J. Membrane Biol. 63, 61–70 (1981)

The Journal of
Membrane Biology

General and Transport Properties of Hypotonic and Isotonic Preparations of Resealed Erythrocyte Ghosts

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Summary. Resealed human erythrocyte ghosts are regarded as valuable tools for the study of membrane properties. In order to investigate to what extent preparation procedures affect the yield of ghosts, their general properties, and their permeability, ghosts prepared by lysis at low (hypotonic media) and high (isotonic media) ionic strength were compared with each other and with native erythrocytes. For isotonic lysis, cells were either subjected to dielectric breakdown or suspended in isotonic NH_4Cl solutions. In spite of very different characteristics of the lysis and the resealing process in the three types of preparations, the resulting ghosts do not differ in a number of features except for somewhat varying yields and for properties resulting from the mode of lysis.

Specific transport properties, as characterized by the mediated fluxes of *m*-erythritol, L-arabinose, L-lactate, and sulfate, proved to be unaltered with a few unsystematic exceptions. The simple nonmediated fluxes of all these permeants, as measured in the presence of inhibitors, however, were enhanced between 1.5- and 4-fold, indicating a somewhat increased ground permeability (of the lipid domain) in all ghost membranes.

Key words: Hemolysis, electric breakdown, erythrocyte ghosts, anion permeability, nonelectrolyte permeability, membrane transport

Isolated, resealed erythrocyte membranes (ghosts) are valuable tools for studies of membrane transport. The most commonly used ghost preparation is obtained by osmotic lysis of the cells in hypotonic solutions, followed by reconstitution of the hemolysate to isotonicity and a resealing period at 37 °C. In addition to restoring the barrier properties of the membrane, this step minimizes heterogeneities of the resulting ghost populations [15, 18, 24]. Nevertheless, irreversible alterations of the membrane might occur in this preparation due to temporary exposure of the membrane to low ionic strength [13].

These possible harmful effects stimulated the interest in resealed ghosts prepared under isotonic conditions. Besides procedures [4, 5] involving the temporary exposure of cells to hypertonic solutions of permeable nonelectrolytes, a reversible increase of membrane permeability after exposure of red cell suspensions to high electric field strengths [36, 37] has been used for this purpose. This increase of permeability, which has been explained by a "dielectric breakdown" of the membrane, is followed by colloid-osmotic [34] lysis [26]. Ghosts produced by electric lysis can be resealed. Their disadvantage is the requirement of expensive equipment and the possibility of irreversible damage to the membranes by the high electric fields applied.

A new and very simple preparation of isotonic ghosts is reported in this paper. As has been known for a long time [21], red cells suspended in an isotonic solution of NH_4Cl hemolyse in a two-step process. First, NH_3 formed by dissociation of NH_4^+ enters the cell and combines with H^+ to form NH_4^+ again. The transmembrane proton gradient resulting from this first step is dissipated in the second step by an efflux of hydroxyl ions in a coupled exchange for extracellular chloride [36]. This exchange is greatly accelerated by the presence of CO_2 or HCO_3^- [17], the underlying mechanism being the reversible formation of HCO_3^- from OH^- and CO_2. The reaction is catalyzed by carbonic anhydrase within the red cell but proceeds at its slow uncatalyzed rate in the extracellular medium. HCO_3^- is a much better exchange partner for chloride than OH^- [32]. The net result is an uptake of NH_4Cl into the cells which induces osmotic swelling and hemolysis. In the present work this type of lysis is used for the first time

0022-2631/81/0063-0061 $02.00

to prepare resealed ghosts. As will be shown, NH_4Cl ghosts reseal to small molecules and ions. Some properties of this preparation will be described and compared to those of the hypotonic and the isotonic electric ghost preparation. Transport studies showed no significant differences between the different preparations.

Materials and Methods

Preparation of Resealed Ghosts

A) Hypotonic and Dielectric Ghosts

Resealed hypotonic ghosts were prepared according to Schwoch and Passow [28] and washed three times in 20 vol of a 165 mmol/liter KCl solution at 0 °C. Preparation of ghosts by dielectric breakdown was based on the technique of Zimmermann et al. [37]. Washed erythrocytes were suspended (hematocrit 9%) in a medium containing (concentrations in mmol/liter): KCl 130, NaCl 20, $MgCl_2$ 4, HEPES[1] 5, pH 7.35, and exposed to a voltage pulse of 40 μsec duration (usually 9 kV/cm) at 0 °C. After complete lysis the suspension was incubated for resealing at 37 °C for 60 min. The resealed ghosts were washed three times in a solution containing 140 mmol/liter NaCl, 15 mmol/liter Na_2HPO_4/NaH_2PO_4, pH 7.35.

B) NH_4Cl-Ghosts

Washed erythrocytes were suspended in 165 mmol/liter KCl at a hematocrit of 50% and cooled to 0 °C. 1 vol of the suspension was mixed with 10 vol. of ice-cold lysing solution (concentrations in mmol/liter): NH_4Cl 150, $MgSO_4$ 4, $NaHCO_3$ 2, $CaCl_2$ 0.05, pH 7.4. This suspension was incubated at 0 °C until complete lysis had occurred (usually after about 45 min) and subsequently warmed up to 37 °C for a resealing period of 60 min. The pH was controlled and readjusted to 7.4 if necessary. After centrifugation (10 min, 0 °C, 20,000 × *g*) the sediment was washed three times with the lysing medium.

Determination of the Time Course of Lysis

The time course of NH_4Cl-induced lysis was determined by adding 1 vol of 50% erythrocyte suspension in isotonic KCl to 10 vol of $NH_4Cl/NaHCO_3$ solution and measuring the hemoglobin content in the supernatant after different time intervals.

The time course of electric lysis was followed in an analogous way in cell suspensions subjected to the voltage pulse at 0 °C (hematocrit 20%) and immediately after brought to the desired temperature by 1:1 dilution with suspension medium of an appropriate temperature.

Estimation of Ghost Volumes

The mean ghost volume was calculated from the number of ghosts in a defined volume, corrected for the trapped intercellular volume by isotope dilution of an impermeable probe (^{14}C-sucrose).

[1] *Abbreviations used:* HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PCMBS = *p*-chloromercuriphenyl-sulfonate; SITS = 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonate; DIDS = 4,4′-diisothiocyano-stilbene-2,2′-disulfonate.

Determination of the Fractional Yield of Resealed Ghosts

Resealing of a ghost membrane can only be defined with respect to a particular solute. We used ^{14}C-sucrose, which was added to the lysing medium and trapped within the ghosts during lysis and resealing. Leaky ghosts will lose sucrose during washing after the resealing period. The fractional yield of resealed ghosts was calculated from the ratio, *R*/Hb, of the radioactivity *R* and the hemoglobin contents Hb in ghosts relysed in H_2O, relative to the ratio *R*/Hb measured in the lysate immediately after lysis in the ^{14}C-sucrose containing medium.

Measurements of the Leakiness of Ghosts to Sucrose

Ghosts were loaded with ^{14}C-sucrose prior to resealing. After resealing they were incubated (Hct 5%, 30 °C) in one of the following media: hypotonic ghosts – 157 mmol/liter KCl, 6 mmol/liter Na_2HPO_4/NaH_2PO_4; dielectric ghosts – washing medium; NH_4Cl ghosts – lysing medium. Streptomycin (7.5 mg%) + penicillin (2 mg%) were added to prevent growth of bacteria. At various times during 24 hr the radioactivity in the supernatant was measured. The rate of leakage of sucrose (k) from resealed ghosts was calculated from

$$k = -\frac{1}{t} \cdot \log\left(1 - \frac{a_t}{a_\infty}\right)$$

where a_t = radioactivity of ^{14}C-sucrose (cpm/ml) in the supernatant at time t, and a_∞ = radioactivity in the total suspension after lysis and protein precipitation by 60% $HClO_4$, taken to represent external radioactivity at tracer equilibrium.

Determination of Nonelectrolyte and Anion Permeabilities

For transport studies the rates of tracer efflux of various nonelectrolytes and anions (L-(^{14}C)-arabinose, L-(^{14}C)-lactate, ^{14}C-erythritol, ^{35}S-sulfate (Amersham Buchler, Braunschweig), from preloaded ghosts or erythrocytes were measured under steady-state conditions as described previously [10], using media of the following composition (Hct 5%, 37 °C, pH 7.4, concentrations in mmol/liter): Hypotonic ghosts – KCl 150, $Na_2HPO_4/NaH_2/PO_4$ 6.5, test solute 5; Electric ghosts – NaCl 135, Na_2HPO_4/NaH_2PO_4 15, test solute 5; NH_4Cl ghosts – NH_4Cl 150, $MgSO_4$ 4, $NaHCO_3$ 2, $CaCl_2$ 0.05, HEPES 10, test solute 5; Erythrocytes – NaCl 145, Na_2HPO_4/NaH_2PO_4 6.5, test solute 5.

Materials

4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) was from Serva, Heidelberg; 4,4′diisothiocyano-stilbene-2,2′-disulfonate (DIDS) was a kind gift from Prof. J.O. Wieth, Copenhagen. P-chloromercuriphenylsulfonate (PMBS) and cytochalasin B were from Sigma, Munich; phloretin from Karl Roth, Karlsruhe.

Results

Time Course of NH_4Cl Lysis: Dependence on HCO_3^--Concentration and Temperature

The time course of lysis of erythrocytes in NH_4Cl solutions depends on various parameters. Lysis can

be accelerated by the addition of HCO_3^- [17, 25]. In the absence of added HCO_3^- complete lysis requires about 4 hr. With increasing bicarbonate concentration, onset of lysis occurs earlier and the time interval between lysis of the first and the last cells becomes shorter. At 10 mmol/liter HCO_3^- complete lysis can be obtained within 10 min.

Lysis furthermore strongly depends on temperature (Fig. 1). With increasing temperature lysis starts earlier and reaches completion within shorter time intervals. The maximal percentage of hemoglobin release decreases with increasing temperature. Provided that the lysis of a single cell is an all-or-none process, incomplete release of hemoglobin would indicate the presence of a residual amount of intact erythrocytes. Since the lysate did not contain intact cells, the incomplete release of hemoglobin must be due to an uneven distribution of hemoglobin between the lysed cells and the surrounding medium.

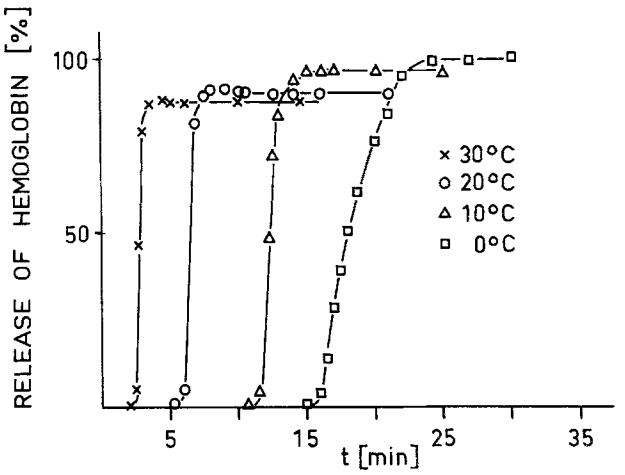

Fig. 1. Influence of temperature on the time course of lysis of erythrocytes in isotonic NH_4Cl-solutions containing 2 mmol/liter $NaHCO_3$. The percentage of hemolysis was calculated by measuring the hemoglobin content in the supernatant of a sample of the suspension after centrifugation, relative to the hemoglobin content of the suspension after complete lysis by Triton X-100. 100% release of hemoglobin corresponds to the hemoglobin content of the suspension after complete lysis by Triton X-100 (*see* Methods)

Time Course of Electric Lysis: Dependence on Temperature

Incomplete release of hemoglobin is not a general property of isotonic ghost preparations. Ghosts produced by dielectric breakdown release the same amount of hemoglobin at all temperatures (Fig. 2). In contrast to NH_4Cl lysis, however, electric lysis occurs more slowly at *higher* temperatures. At 30 °C complete lysis cannot be obtained within 4.5 hr.

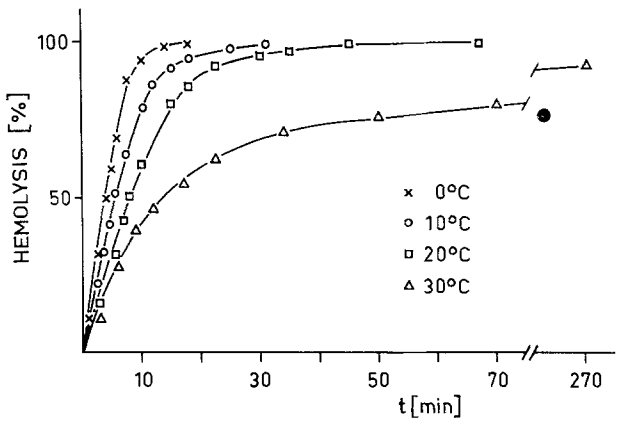

Fig. 2. Influence of temperature on the time course of lysis of erythrocytes after exposure to an electric field. Erythrocyte suspensions (Hct. 18%) were exposed at 0 °C to a 40-μsec pulse (9 kV/cm) and warmed up immediately afterwards to the temperature desired by 1:1 dilution with suspension medium of an appropriate temperature

Time Course of Electric Lysis: Role of the Anion Transfer System

Lysis of cells after dielectric breakdown of their membranes is preceded by a net uptake of cations in the course of the establishment of a Donnan distribution for small ions [19]. For reasons of electroneutrality, an uptake of anions (i.e., Cl^-) has to accompany cation influx. In order to establish whether this uptake occurs via the leak induced by breakdown or via the anion transport system [20] of the membrane, the influence of DIDS, an inhibitor of the anion transport system in the band 3 protein [27], on the time course of electric lysis was studied. Pretreatment of cells with the covalent inhibitor did not retard lysis induced by an electric pulse (Fig. 3), indicating that at least the DIDS-sensitive components of the anion transport system are not involved in the prelytic anion uptake.

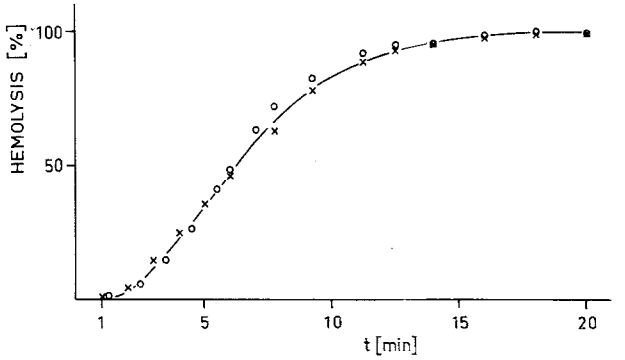

Fig. 3. Lack of influence of an inhibition of the anion transport system on the time course of electric lysis. ×, controls; ○, cells treated with DIDS (10 μmol/liter suspension, cytocrit 20%, 60 min, 37 °C). After pretreatment, cells were washed once, resuspended in isotonic KCl, and subjected to a 40-μsec pulse (7 kV/cm, 0 °C)

Yield of Resealed Ghosts

In order to compare basic properties of the resealed ghosts, the fractions of the total population that had resealed to hemoglobin were determined by relating the hemoglobin content of a defined volume of

Table 1. Fraction of ghosts resealed to ^{14}C-sucrose after lysis and reconstitution at 0 °C[a]

	Type of ghost preparation		
	Hypotonic ($n=4$)	Electric ($n=4$)	NH_4Cl ($n=10$)
Fraction of total number of ghosts resealed to ^{14}C-sucrose (%)	5.6 ± 4.3	40.8 ± 3.6	23.6 ± 4.8
Time of exposure (min)	20 (10)	20 (20)	40 (40)

[a] The data were calculated from the difference between the fraction of sucrose-tight ghosts obtained when the label was added at the moment of mixing cells and lysing medium and the fraction obtained when the label was only added after complete lysis (electric and NH_4Cl) or after lysis and reconstitution (hypotonic ghosts). Mean values ± SD. The numbers below the fractional yields indicate the total time of exposure at 0 °C; time of exposure at high ionic strength in parentheses.

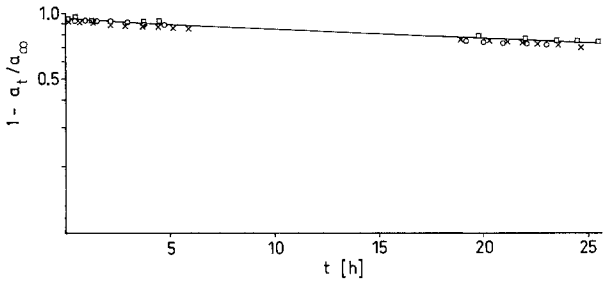

Fig. 4. Loss of entrapped ^{14}C-sucrose from different types of ghosts prepared by the respective standard procedures. □, hypotonic; ○, electric; ×, NH_4Cl. For preparations of hypotonic and NH_4Cl ghosts, ^{14}C-sucrose was added to the lysing medium before lysis was started; for the electric preparation, sucrose was added immediately after application of the electric pulse. Resealed ghosts were transferred into their respective nonradioactive washing media (*see* Methods) at 30 °C, and the radioactivity released into the media was measured after different time intervals. a_t, a_∞: radioactivity at time t and after attainment of equilibrium for ^{14}C-sucrose

packed ghosts before washing to the hemoglobin content of the same volume after washing (centrifugation for 10 min, 20,000 × *g*). The difference between these two values proved to be equal to the intercellular trapped volume of packed ghosts as determined by ^{14}C-sucrose (*see* below and Table 2). It therefore seems safe to assume that the hemoglobin lost during the washing procedure came only from the intercellular space. The different types of ghosts become tight to hemoglobin to 100%.

Hemoglobin could therefore be used as a reference for the determination of the fraction of ghosts that had resealed to sucrose. After hemolysis at 0 °C and resealing at 37 °C about 80% of the electric ghosts regained their impermeability to sucrose, while only 60% of the NH_4Cl ghosts and 70% of the hypotonic preparation resealed to that test molecule. These values could not be improved by a prolonged resealing period. The yield of resealed NH_4Cl ghosts could be raised from 60 to 70% when calcium was omitted from the lysing medium. This improvement, however, went along with a deterioration of the transport properties. Therefore no further use was made of this observation. The medium to cell ratio during NH_4Cl lysis also influences the yield of resealed ghosts: at ratios greater than 40:1 it decreased markedly; at a ratio of 100:1 only 30% of the ghosts resealed to sucrose.

In order to establish the influence of temperature on the resealing process the fraction of ghosts resealed to sucrose was determined at the end of the exposure to 0 °C, i.e., after lysis (electric ghosts: 20 min; NH_4Cl ghosts: 40 min), or after lysis and reconstitution (hypotonic ghosts: 10 min lysis and 10 min reconstitution) (Table 1). After these periods of time only 6% of the hypotonic ghosts had resealed to sucrose, while 40% of the electric ghosts and 24% of the NH_4Cl ghosts regained their impermeability. Prolongation of the reconstitution period (0 °C, high ionic strength) only slightly increased the fraction of resealed hypotonic ghosts. In this respect the resealing properties of hypotonic and isotonic ghost preparations are obviously different.

Release of Entrapped ^{14}C-Sucrose

The extent of restoration of membrane impermeability to sucrose was also tested by measuring the release of entrapped ^{14}C-sucrose. As shown in Fig. 4, loss of sucrose from resealed ghosts is a very slow process. Within the resolution of our experiments it can be described by a single exponential. The rates of leakage calculated from the slopes of the regression lines are 0.008 hr^{-1} for hypotonic ghosts, 0.011 hr^{-1} for electric ghosts, and 0.009 hr^{-1} for NH_4Cl ghosts (standard deviation for all preparations ± 0.003 hr^{-1}).

Ghost Volumes

For calculations of equilibrium exchange fluxes, the mean ghost volume has to be known. Table 2 provides these data as well as intercellular trapped volumes for the different types of ghosts. As compared to the intact cell, the volume of our hypotonic ghosts is almost normal, whereas that of both isotonic preparations is increased. At the moment of osmotic lysis the mean cell volume for all preparations corresponds to the critical hemolytic volume, i.e., about 140 μm^3. The relative shrinking of the hypotonic ghosts is due to solute and water movements occurring after reconstitution and before resealing [11]; in case of the elec-

Table 2. Mean volumes and intercellular trapped volumes for different ghost preparations and for erythrocytes[a]

Type of ghost	Mean volume (μm^3)	Intercellular volume (%)
Hypotonic	94±8 (12)	12.0±2.0
Electric	109±4 (12)	10.9±2.9
NH_4Cl	134±8 (13)	12.7±4.7
Erythrocytes	92±8 (8)[b]	3.8±1.0 (11)

[a] The mean volume was calculated from the number of washed packed ghosts after centrifugation at 8,000 × *g* for 4 min in a defined volume corrected for the intercellular trapped volume measured as the ^{14}C-sucrose accessible space between the cells (for details *see* Methods). The data present mean values ±SD, number of experiments in parentheses.
[b] [11].

tric ghosts, small differences in the osmolarity of the lysing and the washing medium are responsible. The intercellular trapped volumes are considerably higher for ghost preparations than for erythrocytes but do not differ significantly from each other.

Osmotic responses differ when hypotonic and electric ghosts, on the one hand, and NH_4Cl ghosts, on the other hand, are incubated in media containing low concentrations of an impermeable solute (sucrose or HEPES) at 37 °C. An increase of the ideal osmolarity by 4–20 mosmol/liter does not bring about detectable (> 5%) volume changes of hypotonic and dielectric ghosts in agreement with simple osmometer properties of these ghosts. NH_4Cl ghosts, on the other hand, shrink progressively (Fig. 5) with time and the concentration of the impermeable solute.

Permeabilities to Nonelectrolytes and Anions

In order to establish to what extent permeability properties of the erythrocyte membrane are retained after the preparation of the different ghosts, tracer fluxes were measured for test substances transported via the inorganic anion exchange system (sulfate), the monosaccharide transfer system (L-arabinose, *m*-erythritol), and the monocarboxylate carrier system (L-lactate). In addition, the susceptibility of these carrier-mediated transfer processes to specific inhibitors was studied. After inhibition of its transfer via the monosaccharide transfer system, erythritol could also be used as a probe for the permeability of the lipid domain [23, 31].

Within the accuracy of our measurements, tracer efflux kinetics followed a single exponential. The regression lines, however, indicated that part of the radioactivity transferred with the isotope-loaded cells to the efflux medium was immediately present in the external space. Part of this radioactivity comes from the intercellular trapped volume (about 10% according to occasional measurements), while the remaining fraction, between 1% for electric ghosts and 20% for NH_4Cl ghosts, probably represents ghosts not resealed to the test permeant. The rate coefficients of equilibrium exchange are listed in Table 3 for the total exchange as well as for the exchange in the presence of maximally effective concentrations of inhibitors of the different transport processes.

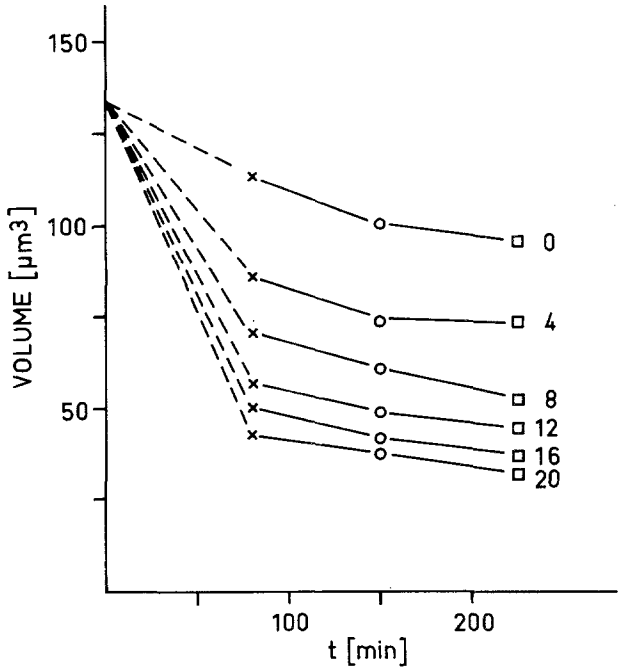

Fig. 5. Time-dependent shrinking of NH_4Cl ghosts after exposure to external sucrose. Ghosts prepared by the standard procedure were incubated at 37 °C in their washing medium, and sucrose was added at the concentrations (mmol/liter) given in the diagram. ×, ○, □: samples obtained after 80, 150, 225 min. Ghost volumes were estimated as described in the Methods

Tables 4 and 5 present fluxes calculated from the rate coefficients in Table 3. The inhibitor-sensitive fluxes were calculated from the difference between the total flux and the inhibitor-insensitive flux measured in the presence of inhibitors.

A) L-Arabinose and Erythritol

Total and inhibitor-sensitive fluxes of L-*arabinose* are the same for erythrocytes, hypotonic, and electric ghosts, while fluxes in NH_4Cl ghosts are significantly lower. Inhibitor-*in*sensitive fluxes are increased in all ghost preparations. Total *erythritol* flux is slightly increased in isotonic ghosts due to an enhancement of erythritol permeation via the inhibitor-*in*sensitive pathway.

B) L-Lactate and Sulfate

The total equilibrium exchange of L-lactate – as measured at an extracellular concentration of 5 mmol/liter

Table 3. Rate coefficients ($k \cdot 10^2$, min^{-1}) of the self-exchange of various test permeants in native erythrocytes and in different types of resealed ghosts[a]

A) Total transport

	L-Arabinose (10 °C)	*m*-Erythritol (30 °C)	L-Lactate (15 °C)	Sulfate (35 °C)
Erythrocytes	9.64±1.25 (4)	6.02±0.84 (5)	7.77±1.40 (6)	3.15±0.26 (3)
Hypotonic ghosts	5.77±0.76 (3)	4.63±0.74 (10)	5.94±0.90 (8)	1.03±0.12 (5)
Electric ghosts	4.87±0.17 (3)	4.54±0.18 (3)	3.94±0.41 (4)	1.03±0.05 (3)
NH_4Cl ghosts	4.19±0.62 (4)	6.52±0.98 (16)	6.40±1.21 (12)	1.59±0.28 (8)

B) Transport in the presence of inhibitors

	L-Arabinose +cytochalasin B (10 µmol/liter)	*m*-Erythritol +cytochalasin B (10 µmol/liter)	L-Lactate		Sulfate	
			+PCMBS (320 µmol/liter)	+Phloretin (360 µmol/liter)	+SITS (31 µmol/liter)	+Phloretin (360 µmol/liter)
Erythrocytes	0.13±0.04 (4)	1.97±0.13 (5)	0.43±0.14 (6)	0.16±0.09 (6)	0.16±0.09 (3)	0.30±0.04 (3)
Hypotonic ghosts	0.47±0.34 (3)	1.78±0.23 (10)	0.37±0.08 (7)	0.30±0.03 (6)	0.10±0.08 (5)	0.09±0.02 (5)
Electric ghosts	0.30±0.12 (3)	1.99±0.12 (3)	0.43±0.07 (4)	0.22±0.08 (4)	0.23±0.06 (3)	0.12±0.04 (3)
NH_4Cl ghosts	0.27±0.05 (4)	2.87±0.43 (16)	0.54±0.14 (12)	0.35±0.08 (7)	0.23±0.11 (8)	0.16±0.06 (9)

[a] Rate coefficients were determined as described in the Methods; mean values ±SD, number of experiments in parentheses.

Table 4. Equilibrium exchange fluxes (J^{ee}) of nonelectrolytes (pH 7.4) in native erythrocytes and in different types of resealed ghosts[a]

	$J^{ee} \cdot 10^{12}$ [mol/cm² · sec]		
	Total	Inhibitor-	
		-Sensitive	-Insensitive
		(Cytochalasin B, 10 µmol/liter)	
L-Arabinose			
Erythrocytes	20.0±2.6	19.7 ±2.6	0.27±0.08
Hypotonic ghosts	18.3±2.4	16.8 ±3.0	1.49±1.09
Electric ghosts	18.1±0.7	17.0 ±1.0	1.11±0.44[b]
NH_4Cl ghosts	10.6±1.6[c]	10.1 ±1.6[c]	0.68±0.13[c]
m-Erythritol			
Erythrocytes	12.5±1.8	8.35±1.93	4.08±0.29
Hypotonic ghosts	14.7±2.4	9.06±1.81	5.66±0.74
Electric ghosts	16.9±0.7[c]	9.47±1.04	7.39±0.44[c]
NH_4Cl ghosts	16.5±2.5[b]	9.23±1.75	7.27±1.09[c]

[a] Total and inhibitor-insensitive fluxes were calculated from the rate coefficients given in Table 3 and the extracellular concentration of the permeants assuming a distribution ratio of 1.0, a mean cell surface area of 142 µm², and mean solvent volumes (µm³) of 58 for native erythrocytes, 89 for hypotonic, 104 for electric, and 71 for NH_4Cl ghosts. Volumes of erythrocytes and ghosts take into consideration the respective hemoglobin contents. The low volume for NH_4Cl ghosts, in addition, reflects a shrinking of these ghosts due to the properties demonstrated in Figs. 5 and 7. Inhibitor-sensitive fluxes were computed from the mean values of the differences between total and inhibitor-insensitive fluxes. This way of calculation accounts for the fact that the sum of sensitive fluxes not always equals the total flux. *P* values refer to the significance of the difference from the value for erythrocytes, calculated by Student's *t*-test. Mean values ±SD from 4–8 experiments.

[b] $0.0025 < P < 0.01$.

[c] $P < 0.0005$.

– is increased significantly in hypotonic and NH_4Cl ghosts and probably accelerated in electric ghosts. Inhibitor-sensitive fluxes of lactate are increased in all ghost preparations. This is true for the PCMBS-sensitive fraction, which corresponds to lactate transfer via the monocarboxylate carrier, as well as for the phloretin-sensitive fraction, which in addition includes lactate movements via the inorganic anion exchange system [9]. The inhibitor-insensitive fractions, corresponding to nonionic diffusion of lactic acid (phloretin-insensitive) or nonionic diffusion plus transfer via the inorganic anion exchange system (PCMBS-insensitive) are also increased significantly.

Total, inhibitor-sensitive, and inhibitor-insensitive sulfate exchange fluxes are slightly, but not significantly, enhanced in the isotonic ghost preparations. Fluxes in hypotonic ghosts are essentially similar to those measured for intact cells.

Discussion

Lysis

In contrast to fast ($t_{1/2} < 2$ sec) hypotonic lysis [1, 18], both types of isotonic lysis occur slowly ($t_{1/2} >$ 10 min). The reasons for the slow progress are different in the two preparations. Electric lysis results from salt leaks [35], whose number and properties determine the rate of lysis. In NH_4Cl lysis, transport properties of the normal membrane, i.e., Cl^-/HCO_3^--exchange capacity, and/or the availability of catalysts (CO_2, HCO_3^-) are rate limiting.

Increase of temperature changes the rate of lysis in the two isotonic preparations in opposite direc-

Table 5. Equilibrium exchange fluxes (J^{ee}) of anions (pH 7.4) in native erythrocytes and in different types of resealed ghosts[a]

L-*Lactate*	$J^{ee} \cdot 10^{12}$ [mol/cm^2 · sec]				
	Total	Inhibitor-			
		-Sensitive		-Insensitive	
		PCMBS (320 μmol/liter)	Phloretin (360 μmol/liter)	PCMBS (320 μmol/liter)	Phloretin (320 μmol/liter)
Erythrocytes	11.3 ±2.0	10.6 ±1.9	10.9 ±2.0	0.62±0.21	0.38±0.09
Hypotonic ghosts	17.9 ±2.7[c]	16.4 ±2.6[c]	17.7 ±2.5c	1.12±2.4[b]	0.91±0.10[c]
Electric ghosts	13.9 ±1.4	12.4 ±1.6	13.2 ±1.8	1.52±0.26[c]	0.78±0.28[b]
NH_4Cl ghosts	15.4 ±2.9[b]	13.9 ±2.6[b]	12.9 ±3.4	1.30±0.34[c]	0.84±0.19[c]
Sulfate	Total	Inhibitor-			
		-Sensitive		-Insensitive	
		SITS (30 μmol/liter)	Phloretin (360 μmol/liter)	SITS (30 μmol/liter)	Phloretin (360 μmol/liter)
Erythrocytes	2.61±0.21	2.48±0.17	2.35±0.19	0.12±0.07	0.25±0.03
Hypotonic ghosts	2.97±0.32	2.66±0.32	2.92±0.35	0.29±0.35	0.26±0.05
Electric ghosts	3.44±0.17[b]	2.67±0.13	3.00±0.12[b]	0.77±0.22[b]	0.42±0.15[b]
NH_4Cl ghosts	3.62±0.65[b]	3.03±0.61	3.31±0.7	0.53±0.23	0.36±0.13

[a] The values were calculated as described in Table 4, except that the following values for the distribution ratios for anions were used: erythrocytes – lactate 0.7, sulfate 0.4; ghosts – lactate 0.95, sulfate 0.9.
[b] $0.0025 < P < 0.01$. [c] $P < 0.0005$.

tions: NH_4Cl lysis is enhanced, dielectric lysis retarded. For NH_4Cl lysis an apparent activation energy of 10 kcal/mole can be obtained (Fig. 6). Although this number closely agrees with that of about 12 kcal/mol [7] for the uncatalyzed dehydration of HCO_3^- to CO_2, it cannot be inferred that this step limits NH_4Cl lysis, since other evidence points to a possible rate limiting rôle of Cl^-/HCO_3^--exchange [2].

The rate of electric lysis *de*creases with increasing temperature. Therefore direct conclusions cannot be drawn from its temperature dependence on the activation energy of the underlying process. Electrical lysis is induced by salt leaks. Diffusion through such leaks should increase with temperature. The observed decrease of the rate of lysis with temperature probably results from superimposed temperature-dependent resealing of the salt leaks, which was described previously [25]. The temperature dependence of the resealing process must be larger than that of the diffusion through the leak, since otherwise a negative temperature dependence would not result.

The leaks induced by electric breakdown of the membrane are pervious to cations and anions (*cf.* Fig. 3). This finding accounts for the observation [35] that divalent anions (phosphate, sulfate), although highly impermeable in the native membrane, only temporarily suppress electric lysis. The magnitude of the electrical leak(s) can be inferred from the time course of lysis: In order to reach their lytic volume

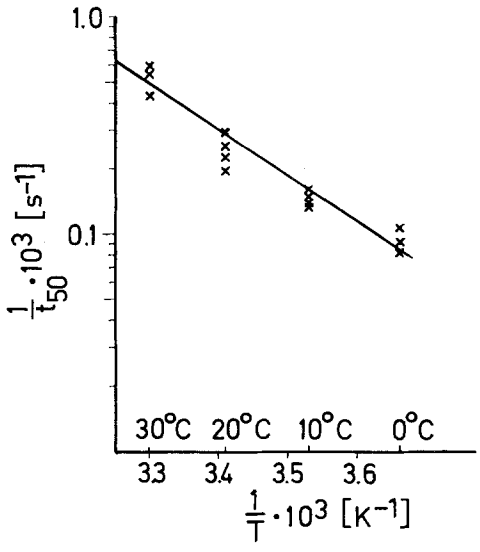

Fig. 6. Arrhenius diagram for NH_4Cl lysis. Ordinate: reciprocal of time t_{50} required for the lysis of 50% of the cells, taken from Fig. 1. Kinetics were corrected by setting the maximal amount of hemoglobin released at every temperature equal to 100% lysis

by colloid-osmotic swelling, erythrocytes have to take up 90 mmol salt (NaCl + KCl) per liter of initial cells. From the half time of lysis at 0 °C (6.1 min, after breakdown at 7 kV/cm), an average salt influx (anions plus cations) of 5×10^{-17} mol/cell · sec can be computed, equivalent to 3×10^7 ions/cell · sec. This number roughly corresponds to the maximal conductance (at

100 mV in saturated NaCl) of a gramicidin channel [14]. For the conditions of our experiments (0.15 M salt, approx. 10 mV) a gramicidin channel would have a conductance of 5×10^4 ions/sec. In terms of channel properties, electric lysis could thus be induced by 1–600 lesions per cell.

Independent of models, an approximate K^+ permeability of 2×10^{-7} cm/sec (0 °C) can be calculated from the prelytic salt influx and the mean gradient driving the influx, obtained according to ref. 30. K^+-permeability is thus at least 10^3 times higher in the modified than in the normal erythrocyte membrane [3].

Resealing

The time course of resealing to sucrose at 37 °C is not very different for the various types of ghosts, in spite of the different yields (data not shown). In contrast, at 0 °C the time course is much faster for isotonic ghosts (Table 1). High ionic strength *during* lysis obviously enables a large number of ghosts to reseal rapidly, even at low temperature, while reconstitution of high ionic strength *after* lysis is not sufficient for rapid resealing at 0 °C.

A further aspect of resealing in isotonic media deserves comment. Hemoglobin does not fully equilibrate between ghosts and media during NH_4Cl lysis at temperatures >0 °C (Fig. 1). Membrane binding of hemoglobin cannot explain this observation for quantitative reasons [29]. More likely [1], hemoglobin is trapped due to a very fast resealing after the release of only a fraction of the hemoglobin. Trapping of hemoglobin, however, is no general property of isotonic ghosts, since it was not observed in electric preparations.

Properties of Resealed Ghosts

A) Yields

While no data to compare with are available for electric and NH_4Cl ghosts, our yield of hypotonic ghosts (70%), as determined by sucrose trapping, can be compared to values reported by other authors [6, 11, 24]. Using various probe molecules, they obtained yields greater than 90%. Application of their techniques to our ghost preparations also resulted in yields >70%. It is not clear as yet why low yields were consistently observed with our sucrose-trapping technique. The sequence of yields (electric > hypotonic > NH_4Cl) indicates that ionic strength during lysis does not have a decisive effect on the yield of resealed ghosts.

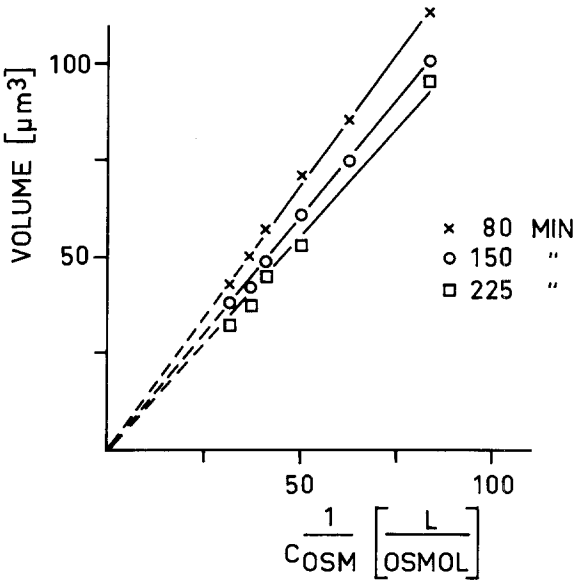

Fig. 7. Osmometer properties of NH_4Cl ghosts. Ghost volume decreases linearily with the osmolar concentration of external impermeant solutes, i.e., $MgSO_4$, $NaHCO_3$ and added sucrose or HEPES in the outside medium

B) Osmotic Properties of NH_4Cl Ghosts

Resealed NH_4Cl ghosts shrink slowly when an impermeable solute is added to the suspension medium (Fig. 5). This shrinkage can be explained by the fact that NH_4Cl, although appropriate for the preparation of stable resealed ghosts, is a permeable electrolyte. Since the osmotic balance of ghost suspensions is only governed by the impermeable solutes, the osmotic response of NH_4Cl ghosts must be described by relating volume changes to the reciprocal of the external concentration of truly impermeable solutes, i.e., not taking into account NH_4Cl. The linear relationship obtained (Fig. 7) is consistent with normal osmometer properties comparable to those of hypotonic ghosts [22].

C) Transport Properties

Ghosts completely reconstituted to the state of the native cell membrane should exhibit the transport properties of the native cell. This postulate is fulfilled to a considerable extent in our preparations.

Pathways of Nonmediated Transport. Nonmediated transport processes assumed to occur via the lipid domain of the membrane [8] are slightly enhanced in all preparations of ghosts. Sucrose permeability, which is virtually zero in the native cell [33], assumes a value of 2×10^{-10} cm/sec at 30 °C (calculated from Fig. 4). Mannitol permeability increases from 3.7×10^{-10} cm/sec (39 °C) in the native cell [16] to 5.3 ×

10^{-10} cm/sec (data not shown), in agreement with data for hemoglobin-free ghosts [6]. The inhibitor-*insensitive* fractions of the transport of L-arabinose, erythritol, and L-lactate (=nonionic diffusion of lactic acid) are enhanced between 1.5- and 4-fold in resealed ghosts (Tables 4 and 5). Whether this enhancement is related to the perturbation of the lipid domain observed in resealed ghosts [12] remains to be elucidated.

Pathways of Mediated Transport. The inhibitor-sensitive fractions of the transport of L-arabinose and erythritol, which involve the monosaccharide carrier [23, 31], are not significantly altered in ghosts except for a 50% inhibition of arabinose movements in NH_4Cl ghosts. It is unlikely that a direct inhibitory effect of NH_4^+ ions accounts for this effect, since in native cells exposed to NH_4Cl (300 mM) fluxes are normal (data not shown).

The transport of divalent anions, like that of monovalent inorganic anions [11], is unaffected by the process of ghost preparation. Lactate fluxes, on the other hand, are increased in all preparations, although not generally at the level of significancy. Lactate transport via the PCMS-sensitive monocarboxylate carrier occurs by a lactate-H^+-cotransport or a lactate/OH^--exchange [9] and is strongly dependent on the gradients of lactate and H^+ over the membrane. It is not yet possible to decide whether changes of these gradients or of the intrinsic properties of the transport system are responsible for the effects.

In conclusion, the comparative analysis of transport functions in hypotonic and isotonic ghosts demonstrates that no major differences can be demonstrated between these two preparations with respect to their suitability for transport studies. The choice of one or the other preparation can thus be based on their differing advantages resulting from the mode of preparation.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160/C3). The authors are indebted to Prof. U. Zimmermann, Jülich, for his advice and support in setting up the equipment for the dielectric breakdown. The excellent help of Mrs. H. Thomas and F.-J. Kaiser in preparing the manuscript is gratefully acknowledged.

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Received 30 October 1980; revised 6 March 1981