J. Membrane Biol. 90, 123–135 (1986)

The Journal of
Membrane Biology

A New Method for the Reconstitution of the Anion Transport System of the Human Erythrocyte Membrane

Uwe Scheuring, Klaus Kollewe, Winfried Haase, and Dieter Schubert
Max-Planck-Institut für Biophysik, D-6000 Frankfurt am Main 71, West Germany

Summary. The anion transport protein of the human erythrocyte membrane, band 3, was solubilized and purified in solutions of the non-ionic detergent Triton X-100. It was incorporated into spherical lipid bilayers by the following procedure: (1) Dry phosphatidylcholine was suspended in the protein solution. Octylglucopyranoside was added until the milky suspension became clear. (2) The sample was dialyzed overnight against detergent-free buffer. (3) Residual Triton X-100 was removed from the opalescent vesicle suspension by sucrose density gradient centrifugation and subsequent dialysis. Sulfate efflux from the vesicles was studied, under exchange conditions, using a filtration method. Three vesicle subpopulations could be distinguished by analyzing the time course of the efflux. One was nearly impermeable to sulfate, and efflux from another was due to leaks. The largest subpopulation, however, showed transport characteristics very similar to those of the anion transport system of the intact erythrocyte membrane: transport numbers (at 30°C) close to 20 sulfate molecules per band 3 and min, an activation energy of approx. 140 kJ/mol, a pH maximum at pH 6.2, saturation of the sulfate flux at sulfate concentrations around 100 mM, inhibition of the flux by H_2DIDS and flufenamate (approx. K_I-values at 30°C: 0.1 and 0.7 μM, respectively), and "right-side-out" orientation of the transport protein (as judged from the inhibition of sulfate efflux by up to 98% by externally added H_2DIDS). Thus, the system represents, for the first time, a reconstitution of all the major properties of the sulfate transport across the erythrocyte membrane.

Key Words anion transport system · human erythrocyte membrane · band 3 protein · reconstitution · right-side-out vesicles

Introduction

The human erythrocyte membrane allows a rapid exchange of anions between the cell interior and the external medium. The exchange is mediated by band 3, the membrane's most abundant integral protein. This protein has been thoroughly studied, both in the membrane and in solubilized form (for recent reviews, *see* [4, 15, 17, 23, 25].

Studies performed on solubilized band 3 protein include numerous attempts to reconstitute the anion transport system into lipid vesicles. The resulting proteoliposomes displayed various properties of the anion transport system of the native human erythrocyte membrane [5, 6, 9, 10, 18–21, 26–28, 37, 38]. In the most successful attempts, those properties include, besides high rates of anion transport and sensitivity towards typical inhibitors of anion transport across erythrocyte membranes, an activation energy of about 138 kJ/mol (33 kcal/mol) [18–20], a decrease of sulfate flux with increasing pH for pH > 6.2 [5, 6, 18–20, 38], and saturation of sulfate flux at high substrate concentration [38]. In none of these studies, however, has the joint reconstitution of all the properties mentioned been demonstrated. Furthermore, in all previously reported cases the sensitivity of anion transport towards the classical inhibitors of anion transport, the stilbene disulfonate H_2DIDS[1] or related compounds, was distinctly lower in the reconstituted system than in the native membrane. This was observed with respect to both the maximum inhibition and the inhibitor concentration required for half-maximum inhibition. In addition, none of the reconstituted systems showed the steep decrease in sulfate flux with decreasing pH at pH < 6.2, which is typical for human erythrocyte membranes [30, 31]. Thus, commendable progress towards a reconstitution of the anion transport system has been achieved, but a fully satisfactory reconstitution has not yet been reported.

We have developed a new method for the incorporation of band 3 protein into liposomal membranes. In this method, solubilization and purification of the protein was carried out in solutions of the nonionic detergent Triton X-100, as in most of the studies described above. However, recombination of the protein with added lipid was performed in a

[1] *Abbreviations:* H_2DIDS, 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate; SDS, sodium dodecyl sulfate.

mixture of Triton X-100 and octylglucopyranoside, a detergent with high critical micelle concentration which can easily be removed from the sample by dialysis. The Triton concentration chosen was less than necessary for complete solubilization of the lipid. Assembly of band 3 protein in the bilayer of the lipid vesicles by dialysis and subsequent removal of the bulk of Triton X-100 by density gradient centrifugation leads to the reconstitution of an anion transport system which much more closely resembles that of the human erythrocyte membrane than the reconstituted systems described previously. In addition, it leads to a virtually completely unidirectional incorporation of the transport protein into the vesicle membranes, resulting in proteoliposomes with "right-side-out" orientation of band 3. A brief description of our reconstitution method has already been published [29].

In addition to the description of the new reconstitution method and the properties of the reconstituted system, the present paper will deal with the analysis of efflux measurements made on functionally nonhomogeneous vesicle populations.

Materials and Methods

Materials

Phosphatidylcholine from egg yolk was purchased from Lipid Products (South Nutfield, Surrey, U.K.) ("grade I," purity >99%). Triton X-100 ("peroxide-free"), octylglucopyranoside, HEPES and Tris were from Boehringer (Mannheim, F.R.G.). Dialysis bags (Visking 20/32) were obtained from Serva (Heidelberg, F.R.G.), and Biogel P6-DG was from Bio-Rad (München, F.R.G.). H_2DIDS and DNDS were a kind gift from Prof. H. Passow, and flufenamic acid was from EGA Chemie (Steinheim, F.R.G.). [^{3}H]Triton X-100 and [^{3}H]iodoacetic acid were purchased from New England Nuclear (Dreieich, F.R.G.) and $Na_2{}^{35}SO_4$ from Amersham-Buchler (Braunschweig, F.R.G.). Liquid scintillation cocktail ("Quickszint 2000") was obtained from Zinsser Analytic (Frankfurt, F.R.G.). For the sources of all other materials used *see* [24].

Reconstitution Procedure

Band 3 was purified by pre-extraction of human erythrocyte membranes in 1% Brij 58, solubilization of the protein in 0.5% Triton X-100 and subsequent ion exchange chromatography. It was eluted from the column in 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.4% (wt/wt) Triton X-100 [32, 39]. The purity of band 3 isolated in this way is >95% [24, 32, 39]. Protein concentration was determined according to [32]. Following chromatography, 3–4 ml of the eluate containing 1.3 mg band 3 (or, occasionally 0.2–1.0 mg) were made up to 10 ml by addition of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and the concentration of Triton X-100 in the sample was adjusted to 0.2%. The protein is predominantly monomeric under those conditions [32]. The protein solution was then added to 20 mg of phosphatidylcholine which had been dried from a solution in chloroform in a rotary evaporator, and the sample was shaken for 10 min in the presence of a few glass beads. To the milky suspension, a 10% (wt/wt) solution of octylglucopyranoside (in water) was added until the sample became clear (final concentration of octylglucopyranoside: approx. 0.7%). The sample was then dialyzed overnight against 3 liters of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.5 mM EDTA, 15 mM β-mercaphoethanol. During dialysis it became opalescent, which is indicative of the formation of (protein-) lipid vesicles. However, the mixture still contained approx. 0.13% Triton X-100.

The sample was now brought to a sucrose concentration of approximately 17% (wt/wt) and placed into a cellulose nitrate (or ultra-clear) tube of the Spinco SW 28 rotor. It was overlayed with 3.5 ml of 12.5% sucrose in buffer A (10 mM HEPES (titrated to pH 7.2 by addition of NaOH), 10 mM Na_2SO_4, 0.5 mM EDTA) containing 15 mM β-mercaptoethanol and then with 24 ml of a linear gradient of 0.05–0.10 g sucrose per ml made up in the same solvent. The tubes were then centrifuged for 6 hr at 28,000 rpm in a Spinco L 5-75 ultracentrifuge. After centrifugation the broad opalescent band of V = 8–10 ml, located near the middle of the gradient, was removed and dialyzed overnight against 1 liter of buffer A containing 15 mM β-mercaptoethanol. After centrifugation for 10 min at 15,000 rpm, which removed minute quantities of precipitated material, the vesicles were pelleted by centrifugation in a Spinco 70 Ti rotor (90 min at 50,000 rpm). The pellet was suspended in 3–6 ml of the same buffer and dissolved by gently shaking for 10 min. The sample was then dialyzed against 100 volumes of the buffer used in the flux measurements (plus 15 mM mercaptoethanol). Dialysis was performed either overnight, or was extended for up to 64 hr if the buffer composition differed greatly from that of buffer A. Sample temperature during the whole preparation procedure was between 0° and 5°C.

In some experiments, ^{3}H-labeled band 3 was used to follow protein losses during the reconstitution procedure and to determine protein concentration in the final sample (e.g., for the determination of transport numbers). The protein was derived from erythrocyte ghosts which had been incubated, in 10 mM Tris-HCl (pH 7.5) at 22°C, for 30 min with 25 nmol of [^{3}H]iodoacetic acid (specific activity 233 mCi/mmol) and then for another 20 min with an additional 200 nmol of unlabeled iodoacetic acid per ml of ghosts. To determine the specific activity of the ^{3}H-labeled band 3, a small amount of the protein was purified in solutions of nonaethylene glycol lauryl ether [24] and protein concentration quantified by absorbance measurements [11]. In other experiments, [^{3}H]Triton X-100 was added to the Triton solutions used, in order to determine the content of residual detergent in the vesicle preparations.

Flux Measurements

The transport properties of the proteoliposomes were studied by measuring sulfate efflux from $^{35}SO_4^{2-}$-preloaded vesicles under equilibrium exchange conditions, following (with some modifications) the filtration method of Köhne et al. [18–20]. To 1 ml of the proteoliposome sample described, 50 μl of 1 mM Na_2SO_4 containing 50 μCi [^{35}S] sulfate were added, and the vesicles were loaded with the radioisotope by incubation (overnight at 15°C or, occasionally, for 3 hr at 30°C). The sample was then applied to a column of Biogel P6-DG (17 × 1.5 cm) and eluted, at 5°C, with the buffer used during final dialysis (but without β-mercaptoethanol) or other desired buffers. Fractions of 1 ml were col-

lected. Usually, the proteoliposome peak consisted of three fractions and was fully separated from the peak of extravesicular radioactivity. The three fractions were pooled and diluted with 12 ml buffer of the same composition but such a temperature that, after mixing, the desired sample temperature was obtained. The efflux experiment was started immediately by pipetting 7.5 ml samples into thermostatted tubes, one of which usually contained 75 μl 1 mM H_2DIDS. At various time intervals, 500 μl of the solutions were removed from each of the tubes and filtered through cellulose nitrate filters of diameter 2.5 cm and pore size 0.2 μm (SM 11307, Sartorius, Göttingen, F.R.G.). The filters were washed with 4 ml of ice-cold buffer, and the intravesicular radioactivity retained by them was determined by liquid scintillation counting (after dissolving the filters in the scintillation cocktail).

Evaluation of the Flux Measurements

General Considerations

Reconstitution procedures of the type described above are most likely to lead to heterogeneous populations of vesicles. Four main vesicle populations can be expected to occur (Fig. 1): (1) sealed vesicles containing the reconstituted anion transport system; (2) sealed vesicles which only show protein- or detergent-induced anion leak flux [22, 36]; (3) sealed vesicles containing neither of the transport pathways of populations 1 and 2; and (4) unsealed vesicles, i.e., vesicles which are so leaky that all the intravesicular radioactivity is lost before the beginning of the flux measurements. The vesicles of population 1 may or may not show leak flux and will thus consist of two subpopulations. Population 4 will not contribute to the flux measurements, and transport across the membranes of vesicle population 3 may be negligible as compared to populations 1 and 2.

If it is assumed that leak flux from one of the subpopulations of population 1 and from population 2 can be described by the same rate constant k_2 (which is supported by the experimental data; *see below*) and that efflux from vesicle population 3 can be neglected, the counting rate $n(t)$ of $^{35}SO_4^{2-}$ trapped in the vesicles of Fig. 1 will follow Eq. (1)

$$n(t) = n_{1a}e^{-k_1 t} + n_{1b}e^{-(k_1+k_2)t} + n_2 e^{-k_2 t} + n_3. \tag{1}$$

Meaningful nonlinear least squares fits to the experimental data according to Eq. (1), in which all six parameters were allowed to float, did not seem to be feasible. We have therefore tried to determine some of the parameters separately: (a) k_2 was determined from experiments in which the reconstituted anion transport pathway was blocked by the addition of a suitable inhibitor. This condition leads to Eq. (2)

$$n_I(t) = n_{1a} + (n_{1b} + n_2)e^{-k_2 t} + n_3 = n_{I2}e^{-k_2 t} + n_{I3} \tag{2}$$

where the subscript I indicates complete inhibition of the band 3-mediated anion transport. The condition could be realized by adding to the samples, at zero time of the flux measurements, H_2DIDS at a concentration of 10–20 μM (*see below*). (b) When k_2 is known, n_3 and n_{I3} can be taken from the experimental data, since $n(t)$ is virtually equal to n_3 (or n_{I3}) for $k_i t > 3$. We have therefore first calculated k_2 from Eq. (2), using an approximate value for n_{I3} in a first step of the calculation and a corrected value (t determined according to (b)) in the second step. This yields,

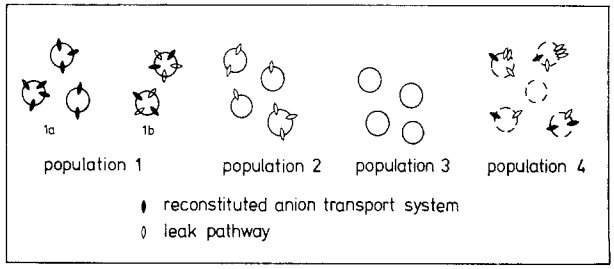

Fig. 1. The main vesicle subpopulations which may be expected to occur in reconstitution experiments

besides k_2 and n_{I3}, n_3 and $n_{1a} = n_{I3} - n_3$ and thus reduces the number of parameters in Eq. (1) to be fitted simultaneously to three. In addition, we have reduced the number of exponentials to be used in the fits by combining the first two terms of Eq. (1) into a single one

$$\bar{n}(t) = n_1 e^{-k_{1\text{eff}}t} + n_2 e^{-k_2 t} + n_3 \tag{3}$$

with $n_1 = n_{1a} + n_{1b}$ and

$$k_{1\text{eff}} = \frac{n_{1a}k_1 + n_{1b}(k_1 + k_2)}{n_1}. \tag{4}$$

$\bar{n}(t)$ has the same initial and final value and the same initial slope as $n(t)$ but differs from $n(t)$ at intermediate values of t.

During the first 1–2 hr of the flux measurements the experimental curves of the counting rate $n_e(t)$ of $^{35}SO_4^{2-}$ trapped in the liposomes could be fitted both by Eqs. (1) and (3), in both cases in combination with procedures (a) and (b). In virtually all experiments the parameters n_i calculated were positive and thus physically meaningful. However, for obvious reasons an unambiguous determination of k_1, $k_{1\text{eff}}$, n_{1b}, n_1 and n_2 was possible only if k_1 was much larger than k_2. In all other cases, the values determined were very sensitive to variations in the number of data points, the values of k_2 and n_{1a} inserted into Eqs. (1) or (3) and to the starting values used in the calculations. As a consequence, we have looked for parameters which would allow a reliable characterization of the anion transport properties of the vesicles over a wide range of experimental conditions. Finally, we have introduced "effective" rate constants defined, analoguously to Eq. (4), by

$$k_{\text{eff}} = \frac{n_{1a}k_1 + n_{1b}(k_1 + k_2) + n_2 k_2}{n_{1a} + n_{1b} + n_2} \tag{5}$$

(if Eq. (1) was applied), or (after application of Eq. (3)) by

$$k_{\text{eff}} = \frac{n_1 k_{1\text{eff}} + n_2 k_2}{n_1 + n_2}. \tag{6}$$

Again, a curve $(n_1 + n_2)\exp(-k_{\text{eff}}t) + n_3$ would have the same initial value and slope and the same final value as the original curves. Since definitions (5) and (6) both measure the initial slope of the corresponding curves, the two values of k_{eff} will be virtually identical.

We have further introduced

$$k_{1\text{eff}} = \frac{n_{I2}k_2}{n_{I2} + (n_{I3} - n_3)} = \frac{(n_{1b} + n_2)k_2}{n_1 + n_2} \tag{7}$$

as the "effective" rate constant in the presence of high concentrations of H_2DIDS (i.e., for $k_1 = 0$), and

$$\Delta k_{\text{eff}} = k_{\text{eff}} - k_{1\text{eff}} \tag{8}$$

as the final description of the anion transport system. As can be easily shown, k_1 and Δk_{eff} are related by

$$\Delta k_{\text{eff}} = \frac{n_1}{n_1 + n_2} k_1 = \frac{n_1}{n(0) - n_3} k_1 \tag{9}$$

which is virtually equal to $k_1(n_1/n(0))$ (*see below*). We have found that Δk_{eff} is much less sensitive to the conditions of the numerical analysis than k_1 or $k_{1\text{eff}}$.

Description of Anion Transport in the Presence of Inhibitors

In the following, we will analyze the experimental data by Eq. (3) without reference to the model of Fig. 1. We will, however, assume that the second term of Eq. (3) is due to leak flux and restrict our analysis to cases where $k_1 \gg k_2$ (in the absence of inhibitors), so that $k_{1\text{eff}}$, n_1 and n_2 can be determined reliably. We will further presume that the flux measurements in the absence and after addition (at $t = 0$) of the inhibitor used identical proteoliposome samples, so that $n(0) = n_I(0)$, etc.

In the presence of inhibitors, we will write instead of Eq. (3)

$$n'(t) = n_1' e^{-k_1' t} + n_2' e^{-k_2' t} + n_3'. \tag{10}$$

According to our model, we will set $k_2' = k_2$. In general, however, n_2' and n_3' will be larger than n_2 or n_3, respectively, especially at high inhibitor concentrations (*see above*). The usual definition of the degree of inhibition I of flux component 1, $I = 1 - k_1'/k_1$, will therefore not be applicable. A suitable, more general definition should follow and take into account the apparent loss of the more strongly inhibited subpopulations from population 1. It should, however, still consider only those vesicles which, in the control, show rapid anion transport. It should thus start, instead of from $n'(t)$, from an expression from which the known unspecific contributions to anion flux have been substracted:

$$g'(t) = n'(t) - n_2 e^{-k_2 t} - n_3 = n_1' e^{-k_1' t} + (n_2' - n_2) e^{-k_2 t} + (n_3' - n_3). \tag{11}$$

In analogy to definitions (4), (6) and (7) we now define

$$I = 1 - k^*/k_{1\text{eff}} \tag{12a}$$

with

$$k^* = \frac{n_1' k_1' + (n_2' - n_2)k_2}{n_1' + (n_2' - n_2) + (n_3' - n_3)}. \tag{12b}$$

k^* as calculated via Eqs. (10) and (12b) was found, similar to k_{eff}, to be virtually independent of the starting values and of the exact value of k_2 used in the calculations (in contrast to k_1').

By definition (12), leak flux from the vesicle population 1 will be treated as incomplete inhibition. This will lead to an underestimation of the degree of inhibition of transport system 1. On the other hand, $k_{1\text{eff}}$ represents a slight overestimate of the rate constant of this transport system (since it also includes leak flux from vesicle population 1), so that the two errors in part compensate each other.

Intravesicular Volume and Transport Numbers

The internal volume v_i of the vesicles was determined during the efflux experiments, by measuring the content of [^{35}S]sulfate in the vesicle samples after and before gel filtration on the Biogel P6-DG column. The radioactivity ratio was related to the phospholipid content of the samples. Efflux of sulfate from the vesicle populations 1–3 during gel filtration was <5% of the intravesicular sulfate and was neglected in the determination. Apparent average vesicle diameters were calculated from the v_i-values according to [13], assuming a membrane thickness of 4 nm.

Transport numbers τ (number of sulfate ions transported per band 3 molecule per minute) were determined from the equation [19, 27]

$$\tau = \frac{k_1 \cdot c \cdot v_i}{\alpha} \tag{13}$$

(c, sulfate concentration [M]; v_i, intravesicular volume in l per mol of phospholipid; α, molar ratio of band 3 to phospholipid in the vesicles). In applying Eq. (13) it is assumed that all vesicles have the same diameter. Furthermore, it is assumed that all liposomes take part equally in H_2DIDS-sensitive transport.

Electron Microscopy

Band 3 containing liposomes were prepared for electron microscopy either by negative staining, on Formvar coated grids, with 5% ammonium molybdate (pH 7.2), or by freeze-etching. In the latter case, the liposome suspension was frozen, without chemical fixation, by spraying into liquid propane [1]. Freeze-cleaving, etching (for 5 sec) and shadowing with platinum/carbon were performed in a Bioetch 2005 apparatus (Leybold-Heraeus, Cologne, F.R.G.), with the specimen stage at −135°C. The samples were examined in a Philips 300 electron microscope.

Other Methods

Phospholipid concentration was determined according to Bartlett [2]. Gel electrophoresis of samples solubilized in 3% SDS was performed essentially as described by Fairbanks et al. [12]. SDS concentration in the buffers was 0.1%. The osmolarity of buffer solutions was determined by means of a Knauer "Halbmikro"-osmometer (Knauer, W. Berlin, F.R.G.).

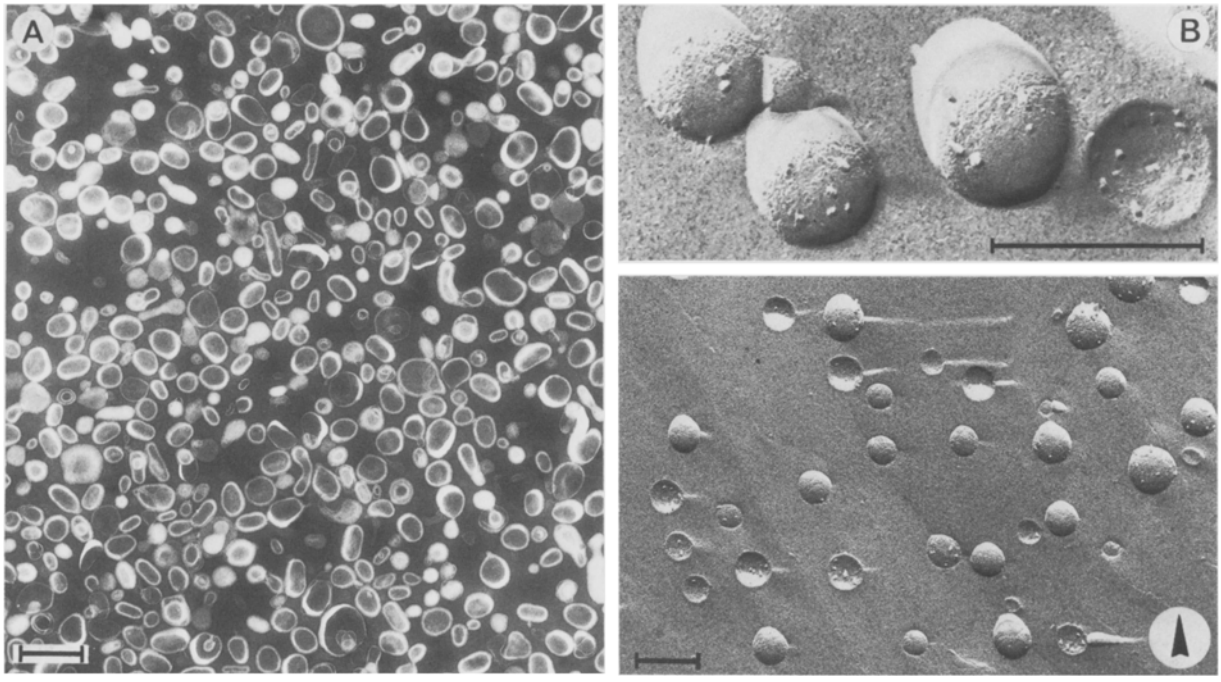

Fig. 2. Morphology of the band 3-containing lipid vesicles, as revealed by electron microscopy following sample preparation by negative staining (*A*) or freeze-fracturing (*B*). The bars indicate 200 nm, and the arrow in *B* indicates the direction of shadowing

Results

Formation, Isolation and Structural Properties of Band 3-Phospholipid Vesicles

In the reconstitution method applied by us, lipoprotein aggregates are formed from a band 3/phospholipid/Triton X-100/octylglucopyranoside mixture by removing the bulk of the latter detergent by dialysis. The concentration of Triton X-100 is reduced in this step by approx. one third. In order to further reduce the detergent content, the sample was applied to the bottom of a sucrose gradient. After centrifugation, 15–35% of the applied lipid, 40–70% of the protein and approx. 50% of the Triton remained at the bottom of the tube. Thus, this material did not take part in the formation of large lipid or lipoprotein particles. However, 60–80% of the lipid and 20–50% of the protein were found in an opalescent band near the center of the tube. The variability of the yield seems to depend on still unidentified differences in the protein batches used. The amount of Triton X-100 in the lipoprotein band was still approx. 25% of that applied to the tube. Further losses of lipid and protein occurred when the material was pelletted by high-speed centrifugation, whereas further loss of Triton occurred both during pelletting and dialysis. The final preparation contained 30–40% of the lipid and 10–30% of the protein initially present. No serious attempts were made to achieve higher yields, since both protein and lipid are easily available. The content of Triton X-100 was approx. 0.03–0.05 mg per mg of lipid (as compared to 1.0 mg in the starting mixture).

The morphology of the isolated lipoprotein material was studied by electron microscopy. Samples prepared by negative staining showed closed vesicles, apparently predominantly made up of a single lipid bilayer. Vesicle diameters were in the range of 23–140 nm, the bulk of them being around 70 nm (Fig. 2*A*). Electron micrographs obtained by the freeze-fracturing technique supported the predominantly unilamellar character of the vesicles. In addition, they showed that the inner faces of the two lipid monolayers (both the convex and the concave ones) were covered by the typical "membrane particles" which indicate membrane-spanning protein. Most of the particles did not form clusters but seemed to be randomly distributed (Fig. 2*B*).

SDS gels stained with Coomassie Blue on which either purified band 3, as used in the reconstitution mixture, or the isolated lipoprotein vesicles were run, showed no significant differences (Fig. 3).

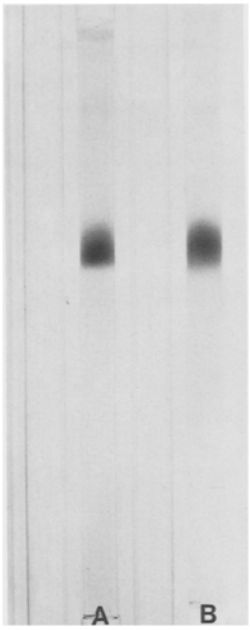

Fig. 3. SDS gel electrophoresis of isolated band 3 protein as used for the reconstitution experiments (*A*), and of the reconstituted band 3-lipid vesicles as present after pelleting (*B*). The gels were stained with Coomassie Blue

Thus, there is no enrichment of impurities of the band 3 preparations during reconstitution.

Sulfate Transport Across the Vesicle Membranes

General Findings

When protein-free liposomes, prepared in the same manner as the proteoliposomes described, were loaded with [^{35}S]sulfate and afterwards transferred into tracer-free buffers, they released their intravesicular radioactivity with a half time of many hours (Fig. 4). On the other hand, a much more rapid sulfate efflux could be demonstrated for band 3-containing vesicles. The time course of the efflux was reproducible for at least one week. The flux could be effectively inhibited by micromolar concentrations of H_2DIDS or flufenamate which, in this concentration range, are specific inhibitors of anion transport across the human erythrocyte membrane [4, 17, 25] (Fig. 4). This strongly suggests that the vesicle membranes contain a reconstituted anion transport system.

The experimental curves relating the counting rate $n(t)$ of [^{35}S]sulfate trapped in the liposomes to time were analyzed as described in detail above. The time range covered was 0 to approx. 60 min. The curves obtained in the presence of high concentrations of inhibitors of anion transport could be fitted by a single exponential plus a constant term n_{I3} according to Eq. (2). On the other hand, the data obtained in the absence of inhibitors did not, in most cases, follow a single exponential. They could, however, be fitted satisfactorily by Eqs. (1) and (3) which contain three and two exponentials, respectively, plus a constant term $n_3 \ll n_{I3}$. In general, the latter calculations used the figures for k_2 as determined by means of Eq. (2). When, however, k_2 was determined from Eq. (3), for curves which were characterized by $k_1 \gg k_2$ and which showed little experimental scatter the value obtained was virtually identical to that determined by means of Eq. (2). These findings strongly support the assumptions made above (a) that the flux component characterized by k_2 and n_2 represents leak flux and thus corresponds to vesicle population 2 of Fig. 1, and (b) that leak flux from vesicle population 1 can also be described by k_2. The leak character of the flux component characterized by k_2 is further supported by the findings that the dependence of k_2 on temperature differs strongly from that of k_1, and that neither n_{I2} nor k_2 (as determined in the presence of 10–20 μM H_2DIDS) could be further reduced by addition of 10 μM flufenamate (*see below*). These results also strongly suggest that the model underlying Fig. 1 and Eq. (1) represents an adequate description of the anion transport properties of the vesicle system.

In the case $k_1 \gg k_2$, the values of k_1, $k_{1\text{eff}}$, n_{1b}, $n_1 = n_{1a} + n_{1b}$ and n_2 calculated by means of Eqs. (1) or (3) were unique. In addition, the values for k_1 and $k_{1\text{eff}}$ differed only by a few percent. However, all these figures became unreliable if k_1 and k_2 were of the same order of magnitude. This problem was circumvented by introducing "effective" rate constants k_{eff} and $k_{I\text{eff}}$ and their difference Δk_{eff} as defined by Eqs. (5)–(8). Most Δk_{eff}-values were obtained applying Eq. (3). Δk_{eff} is proportional to k_1, as is shown by Eq. (9) and demonstrated below for the case $k_1 \gg k_2$, i.e. when k_1 can be determined reliably (Fig. 7). The proportionality factor in most cases was found to be around 0.8 and did not depend significantly on the conditions of the flux measurements (*see below*). Δk_{eff} is a reliable parameter even when k_1 cannot be determined reliably. A representative example of an analysis, which also indicates typical initial counting rates, is given in the Table.

The different n_i-values ($i = 1, 2, 3$), obtained in cases where $k_1 \gg k_2$, provide information on the relative sizes of the vesicle populations defined by Fig. 1 (Table). At 30°C, pH 7.2 and in the absence of

inhibitors of anion transport, the contribution of sulfate efflux from vesicle population 1 (represented by $n_1/n(o)$) accounted for approx. 80% of the sulfate transported. Two thirds of this contribution resulted from subpopulation 1a, which does not show anion leak flux. 10–25% was contributed by vesicle population 2 $(n_2/n(o))^2$. Vesicle population 3, which may consist of protein-free liposomes, contained around 3% of the radioactivity initially present in the vesicles. The relative size of the different populations seemed to depend mainly on the vesicle batch. It depended little on the parameters of the flux experiments, except that the size of vesicle subpopulation 1a decreased at $T > 35$°C and pH < 6.8, with a concomitant increase in the size of subpopulation 1b. As mentioned above, vesicle population 4 of Fig. 1, which consists of vesicles which are so leaky that all intravesicular radioactivity is already removed during gel filtration, will not show up in the flux measurements.

It is thus clear that, at physiological pH and temperature, sulfate efflux in the system we studied is dominated by contributions from a transport system which is inhibitable by H_2DIDS and flufenamate. For most purposes, this transport system is sufficiently well characterized by the parameter Δk_{eff}.

Evaluation of the effect of H_2DIDS

10–20 μM H_2DIDS inhibit the band 3-mediated anion transport across intact human erythrocyte membranes by 98–99%. The H_2DIDS binding site on band 3 is accessible only from the outside of the membrane; the reagent has no inhibitory effect when present at the cytoplasmic surface [4, 17, 25]. Therefore, the effect of externally added H_2DIDS on sulfate efflux from the lipoprotein vesicles allows the sidedness of the reconstituted band 3 molecules to be tested.

At pH 7.2 and temperatures between 25° and 40°C, addition of 10–20 μM H_2DIDS reproducibly led to an inhibition of the rapid component of sulfate efflux by >93% and in most cases by 97–98% (as calculated from Eq. (12)). Since the residual flux still includes the leak flux from vesicle population 1, the actual inhibition of the band 3-mediated sulfate

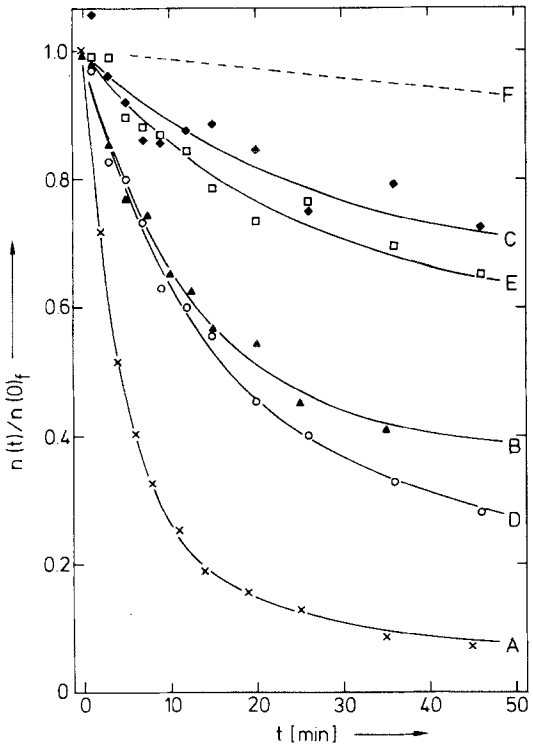

Fig. 4. Efflux of [^{35}S]sulfate from band 3-lipid vesicles under exchange conditions in the absence of inhibitors of anion transport (×), and in the presence of 0.1 μM H_2DIDS (▲), 15 μM H_2DIDS (◆), 1 μM flufenamate (○) or 10 μM flufenamate (□). The solid curves represent the least squares fit to the data according to Eqs. (3) (curves *A*, *B*, *D*) and (2) (curves *C*, *E*). The broken line (*F*) indicates sulfate efflux from vesicles which did not contain band 3 but otherwise were prepared in the same way as the proteoliposomes. All experimental and fitted $n(t)$-data were normalized to the value $n(0)_f$ of the corresponding fitted curve. Solvent: Buffer *A*. T = 30°C

flux will be even higher. This shows that, in virtually all band 3 molecules which take part in rapid sulfate transport across the vesicle membranes, the H_2DIDS binding sites on the protein are not only intact but also accessible to the inhibitor. Since H_2DIDS does not penetrate through the vesicle membranes (at least not at a rate comparable to that of sulfate) [10, 20], these results clearly demonstrate that virtually all functional band 3 molecules are incorporated into the vesicle membranes with their H_2DIDS binding site oriented to the outside (at least in vesicle population 1). Thus, the vesicles show a virtually complete right-side-out orientation of the transport protein. This conclusion is supported by the effects of flufenamate on sulfate efflux: flufenamate, which has a high membrane permeability [8], does not effect a higher inhibition than the nonpenetrating H_2DIDS. This holds both for the rapid and the slow component of the flux (Fig. 4 and

[2] The anion leak flux is apparently induced by protein and not by residual Triton X-100, since vesicles which had been dialyzed for short periods only and thus contained up to five times more residual detergent than others from the same batch which were dialyzed for longer periods, yielded the same n_2 and k_2 (as well as k_1, n_1 and n_3) as the latter ones. This finding agrees with similar results by Ueno et al. on lipid vesicles containing the detergent *n*-dodecyl octaethylene glycol monoether [35]

Table. Representative examples of the analysis of the efflux measurements

Curve/equation used	n_{1a}	n_{1b}	n_1	n_2 n_{I2}	n_3 n_{I3}	k_1	$k_{1\text{eff}}$	k_2	k_{eff} $k_{I\text{eff}}$	Δk_{eff}	k^*
A/1	6285	1010	—	2390	250	0.226	—	—	0.183	0.170	—
A/3	—	—	7290	2395	250	—	0.231	—[a]	0.183	0.170	—
C/2	—	—	—	3470	6460	—	—	0.037	0.013	—	0.005
E/2	—	—	—	4090	5840	—	—	0.042	0.018	0.005	0.010

The data analyzed are those in Fig. 4, curves *A*, *C* and *E*. The units of n_i are counts per 10 min, and those of k_i min^{-1}.

[a] k_2 fitted by Eq. (3) was 0.038 min^{-1}.

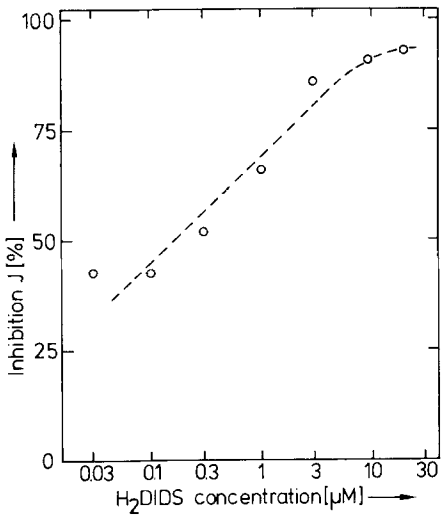

Fig. 5. Dose-response curve for the inhibition of sulfate efflux (under exchange conditions) from the band 3-containing lipid vesicles by H_2DIDS, under conditions of reversible binding of the inhibitor. Solvent: Buffer A titrated to pH 6.5. T = 30°C.

Table). Furthermore, the simultaneous addition, to the sample, of 10 μM flufenamate and 15 μM H_2DIDS does not reduce the efflux from the vesicles as compared to the effect of H_2DIDS alone (*data not shown*).

With intact erythrocyte membranes at pH 7.2, inhibition of anion transport by H_2DIDS can be due to covalent or noncovalent binding of the inhibitor to band 3. The latter reaction precedes the former and is characterized by a K_I-value of 0.1–0.3 μM [4, 17, 25]. We have found that, at pH 7.2, 50% inhibition of the rapid component of sulfate flux across the vesicle membranes is achieved at H_2DIDS concentrations around 0.1 μM (*see* Fig. 4), as with intact erythrocyte membranes. However, since some of the inhibition will probably be due to covalent H_2DIDS binding to band 3, the true K_I-value may be higher. Additional experiments were therefore performed at pH 6.5, where covalent H_2DIDS binding is negligible [16]. The data obtained are shown in Fig. 5. Again 50% inhibition of sulfate efflux was obtained at an H_2DIDS concentration of 0.1–0.2 μM. The reduction in the maximum inhibition by H_2DIDS, which is apparent from the figure, is due to an increase in leak flux from vesicle population 1 at low pH (*see above*). The increase in inhibition with increasing inhibitor concentration, c_I, at $c_I >$ 0.1 μM is, however, less than expected from a simple 1 : 1 interaction of the inhibitor with a uniform population of band 3 molecules, as found with erythrocyte membranes [3, 17] (a Hill plot of the data in Fig. 5 yields a slope of approx. 0.5 instead of 1.0). On the other hand, inhibition at 0.03 (and also at 0.01) μM H_2DIDS was consistently around 40% and thus distinctly higher than extrapolated from the curve of Fig. 5. We assume, as did Köhne et al. with respect to their analogous findings [20], that part of the band 3 molecules in the vesicle membranes may have undergone slight conformational changes. This in turn may have led to a small increase in the apparent K_I-values of these protein subpopulations.

We have also determined the concentration of DNDS and flufenamate needed for 50% inhibition of sulfate efflux from the vesicles and have found values of approx. 2.0 and 0.7 μM, respectively (pH 7.2, 30°C). In the case of DNDS, the value is four times higher than for erythrocyte membranes [3, 17]; with flufenamate it is about seven times lower (at the same temperature) [7, 8]. For both inhibitors, the respective values are reasonably close to each other, especially if the differences in the experimental conditions are taken into account.

The Influence of Other Parameters

Sulfate exchange across the erythrocyte membrane shows characteristic dependencies on temperature, pH and sulfate concentration [4, 17, 23, 25]. In order to compare the reconstituted with the native anion transport system, we have studied the depen-

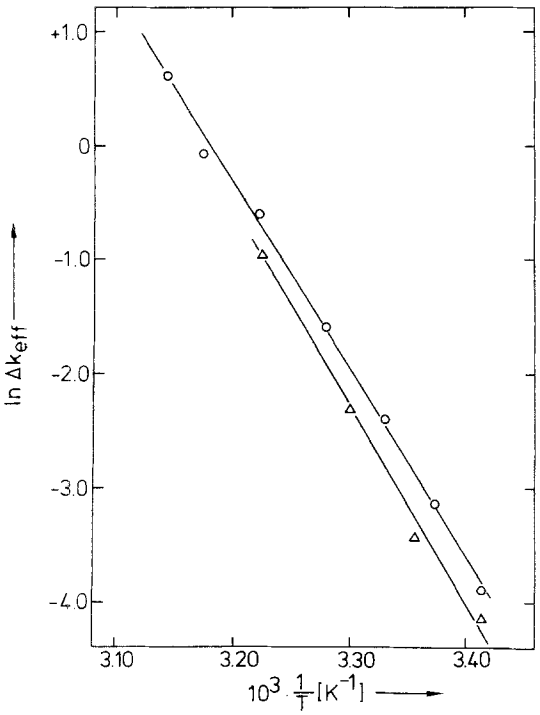

Fig. 6. Temperature dependence of band 3-mediated sulfate flux (described by Δk_{eff}) for two different vesicle preparations in buffer A, presented as an Arrhenius plot

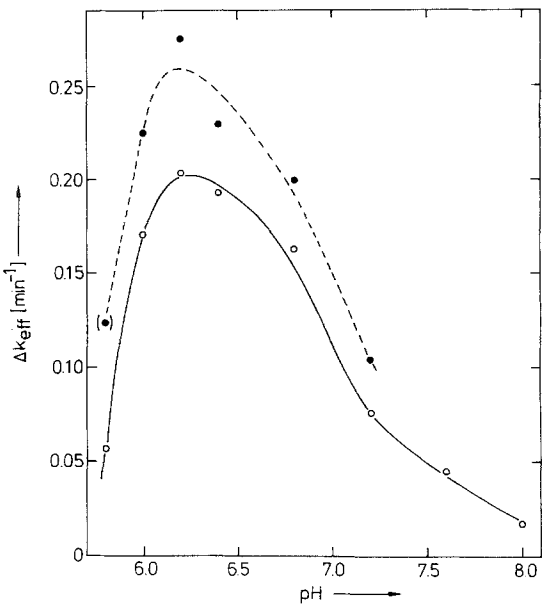

Fig. 7. pH dependence of the band 3-mediated sulfate efflux from the reconstituted vesicles, as described by k_1 (●) and by Δk_{eff} (○). Solvent: 10 mM HEPES, 50 mM Na_2SO_4, 0.5 mM EDTA, in each case titrated to the respective pH. T = 30°C

dency of sulfate equilibrium exchange on the parameters mentioned. We have also performed a few experiments on sulfate net efflux into a medium free of permeable anions.

Arrhenius plots of the dependency of Δk_{eff} on temperature, for data from two different vesicle preparations, are shown in Fig. 6. From the straight lines, values for the activation energy E_a of approx. 137 and 145 kJ/mol can be derived, which compare well with the 138 kJ/mol (33 kcal/mol) reported for intact erythrocyte membranes [17, 25, 31, 33]. It is noteworthy that, according to Fig. 6, the linear range of the Arrhenius plot extends up to a temperature of at least 45°C.

In contrast to Δk_{eff} and k_1, the rate constant k_2 for leak flux only slightly increases with increasing temperature (less than twofold between 10 and 37°C).

The dependency of Δk_{eff} and of k_1 (for cases $k_1 \gg k_2$) on pH is shown in Fig. 7. The data closely follow the pattern found by Schnell et al. in erythrocytes and erythrocyte ghosts [30, 31]: the curve is bell-shaped, with a maximum around pH 6.2, and the decrease towards lower pH is steeper than towards higher ones. The figure also demonstrates the parallelity in the behavior of k_1 and Δk_{eff}.

As shown in Fig. 8, sulfate flux ϕ (Δk_{eff} · sulfate

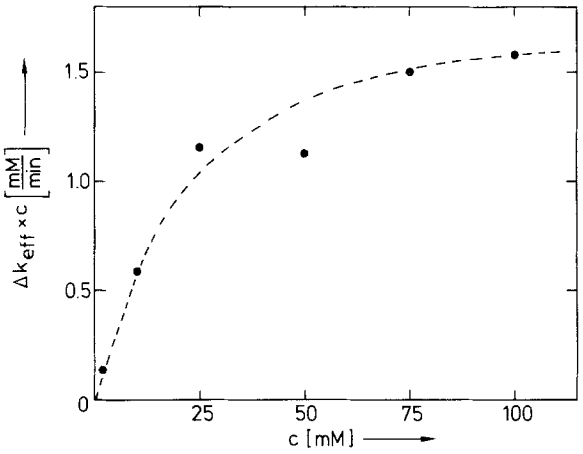

Fig. 8. Dependence of sulfate efflux $\phi = \Delta k_{eff} \cdot c$ on sulfate concentration c at pH 7.2. The buffers used contained 10 mM HEPES (pH 7.2), 0.5 mM EDTA plus the respective sulfate concentrations. T = 30°C. The broken line represents the dependence of sulfate self-exchange flux on the sulfate concentration in amphotericin B-treated red blood cells according to Schnell et al. [31] (arbitrarily normalized to the data point at c = 100 mM)

concentration) across the vesicle membranes shows saturation kinetics, similar to the findings of Schnell et al. for amphotericin B-treated erythrocytes and for erythrocyte ghosts [31]. We could not determine whether or not the reconstituted anion transport system shows the same decrease of ϕ at $c > 150$

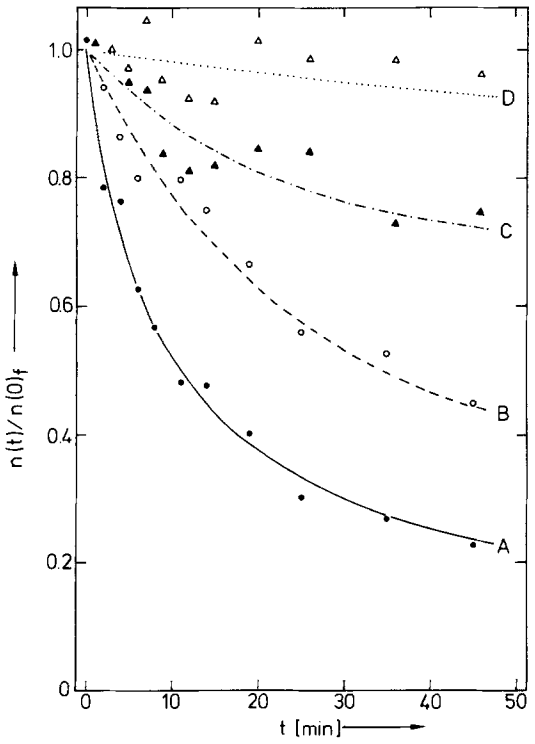

Fig. 9. Sulfate efflux from band 3-lipid vesicles into buffer free of permeable anions, as compared to efflux under exchange conditions. The vesicles were loaded with [^{35}S]sulfate in buffer B (20 mM HEPES (pH 7.2), 50 mM Na_2SO_4, 0.5 mM EDTA) plus 15 mM β-mercaptoethanol. Afterwards, the extravesicular medium was exchanged by gel filtration against buffer C (100 mM HEPES (pH 7.2), 0.5 mM EDTA; this buffer has the same osmolarity as buffer B). The efflux measurements were performed in either buffer B (●), buffer B plus 10 μM H_2DIDS (▲), buffer C (○) or buffer C plus 10 μM H_2DIDS (△). T = 30°C. The curves represent the least squares fit to the experimental data according to Eqs. (2) and (3). In the plot, all data are normalized to the value $n(o)_f$ of the corresponding fitted curve

mM as the native system. This was due to a strong decrease in k_{eff} and a strong increase in the scatter of the experimental data under these conditions, which made the Δk_{eff}-values unreliable.

After replacement of extravesicular sulfate by the nonpenetrating HEPES by gel filtration, sulfate efflux from the vesicles was distinctly decreased. In the experiment shown in Fig. 9, k_{eff} calculated from curve B amounts to only 30% of the corresponding value obtained under exchange conditions (curve A). Calculation of the degree of inhibition I according to Eq. (12) even yields $I > 90\%$ for the rapid flux component. The remaining sulfate efflux was inhibitable by H_2DIDS, the residual flux being smaller than under exchange conditions (Fig. 9, curve D). These results suggest that net movement of sulfate across the vesicle membrane is distinctly slower than sulfate self-exchange, as is known for erythrocyte membranes [4, 17, 25]. However, a quantitative comparison between the two systems does not seem to be useful, due to the problems involved in the quantification of net sulfate fluxes (*see* the discussions in [20, 25]).

Intravesicular Volume and Transport Numbers

The intravesicular volume v_i of the vesicles, as determined from the loss of [^{35}S]sulfate from the sample during the gel filtration step preceding the flux measurements, was 0.5–1.7 liter per mol of phospholipid (depending on the particular vesicle preparation). From these data, effective outer vesicle diameters of 22–55 nm can be calculated [13]. These values, except for the highest ones, are distinctly smaller than the average diameter derived from electron microscopic observations (*see above*). In addition, the large variability of v_i was not paralleled by a similar variability in the size distribution determined by electron microscopy. The relatively low values of v_i thus indicate that a variable fraction of the vesicles are so leaky that all the intravesicular radioactivity is lost during gel filtration (population 4 of Fig. 1). Similar discrepancies between the average vesicle diameters obtained from electron microscopy and from v_i-measurements, respectively, were also observed in other studies on band 3 reconstitution [20, 27, 36].

The transport number τ of band 3 incorporated into the vesicle membranes was determined from measurements of k_1, the protein content of the vesicles and the intravesicular volume v_i according to Eq. (13). At pH 7.2, 30°C and a sulfate concentration of 10 mM we obtained, from three different preparations with $\alpha = 3 \times 10^{-4}$, 7×10^{-5} and 2.5×10^{-5}, respectively, τ = 10, 15 and 22 sulfate ions per band 3 molecule and min. These values for τ are near to the value of 20 ions per band 3 molecule and min for band 3 protein in the intact erythrocyte membrane, as calculated from the data in [19, 31]. However, at least one of the assumptions underlying Eq. (13), namely that all liposomes equally take part in H_2DIDS-sensitive sulfate transport, is certainly incorrect. The τ-values obtained from Eq. (13) thus only represent approximations. Nevertheless, they seem to indicate that the sulfate transport capacity of band 3 in the reconstituted system is close to that in the native membrane.

Discussion

As described in the introduction, numerous papers have already been published on the reconstitution

of the anion transport system of the human erythrocyte membrane in liposomes. The present paper adds to this literature the description of a new reconstitution method and new considerations on the evaluation of the transport measurements.

Reconstitution Method

The present reconstitution method differs from the methods of others in two major aspects: (1) Lipid vesicle formation and concomitant incorporation of transport protein into the vesicle membranes takes place in a mixture of two nonionic detergents, Triton X-100 and octylglucopyranoside, during slow removal of the octylglucopyranoside by dialysis while most of the Triton is retained. (2) The Triton micelles and monomers which remained in the sample are separated from the vesicles by sucrose gradient centrifugation. For this, we have made use of the high density of Triton X-100 (approx. 1.11 g/ml [34]), which is higher than the density of the sucrose solutions used: we have applied the sample at the bottom of the centrifuge tube and centrifuged the vesicles out of the Triton solution towards the top of the tube. This centrifugation step removes from the vesicles all Triton X-100 which is not located either in their membranes or in their interior. The density gradient centrifugation also separates from the vesicles all lipid and protein molecules (due to their small sedimentation coefficient or to their high buoyant density, respectively) which did not take part in vesicle formation. Most of the residual Triton X-100, which remains after centrifugation, is removed by dialysis. However, the vesicle membranes still contain 4–6 detergent molecules per 100 molecules of phospholipid. This is 2–5 times the amount left after extraction of the detergent by the Bio-Beads method [20, 37].

Measurements of sulfate efflux from the vesicle preparations revealed the existence of three populations (a fourth population, consisting of completely leaky vesicles, remains undetected in these measurements). Two of the populations (Nos. 2 and 3 of Fig. 1) are devoid of a reconstituted anion transport system. However, the third, vastly predominant vesicle population (No. 1 of Fig. 1) shows all the major properties of sulfate transport across the intact human erythrocyte membrane: transport numbers (at 30°C) close to 20 ions per band 3 molecule and min, an activation energy of approx. 140 kJ/mol, a bell-shaped pH dependency with a maximum at pH 6.2, saturation of the flux at sulfate concentrations around 100 mM, reduced sulfate efflux when the extravesicular volume is free of transportable anions, inhibition of the flux by H_2DIDS and flufenamate (approx. K_I-values at 30°C: 0.1 and 0.7 μM, respectively), and "right-side-out" orientation of the transport protein (as judged from the inhibition of up to 98% by externally added H_2DIDS). Thus, these vesicles obviously contain the essentially intact anion transport system of the human erythrocyte membrane. The success of the reconstitution method described distinctly exceeds that of the reconstitution procedures published earlier. In particular, it is now possible for the first time to demonstrate the bell-shape of the pH dependency of sulfate efflux and the appropriate K_I-value for the inhibition of the transport system by H_2DIDS.

The most surprising structural property of the reconstituted system is the virtually complete right-side-out orientation of the transport protein. This right-side-out orientation must be the result of the conditions prevailing during vesicle formation; the details of the process are currently under study. Two other groups have also reported the preparation of artificial band 3-lipid vesicles with predominantly unidirectional orientation of band 3 [9, 14]; however, the restoration of anion transport was not demonstrated. The present paper thus represents the first report showing both a unidirectional incorporation of band 3 into lipid bilayers and the functionality of the transport protein.

Evaluation of the Flux Measurements

In our measurements of sulfate efflux, the counting rate $n_e(t)$ of radioactive sulfate trapped in the liposomes as a function of time could be fitted by two exponentials plus an additive constant (Eq. 3). This suggests that three populations of vesicles with grossly differing transport properties contribute to $n_e(t)$. The population with the highest amplitude (n_1) and rate constant ($k_{1\text{eff}}$) contains the reconstituted anion transport system and thus is the population of primary interest. This population consists of two subpopulations, one of which shows an additional anion leak, as is revealed by flux experiments in the presence of high concentrations of H_2DIDS. The latter experiments also demonstrate that anion leak flux in the system can be described by a single rate constant k_2. This quite conclusively leads to the model described by Fig. 1 and Eq. (1).

The "true" rate constant k_1 characterizing the reconstituted anion transport pathway could be reliably calculated from the $n_e(t)$-data, by least squares techniques, only in the case $k_1 \gg k_2$. We have therefore introduced "effective" rate constants k_{eff} and $k_{I\text{eff}}$, which are weighted means of the individual k_i-values of the system in the absence and presence, respectively, of high concentrations of H_2DIDS. Both parameters could be determined much more reliably than the individual k_i. We have used the

difference of the two effective rate constants, Δk_{eff}, for the characterization of the reconstituted anion transport pathway. Δk_{eff} is virtually identical to $k_1 \cdot (n_1/n(0))$ (Eq. (9)). Since $n_1/n(0)$ apparently depends only very weakly on the conditions of the flux experiments, Δk_{eff} is nearly as useful for the characterization of the anion transport system as k_1.

The evaluation of the effect of inhibitors on the reconstituted anion transport system, especially at maximum inhibition of the transport pathway, could not be based on Eq. (1) (which *a priori* assumes that the pathway can be blocked fully by H_2DIDS). The data were therefore analyzed by Eq. (3). We have described the anion transport properties of the vesicle system by the parameter k^*, which is the effective rate constant of the system when the known unspecific contributions to sulfate flux have been subtracted from the $n(t)$-curve and which is directly related to the (appropriately defined) degree of inhibition of the transport system. As described above, k^* is only an approximate measure of the degree of inhibition. It seems, however, to be a suitable measure, especially of the maximum degree of inhibition achieved. The latter figure is fundamental for the determination of the sidedness of the transport system. The virtually complete right-side-out character of the reconstituted anion transport pathway revealed by this determination in turn represents the basis for the separate determination of k_2 from flux experiments performed in the presence of high concentration of H_2DIDS, and thus of our general evaluation procedure.

It should be added that all our calculations are in any case only approximate, since they disregard the size distribution of the vesicles. The isolation of vesicle subfractions with a narrower size distribution by gel filtration may eliminate this problem. Another problem remaining is the existence of vesicles which are so leaky that they do not contribute to $n(t)$ or v_i, which will effect all determinations of transport numbers. This problem could possibly be solved by improvements in the procedure of vesicle isolation.

What use may be made of the reconstituted anion transport system and the method of reconstitution described in this paper? It seems probable that the reconstituted system will offer new possibilities for studies on structure-function relationships of band 3. Its main advantage could be the ease by which, by addition of H_2DIDS, specific and unspecific contributions to the anion flux can be distinguished from each other. The method of reconstitution developed by us may, in addition, be applicable to any transport system that is not irreversibly inactivated by Triton X-100 plus moderate concentrations of octylglucopyranoside and may thus increase the existing collection of promising reconstitution methods. Some of the possible applications described are now under study in our laboratory.

We are very grateful to Professor H. Passow, Professor B. Deuticke and Dr. W. Köhne for helpful advice and stimulating discussions, and to Dr. L. Kampmann for help with the computations. We are also indebted to Mrs. K. Boss and Mrs. K. Heitmann for excellent technical assistance and to the Deutsche Forschungsgemeinschaft for financial support (grants Fa 48 and SFB 169).

References

1. Bachmann, L., Schmitt-Fumian, W.W. 1973. Spray-freezing and freeze-etching. *In:* Freeze-Etching, Techniques and Applications. E.L. Benedetti and P. Favard, editors. pp. 73–99. Soc. Franc. Microscopie Electronique, Paris
2. Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:**466–468
3. Barzilay, M., Cabantchik, Z.I. 1979. Anion transport in red blood cells: II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membr. Biochem.* **2:**255–281
4. Cabantchik, Z.I., Knauf, P., Rothstein, A. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta* **515:**239–302
5. Cabantchik, Z.I., Loyter, A. 1980. Functional characterization of isolated membrane transport systems. The erythrocyte anion transporter as a model. *In:* Membrane Transport in Erythrocytes. U.V. Lassen, H.H. Ussing, and J.O. Wieth, editors. pp. 373–383. Munksgaard, Copenhagen
6. Cabantchik, Z.I., Volsky, D.J., Ginsburg, H., Loyter, A. 1980. Reconstitution of the erythrocyte anion transport system: *In vitro* and *in vivo* approaches. *Ann. N.Y. Acad. Sci.* **341:**444–454
7. Cousin, J.-L., Motais, R. 1979. Inhibition of anion permeability by amphiphilic compounds in human red cell: Evidence for an interaction of niflumic acid with the band 3 protein. *J. Membrane Biol.* **46:**125–153
8. Cousin, J.-L., Motais, R. 1982. Inhibition of anion transport in the red blood cell by anionic amphiphilic compounds: I. Determination of the flufenamate-binding site by proteolytic dissection of the band 3 protein. *Biochim. Biophys. Acta* **687:**147–155
9. Darmon, A., Bar-Noy, S., Ginsburg, H., Cabantchik, Z.I. 1985. Oriented reconstitution of red cell membrane proteins and assessment of their transmembrane disposition by immunoquenching of fluorescence. *Biochim. Biophys. Acta* **817:**238–248
10. Darmon, A., Zangrill, M., Cabantchik, Z.I. 1983. New approaches for the reconstitution and functional assay of membrane transport proteins. Application to the anion transporter of human erythrocytes. *Biochim. Biophys. Acta* **727:**77–88
11. Dorst, H.-J., Schubert, D. 1979. Self-association of band 3 protein from human erythrocyte membranes in aqueous solutions. *Hoppe-Seyler's Z. Physiol. Chem.* **360:**1605–1618
12. Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major peptides of the human erythrocyte membrane. *Biochemistry* **10:**2606–2617
13. Fraley, R., Wilschut, J., Düzgünes, N., Smith, C., Papahad-

jopoulos, D. 1980. Studies on the mechanism of membrane fusion: Role of phosphate in promoting calcium induced fusion of phospholipid vesicles. *Biochemistry* **19:**6021–6029
14. Gerritsen, W.J., Verkley, A.J., Zwaal, R.F.A., Van Deenen, L.L.M. 1978. Freeze-fracture appearance and disposition of band 3 protein from the human erythrocyte membrane in lipid vesicles. *Eur. J. Biochem.* **85:**255–261
15. Jennings, M.L. 1984. Oligomeric structure and the anion transport function of human erythrocyte band 3 protein. *J. Membrane Biol.* **80:**105–117
16. Kampmann, L., Lepke, S., Fasold, H., Fritsch, G., Passow, H. 1982. The kinetics of intramolecular cross-linking of the band 3 protein in the red blood cell membrane by 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid (H_2DIDS). *J. Membrane Biol.* **70:**199–216
17. Knauf, P.A. 1979. Erythrocyte anion exchange and the band 3 protein: Transport kinetics and molecular structure. Curr. *Topics Membr. Transp.* **12:**249–363
18. Köhne, W. 1982. Rekonstitution des Anionentransportsystems der Erythrotyten-Membran durch Einbau des Transportproteins in Lipid-Vesikel. Allgemeine Eigenschaften und Lipidabhängigkeit des rekonstituierten Systems. Ph.D. Thesis. RWTH, Aachen
19. Köhne, W., Deuticke, B., Haest, C.W.M. 1983. Phospholipid dependence of the anion transport system of the human erythrocyte membrane. Studies on reconstituted band 3/lipid vesicles. *Biochim. Biophys. Acta* **730:**139–150
20. Köhne, W., Haest, C.W.M., Deuticke, B. 1981. Mediated transport of anions in band 3-phospholipid vesicles. *Biochim. Biophys. Acta* **664:**108–120
21. Lukacovic, M.F., Feinstein, M.B., Sha'afi, R.J., Perrie, S. 1981. Purification of stabilized band 3 protein of the human erythrocyte membrane and its reconstitution into liposomes. *Biochemistry* **20:**3145–3151
22. Lukacovic, M.F., Toon, M.R., Solomon, A.K. 1984. Site of red cell cation leak induced by mercurial sulfhydryl reagents. *Biochim. Biophys. Acta* **772:**313–320
23. Macara, J.G., Cantley, L.C. 1983. The structure and function of band 3. *In:* Cell Membranes. Methods and Reviews. E. Elson, W. Frazier, and L. Glasier, editors. Vol. 1, pp. 41–87. Plenum, New York
24. Pappert, G., Schubert, D. 1983. The state of association of band 3 protein of the human erythrocyte membrane in solutions of nonionic detergents. *Biochim. Biophys. Acta* **730:**32–40
25. Passow, H. 1986. Molecular aspects of the band 3 protein-mediated anion transport across the red blood cell membrane. *Rev. Physiol. Biochem. Pharmacol.* **103:**61–203
26. Ross, A.H., McConnell, H.M. 1977. Reconstitution of band 3, the erythrocyte anion exchange protein. *Biochem. Biophys. Res. Commun.* **74:**1318–1325
27. Ross, A.H., McConnell, H.M. 1978. Reconstitution of the erythrocyte anion channel. *J. Biol. Chem.* **253:**4777–4782
28. Rothstein, A., Cabantchik, Z.I., Balshin, M., Juliano, R. 1975. Enhancement of anion permeability in lecithin vesicles by hydrophobic proteins extracted from red blood cell membranes. *Biochem. Biophys. Res. Commun.* **64:**144–150
29. Scheuring, U., Kollewe, K., Schubert, D. 1984. A new method for the reconstitution of the anion transport system of the human erythrocyte membrane. *Hoppe-Seyler's Z. Physiol. Chem.* **365:**1056–1057
30. Schnell, K.F. 1972. On the mechanism of inhibition of the sulfate transfer across the human erythrocyte membrane. *Biochim. Biophys. Acta* **282:**265–276
31. Schnell, K.F., Gerhardt, S., Schöppe-Fredenburg, A. 1977. Kinetic characteristics of the sulfate self-exchange in human red blood cells and red blood cell ghosts. *J. Membrane Biol.* **30:**319–350
32. Schubert, D., Boss, K., Dorst, H.-J., Flossdorf, J., Pappert, G. 1983. The nature of the stable noncovalent dimers of band 3 protein from erythrocyte membranes in solutions of Triton X-100. *FEBS Lett.* **163:**81–84
33. Schwoch, G., Rudloff, V., Wood-Guth, I., Passow, H. 1974. Effect of temperature on sulfate movements across chemically or enzymatically modified membranes of human red blood cells. *Biochim. Biophys. Acta* **339:**126–138
34. Tanford, C., Reynolds, J.A. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta* **457:**133–170
35. Ueno, M., Tanford, C., Reynolds, J.A. 1984. Phospholipid vesicle formation using nonionic detergents with low monomer solubility. Kinetic factors determine vesicle size and permeability. *Biochemistry* **23:**3070–3076
36. Van Hoogevest, P., Du Maine, A.P.M., De Kruijff, B., De Gier, J. 1984. The influence of lipid composition on the barrier properties of band 3-containing lipid vesicles. *Biochim. Biophys. Acta* **777:**241–252
37. Van Hoogevest, P., Van Duijn, G., Batenburg, A.M., De Kruijff, B., De Gier, J. 1983. The anion permeability of vesicles reconstituted with intrinsic proteins from the human erythrocyte membrane. *Biochim. Biophys. Acta* **734:**1–17
38. Wolosin, J.M. 1980. A procedure for membrane-protein reconstitution and the functional reconstitution of the anion transport system of the human erythrocyte membrane. *Biochem. J.* **189:**35–44
39. Yu, J., Steck, T.L. 1975. Isolation and characterization of band 3, the predominant polypeptide of the human erythrocyte membrane. *J. Biol. Chem.* **250:**9170–9175

Received 30 May 1985; revised 8 November 1985