were also much more leaky for L-glucose as compared to protein-free vesicles. The L-glucose flux at 15°C appeared to be inhibited by 100 μM DIDS. The comparison of the L-glucose influx curve of the band 3-lipid vesicles with the protein-free vesicles

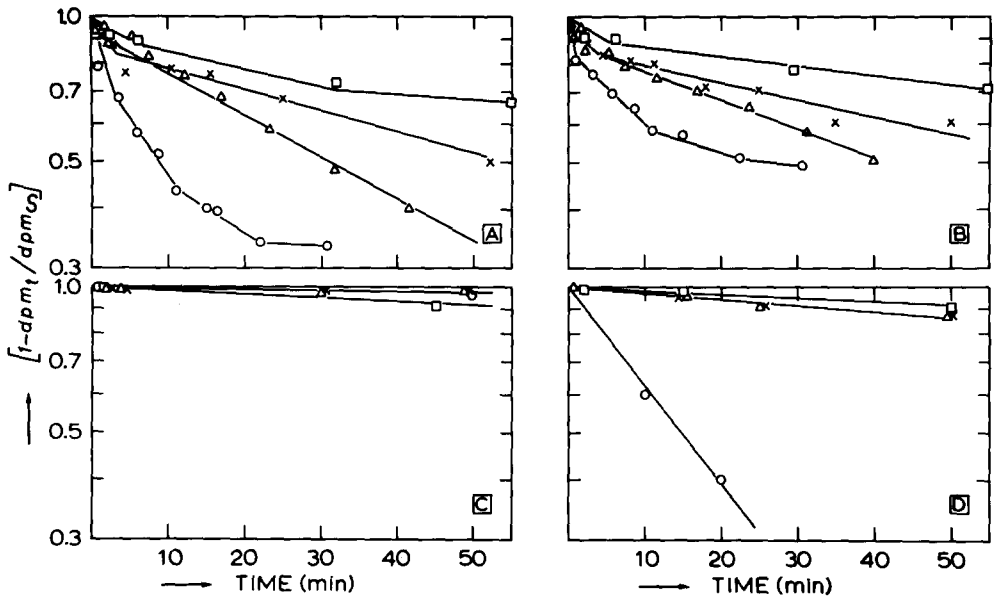

Fig. 5. Specificity of enhanced SO_4^{2-} permeability of vesicles reconstituted with band 3, compared with protein-free vesicles. (A) and (B) represent, respectively, SO_4^{2-} and L-glucose permeability of vesicles reconstituted with band 3. (C) and (D) represent, respectively, SO_4^{2-} and L-glucose permeability of protein-free vesicles. □———□, SO_4^{2-} or L-glucose flux at 3°C; △———△, SO_4^{2-} or L-glucose flux at 15°C; ×——— ×, SO_4^{2-} or L-glucose flux at 15°C with 100 μM DIDS; ○——— ○, SO_4^{2-} or L-glucose flux at 30°C. Band 3-lipid vesicles and protein-free vesicles were made in 10 mM Na_2SO_4/1 mM L-glucose/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0) as outlined in the Materials and Methods section. Final concentrations in the assay medium were 1.25–2.5 μmol phospholipid/ml, $60 \cdot 10^6$ dpm $^{35}SO_4^{2-}$/ml and $300 \cdot 10^6$ dpm L-[1(n)-^{3}H]glucose/ml. All influx rates were related to an arbitrary trap of 1.5 μl/μmol phospholipid. Traps at isotopic equilibrium of band 3-lipid vesicles were, respectively, at 3 and 15°C, 15°C with 100 μM DIDS and 30°C, 2.9, 2.8, 0.02 and 2.2 μl/μmol phospholipid as based on $^{35}SO_4^{2-}$ and 3.2, 2.4, 0.11 and 2.3 μl/μmol phospholipid as based on L-[1(n)-^{3}H]glucose. Traps at isotopic equilibrium of protein-free vesicles were, respectively, at 3 and 15°C, 15°C with 100 μM DIDS and 30°C, 3.5, 2.6, 0.5 and 3.5 μl/μmol phospholipid as based on $^{35}SO_4^{2-}$ and 3.5, 3.1, 4.3 and 3.6 μl/μmol phospholipid as based on L-[1(n)-^{3}H]glucose. The permeability properties of the protein-free vesicles and protein-lipid vesicles at 3°C were followed for 6 h. The permeability properties of band 3-lipid vesicles at 15°C with and without DIDS were followed for 150 min. To allow a good comparison, those influx curves are only partially shown. Half-times of influx curves were derived from the complete influx curves.

at 30°C is complicated due to the complexity of the influx curve of the band 3-lipid vesicles. Moreover, it should be realized that the glucose influx into the protein-free vesicles is related to the same arbitrary trap as used for the band 3-containing vesicles. As the isotopic equilibrium traps indicate that the protein-free vesicles are somewhat larger (compare legend Fig. 5), this will result in an decrease of the rate of the SO_4^{2-} influx. The half-times of L-glucose fluxes into band 3-lipid vesicles at 3 and 15°C, 15°C + 100 μM DIDS and 30°C were, respectively, 110 ± 10 min, 42 ± 2 min; 63 ± 5 min and 28 ± 4 min.

For protein-free vesicles the half-times of L-glucose fluxes at 3 and 15°C, 15°C + 100 μM DIDS and of 30°C were, respectively, 376 ± 12, 281 ± 13, 281 ± 13 and 16 + 6 min, which indicates that DIDS had no influence on L-glucose flux into protein-free vesicles either. The SO_4^{2-} as well as L-glucose permeability of protein-free vesicles shows a peculiar temperature dependency in that the SO_4^{2-} permeability at 3°C is higher than at 15 and 30°C and the L-glucose permeability at 30°C is unexpectedly high as compared with L-glucose permeability at 3 and 15°C. In the past, similar effects were shown for phosphate as well as glucose permeability of liposomes. Moreover, these effects appeared to be strongly lipid-dependent [33,34].

The enhancement of SO_4^{2-} flux through band

3-lipid vesicles as compared to protein-free vesicles can be evaluated when the L-glucose permeability is used as an internal standard for the passive permeability of the band 3-lipid vesicles and protein-free vesicles. At 30°C, protein-free vesicles are 0.02-times as leaky for SO_4^{2-} as for L-glucose, while band 3-lipid vesicles are 3.5-times as leaky for SO_4^{2-} as for L-glucose. From these data, it can be derived that, due to the incorporation of band 3, the SO_4^{2-} permeability at 30°C is 175-times higher than the L-glucose permeability. In the same way, it can be derived that at 15°C this preference for SO_4^{2-} is 12-times greater and at 3°C it is negligible. From Fig. 5 it is also obvious that the influx curves of the band 3 vesicles consist of more phases. Therefore, the assignment of a particular rate constant to each influx curve is very difficult. For this reason, the activation energy of the SO_4^{2-} as well as of L-glucose cannot be determined. But since the sulfate permeability increases more with increasing temperature than the L-glucose permeability at the corresponding temperatures, it can be stated that the activation energy of the sulfate permeation is higher than the activation energy of L-glucose permeation. Protein-free vesicles show influx curves which consist of one straight line.

Heterogeneity of band 3-lipid vesicles

Vesicle heterogeneity is one possible candidate to explain the variability of the absolute SO_4^{2-} permeability of various band 3-lipid vesicle preparations, as presented in Fig. 2, and the fact that SO_4^{2-} influx curves of band 3-lipid vesicles consist, in general, of more phases. This heterogeneity can be a heterogeneity in vesicle size, protein : phospholipid ratio, residual Triton X-100 content or the way band 3 is incorporated in the bilayer.

In order to obtain more insight into the first three possibilities, we investigated the vesicle population by means of stractan density gradient centrifugation. The resulting vesicle bands as traced by [^{14}C]phosphatidylcholine, which was incorporated in the vesicles, were analyzed by freeze-fracture electron microscopy, protein and phospholipid determination and anion influx assays. The results are depicted in Figs. 6, 7 and 8, and Table I.

After the gradient centrifugation procedure, several diffuse vesicle bands, sometimes aligned with clots, were seen. As Fig. 7 and Table I show, three bands usually appeared but sometimes an extra vesicle population appeared at 9.5% stractan. Freeze-fracture electron microscopy showed (Fig. 6) that at 7.5% stractan large unilamellar vesicles of about 2000–4000 Å were present with only a few protein particles, at 8.0% stractan smaller unilamellar vesicles of about 500–1200 Å with considerably more protein particles per vesicle and at 9.0% stractan, very small unilamellar vesicles of about 300–700 Å occupied with protein particles. The ratio of yields of vesicle bands differed for independent reconstitutions (Table I).

The Triton X-100 content, measured by performing a reconstitution with ^{3}H-labeled Triton X-100 appeared to be heterogeneous, too. The vesicles located in 9.0% stractan contained 1.8-times more

TABLE I

HETEROGENEITY OF BAND 3 VESICLES AS REVEALED BY STRACTAN GRADIENT CENTRIFUGATION

Vesicles and gradients were performed in 50 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0) as outlined in the Materials and Methods section. The spread of the amount of phospholipid present in a certain stractan density region represents the variability of the amount of phospholipids in that particular stractan density region of independently reconstituted vesicles. The phospholipid : protein molar ratio is derived from the phospholipid : protein (w/w) ratio using a molecular weight of 95000 for band 3 and 775 for phospholipids.

Percentage stractan	Density of stractan at 4°C in g/ml	Phospholipid : protein molar ratio	Percentage phos- phospholipid present
7.5	1.0320	4364 : 1	15–20
8.0	1.0325	4091 : 1	45–60
9.0	1.0335	2273 : 1	10–35
9.5	1.0340	-	0–10

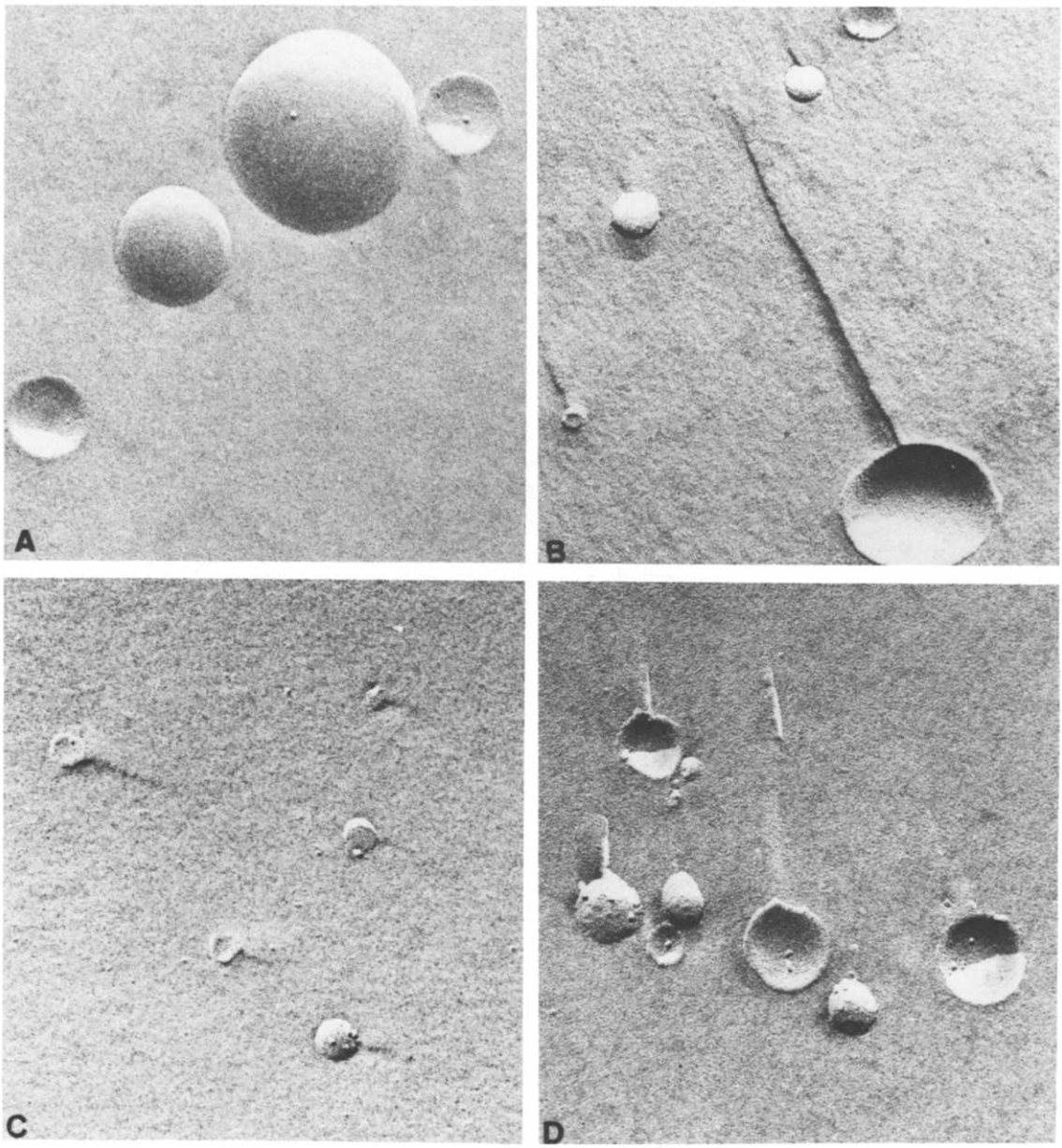

Fig. 6. Freeze-fracture electron microscopic pictures of band 3-lipid vesicles after separation by stractan density gradient centrifugation. (A) Vesicles located in 7.5% stractan; (B) vesicles located in 8.0% stractan; (C) vesicles located in 9.0% stractan; (D) vesicles derived from sonicated mixed micelles, located in 8.0% stractan. Magnification, × 100000. The pictures are typical examples of the vesicle populations as based on several pictures with numerous vesicles.

protein per phospholipid but at the same time the Triton content was 1.4-times higher as compared with vesicles located in the 7.5–8.0% stractan region. This can be explained by the high affinity of band 3 for Triton X-100 [35]. The average residual amount of Triton X-100 after the bead treatment of Gerritsen et al. [24] or Köhne et al. [8], respectively, 0.3 g SM2 beads/ml for 18 h and three times 0.1 g SM2 beads/ml for three subsequent periods of 8 h, was about 2–3 mol% relative to the lipid. The heterogeneity of the vesicle preparation was similar for both methods. Vesicles made from

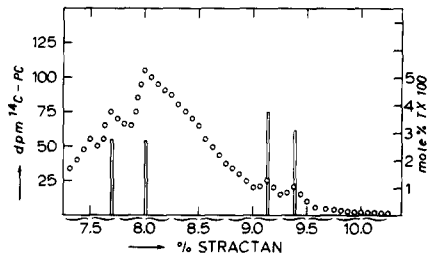

Fig. 7. Distribution of band 3-lipid vesicles over a stractan gradient after 18 h 35000 rpm centrifugation. Vesicles and gradients were made in 50 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0) as outlined in the Materials and Methods section. Vesicles were labeled with [^{14}C]phosphatidylcholine by adding [^{14}C]phosphatidylcholine (PC) to the phospholipid film prior to resuspension in the Triton X-100 extract. Vertical bars represent Triton X-100 (TX) content of the corresponding vesicle fraction as measured with [^{3}H]Triton X-100. Triton X-100 content is expressed as mole percentage relative to phospholipid.

the purified Triton X-100 extract by means of wheat-germ lectin chromatography (see Methods) showed also similar heterogeneity and transport behavior.

The permeability properties of the various vesicle bands was studied by means of the $^{35}SO_4^{2-}$-influx assay on tritium-labeled vesicles (Fig. 8) after the separate vesicle band suspensions had been freed from stractan by means of ultracentrifugation. Related to traps at isotopic equilibrium the half-times of SO_4^{2-} influx were, respectively, for vesicles in 7.5, 8.0 and 9.0% stractan 41 ± 3 min, 178 ± 37 min and 75 ± 8 min (Fig. 8).

When the SO_4^{2-}-influx rates are related to traps which are roughly in agreement with freeze-fracture electron microscopy pictures of the vesicle bands (Fig. 6A, vesicles at 7.5% stractan, diameter 2000–4000 Å, average trap 5 μl/μmol phospholipid; Fig. 6B, vesicles at 8.0% stractan, diameter 500–1200 Å, average trap 2 μl/μmol phospholipid; Fig. 6C, vesicles at 9.0% stractan, diameter 300–700 Å, average trap 0.2 μl/μmol phospholipid), the half-times of SO_4^{2-} influx will become for vesicles located in 7.5% stractan about 225 min, 8.0% stractan about 56 min and in 9.0% stractan about 37 min. This possibly indicates that the vesicles become more leaky for SO_4^{2-} at increasing incorporation of band 3. Vesicles located in 9.5% stractan did not show any influx or trap at isotopic equilibrium of $^{35}SO_4^{2-}$. The influx curves of the separate vesicle bands show one phase. This is in agreement with the increased homogeneity of the vesicle population.

The heterogeneity revealed by the stractan gradient centrifugation seems to originate from a

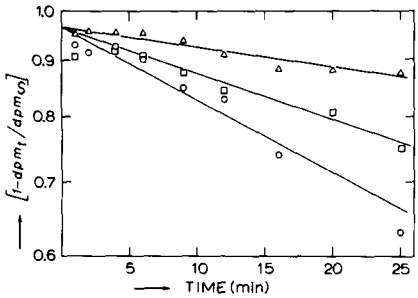

Fig. 8. SO_4^{2-}-influx rates of band 3-lipid fractions from stractan gradient at 30°C. Vesicles were made in 50 mM Na_2SO_4 buffer as outlined in the Materials and Methods section. After the stractan gradient procedure, vesicles were fractionated and freed from stractan by means of ultracentrifugation for 3 h. Subsequently, vesicles were resuspended and assayed in 50 mM Na_2SO_4/0.2 mM NaN_3/0.2 mM EDTA/10 mM Na-Hepes (pH 7.0). Influx rates were related to traps at isotopic equilibrium and were, respectively, for vesicle fractions at 7.5% stractan, 1.05 μl/μmol phospholipid; 8.0% stractan, 2.84 μl/μmol phospholipid ad 9.0% stractan, 0.41 μl/μmol phospholipid. ○———○, Vesicle fraction located in 7.5% stractan; △———△, vesicle fraction located in 8.0% stractan; □———□, vesicle fraction located in 9.0% stractan.

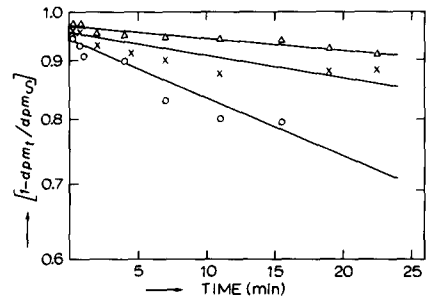

Fig. 9. Effect of DIDS on the SO_4^{2-} permeability of vesicles reconstituted with glycophorin at 12°C. Vesicles of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine with [7(n)-^{3}H]cholesterol (phospholipid/cholesterol molar ratio, 10000 : 1) were reconstituted in a 400 : 1 phospholipid : protein molar ratio, with glycophorin according to the procedure of MacDonald and MacDonald [13] as outlined in the Materials and Methods section. Influx rates at 12°C were related to traps at isotopic equilibrium, which was for all systems 1.5 μl/μmol phospholipid. The assays were performed in 10 mM Na_2SO_4/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). ○———○, Without DIDS; ×———× with 10 μM DIDS; △———△, with 100 μM DIDS.

heterogeneity which already exists in the protein-phospholipid Triton X-100 mixed micelles, as sonication of these micelles during 30 min at 0°C in a bath sonicator, before the reconstitution, resulted in a more homogeneous vesicle population. After stractan gradient centrifugation, only two vesicle bands were detectable at 7.5 and 8.0% stractan. Freeze-fracture electron microscopic studies showed unilamellar vesicles of about 750–2000 Å for both vesicle bands (Fig. 6D). The protein particle density was comparable with vesicles located at 8.0% stractan of the heterogeneous vesicle population, prepared without sonication. The SO_4^{2-} and L-glucose permeability properties of the vesicle preparation derived from sonicated mixed micelles were similar with respect to the preferential uptake of SO_4^{2-} over L-glucose. Gel electrophoresis performed on these band 3-lipid vesicles, according to Fairbanks [3] showed that band 3 was still intact.

SO_4^{2-} and L-glucose permeability of glycophorin-containing vesicles

For the evaluation of the permeability properties of band 3-lipid vesicles it is important to know whether other membrane proteins, which lack transport activity when incorporated in an artificial bilayer, can induce an enhanced permeability for SO_4^{2-}. For this purpose we investigated the permeability properties of glycophorin-containing vesicles.

When glycophorin, by means of the method of MacDonald and MacDonald [13], is reconstituted with 94:6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine in a 400:1 phospholipid:protein molar ratio, the resulting vesicles show an enhanced SO_4^{2-} permeability. At 12°C, the half-time of the SO_4^{2-} flux was 78 ± 13.5 min (protein-free vesicles under the same conditions: 2517 min) (Fig. 9).

Surprisingly, this increased sulfate permeability can be inhibited by 100 μM DIDS, and even 10 μM DIDS caused a significant inhibitory effect. The influx curves with inhibition showed, respectively, half-times of 178 ± 61 min (10 μM DIDS) and 263 ± 66 min (100 μM DIDS) (Fig. 9). Another anion, thiocyanate, showed also a 'competitive' inhibiting action on the enhanced sulfate permeability (Fig. 10). The sulfate permeability without competitive anion had a half-time of 26.1 ± 1 min and with competitive anion of 89 ± 15 min. Both influx curves were related to isotopic equilibrium traps of 1.05 μl/μmol phospholipid.

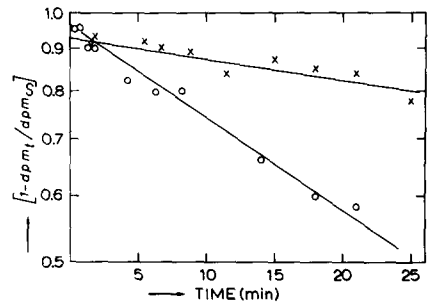

Fig. 10. Effect of 10 mM thiocyanate on the SO_4^{2-} permeability of vesicles reconstituted with glycophorin at 12°C. Vesicles were reconstituted with glycophorin in 10 mM Na_2SO_4/80 mM mannitol/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). 250 μl of the vesicle suspension was mixed with this buffer, while another 250 μl of the vesicle suspension was mixed with 10 mM Na_2SO_4/40 mM mannitol/20 mM KCNS/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0). Influx rates were related to traps at isotopic equilibrium, which were for both system 1.05 μl/μmol. O——O, Influx curve without 10 mM thiocyanate; ×——×, influx curve with 10 mM thiocyanate.

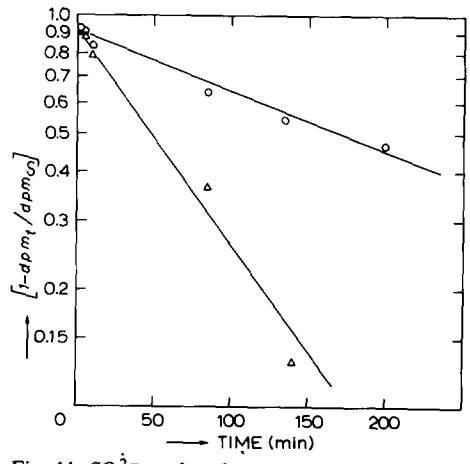

Fig. 11. SO_4^{2-} and L-glucose permeability at 30°C of vesicles reconstituted with glycophorin. Vesicles of 94:6 (molar ratio) egg phosphatidylcholine/bovine heart phosphatidylserine were reconstituted in a 400:1 phospholipid:protein molar ratio in 10 mM Na_2SO_4/1 mM L-glucose/0.2 mM EDTA/0.2 mM NaN_3/10 mM Na-Hepes (pH 7.0), with glycophorin as described in the Materials and Methods section. Influx rates of both solutes were related to their isotopic equilibrium traps of 1.5 μl/μmol phospholipid. O——O, SO_4^{2-} influx; Δ——Δ, L-glucose influx.

The glycophorin-containing vesicles appeared at 30°C to have a higher permeability for L-glucose than for SO_4^{2-} (Fig. 11). Half-times, were respectively, for L-glucose 50 ± 3 min and for SO_4^{2-} 184 ± 14 min, all related to a trap of 1.5 μl/μmol. In contrast, in band 3-lipid vesicles at 30°C there is a preference for SO_4^{2-} over L-glucose (compare Fig. 5A, B).

At isotopic equilibrium, the various glycophorin-lipid vesicle preparations we studied appeared to have a trap of 1–1.5 μl/μmol phospholipid (for L-glucose similar traps have been observed). When the glycophorin-containing vesicles were prepared in a sulfate buffer with $^{35}SO_4^{2-}$ and the vesicles were subsequently freed from non-trapped SO_4^{2-} by means of Sephadex G-75 filtration at 0°C the trapped volume appeared to be 1.5 μl/μmol phospholipid. It is known from freeze-fracture electron microscopy [24] and trap measurements by enclosing ^{3}H-labeled dextran [12] that these glycophorin-lipid vesicles have a trap of about 4–10 μl/μmol phospholipid. Together with the values obtained with $^{35}SO_4^{2-}$ it means that only about 15–35% of the vesicles are sealed to SO_4^{2-}. The influx curves we studied are related to this fraction only. The rest of the glycophorin-lipid vesicle population has an extremely high sulfate and glucose permeability.

Discussion

Recently there have been several reports on the reconstitution of band 3 in lipid vesicles via Triton X-100 SM2 bead methods [5–8]. These vesicles showed an enhanced SO_4^{2-} permeability which could be (partially) inhibited by DIDS, suggesting proper reconstitution of the erythrocyte anion-transport system.

In the present study, these observations could be confirmed. In addition, several interesting new features of these reconstituted vesicles were observed, which we will discuss in turn. First of all, it was noticed, in agreement with Wolosin [6], that the magnitude of the enhanced SO_4^{2-} permeability of independently, identically reconstituted vesicles was highly variable (see, for instance, (Fig. 2). The two possible sources of this variability are the method used to determine the anion transport and the properties of the vesicles themselves. In this study, the anion transport rate is related to the trap of the vesicles at isotopic equilibrium, which can be determined after prolonged incubation of the vesicles at elevated temperatures. Therefore, a potential candidate for the variability of the anion transport rates could be the variation in equilibrium isotope distribution values. Although some scattering in equilibrium isotope distribution values were observed, this could not explain the total variability. Interestingly, the equilibrium isotope distribution was found to be temperature-dependent, as has been described also by Ross and McConnell [35]. This temperature-dependency, which must reflect some unknown transport property of the vesicles, could be greatly reduced by preincubation of the vesicles for 24 h at 4°C or 1 h at 37°C. Similar 'vesicle stabilizations' were used by Köhne et al. [8] and Wolosin [6]. The reported zero activation energy for SO_4^{2-} transport through 'non-stabilized' band 3-containing lipid vesicles [36] could very well be the result of these temperature-dependent equilibrium isotope distributions.

It is more likely that the variations in transport properties are the result of uncontrolled variations in the properties of the band 3 vesicles. These could, for instance, result from variations in the way the protein is incorporated into the bilayer or in vesicle heterogeneity. That these lipid vesicles are indeed very heterogeneous in size and protein content had already been indicated by Gerritsen et al. [11,23] and could be clearly established in this study, using stractan density gradient centrifugation. The various vesicle bands obtained from such gradients not only varied in intensity between different experiments, but showed also a large variation in protein content, vesicle size and SO_4^{2-} permeability. For this reason, although in the literature influx rates have been related to the first apparent equilibrium level of the transported anion [6], transport parameters which are related to the total vesicle population can be obtained only when the anion influx or efflux at a particular time is related to the equilibrium distribution of the anion of the total vesicle population. The vesicle heterogeneity is most likely the source of the complex kinetic behavior of the unfractioned vesicles (see, for instance, Fig. 5A, B). The difficulties in exactly reproducing the way the Triton is removed by the SM2 beads from the protein-phospholipid detergent micelles, or variations in the micelles'

composition could very well be the source of the variations in transport properties of the band 3 vesicles. In future experiments, the vesicle homogeneity has to be improved. One potentially useful method is to sonicate the band 3-lipid mixed micelles prior to removal of the detergent. This, indeed, resulted in a more homogeneously sized vesicle preparation (Fig. 6D). Alternatively, differential centrifugation, to remove the large vesicles [47] or sonication of the final reconstituted vesicles could be attempted. However, both methods result in very small lipid vesicles. The use of such vesicles for permeability studies is, however, questionable [37]. Another, more attractive method could be the use of octyl glucoside instead of Triton X-100. Preliminary reconstitution experiments with this detergent greatly improved vesicle homogeneity.

A crucial criterion for the proper reconstitution of a transport protein is the specificity of the induced transport. The specificity is two-fold: specificity for the protein and specificity for the transport solute.

Inhibitors have been commonly used to assay the specificity of the transport properties of band 3 containing lipid vesicles. As reported by Wolosin [6] and confirmed in this study, the addition of 100 μM DIDS reproducibly inhibits the transport of SO_4^{2-} through the vesicles. However, several critical remarks have to be made about the interpretation of such experiments. Firstly, the concentration of DIDS and DNDS, a reversible acting analog of DIDS [4] inhibiting the anion transport in lipid vesicles, is an order of magnitude higher than the concentration needed to inhibit the anion transport in the erythrocyte membrane [5,6]. Secondly, it is well-known that DIDS can inhibit the transport properties of a large variety of different model and biological membranes. For instance, DIDS at micromolar levels inhibits the $(Na^+ + K^+)$-ATPase of microsomal preparations of both turtle bladder and electric organ of eel [38], the Ca^{2+} transport into liposomes reconstituted with the purified sarcoplasmic reticulum Ca^{2+}-ATPase [39] the ATPase activity of the CA^{2+}-ATPase from the human erythrocyte when reconstituted in lipid vesicles [40] and even the glycophorin-induced SO_4^{2-} flux through lipid vesicles (this study). Therefore, it is highly questionable whether the observed inhibition of the SO_4^{2-} transport by DIDS in band 3-lipid vesicles can be taken as evidence for the proper reconstitution of the band 3 protein.

Other ways to look at the specificity of the reconstituted band 3 system are to use competitive anions [6] and to compare the SO_4^{2-} permeability with that of a molecule which normally is not transported across the erythrocyte membrane. Although the thiocyanate anion inhibited the SO_4^{2-} permeability in the band 3 vesicles, suggesting specificity, similar effects were noticed for glycophorin-containing vesicles (Fig. 10). Since thiocyanate is a rather lipid-soluble anion [43–45], this anion may increase the negative (surface) charge of the lipid bilayer, which could result in a decrease of anion permeability. Therefore, this inhibiting effect of thiocyanate is less suprising than the inhibiting effect of DIDS on the SO_4^{2-} flux into glycophorin-lipid vesicles. That band 3 incorporation increased the permeability of the vesicles also in an nonspecific way was indicated by experiments using L-glucose. This molecule, which cannot readily move across the erythrocyte membrane or protein-free vesicle bilayers, can rapidly pass the band 3-containing lipid bilayers. However, at 15 and 30°C, the bilayer was more permeable towards, SO_4^{2-} as compared to L-glucose. The inhibitory effect of DIDS on the L-glucose permeability further confirms the lack of specificity of this inhibitor as discussed above. These data, together with the lack of self-saturation of the SO_4^{-2} influx in band 3-lipid vesicles [5,8], strongly suggests that the way band 3 is incorporated in the vesicles is different from that in the erythrocyte membrane.

Concerning the nature of the (non)specific increase in permeability of the band 3-lipid vesicles, the studies of Bjerrum and co-workers [40] on the transport properties of erythrocytes at pH 5 are worth mentioning. These authors showed that the aggregation of the intermembranous particles at this low pH is accompanied by the formation of pores for molecules with a size smaller or equal to that of sucrose [41]. Also, the fluxes of molecules through these pores could be inhibited by DIDS. This could indicate that the increase in permeability (pore formation) is related to protein aggregation which could nonspecifically be affected by DIDS.

Previous studies have shown that incorporation of the other major intrinsic protein from the erythrocyte membrane, glycophorin, increases the bilayer permeability towards K^+ and glucose [12]. In this study we have shown that this protein, which has no known transport function, also increases the permeability towards SO_4^{2-} and L-glucose of 94 : 6 (molar ratio) egg phosphatidylcholine-bovine heart phosphatidylserine vesicles. This permeability increase has been attributed to packing defects at the protein/lipid interface or protein aggregation resulting in pore formation [12]. An interesting difference from the band 3-containing vesicles is that the glucose permeability is higher than the SO_4^{2-} permeability, suggesting some anion specificity of the increased bilayer permeability of the band 3-lipid vesicles. Finally, as the increase in SO_4^{2-} flux into the band 3-lipid vesicles appears to result from a combination of specific and nonspecific leakage events an understanding of a possible lipid dependency of the anion transport system and, in general, every other transport system, can be obtained only after the lipid dependency of the protein-induced aspecific leakage is understood.

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