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On the water and proton permeabilities across membranes from erythrocyte ghosts

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The diffusional permeability of water across membranes from bovine and human erythrocyte ghosts was measured by a recently developed method which is based on the different indices of refraction of H_2O and ^2H_2O. Resealed erythrocyte ghosts were prepared by a gel-filtration technique. P_d ($^2H_2O/H_2O$) values of $1.2 \cdot 10^{-3}$ cm/s (human) and $1.7 \cdot 10^{-3}$ cm/s (bovine) were calculated at 20°C. The activation energies of the water exchange were 23.5 kJ/mol (human) and 25.4 kJ/mol (bovine). Treatment of the ghosts with *p*-chloromercuribenzenesulfonic acid (PCMBS) led to a 60–70% inhibition of the diffusional water exchange. The pH equilibration across membranes of erythrocyte ghosts was measured by intracellular carboxyfluorescein. The rates of proton flux after pH-jumps (pH 7.3 to pH 6.1) were about 100-fold lower than those of the water exchange and dependent on the kind of anions present (Cl^-, NO_3^-, SO_4^{2-}). The activation energies of proton flux were 60–70 kJ/mol. 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) inhibited the exchange by 97–98% and lowered the activation energy. The inhibitor of water exchange, PCMBS, increased the proton-permeation rate by a factor of 4–5. It is assumed that the rate-limiting step for the proton permeation is determined by the anion exchange. Under this condition our results are not in accord with one channel as a common pathway for both the passive water and anion transport.

Introduction

From studies on lipid bilayers evidence is accumulating that the permeation rates for water and protons are comparable [1–3]. For the first time we have measured both permeation processes almost simultaneously [3]. The results may be summarized as follows: in the crystalline state of the phosphatidylcholine bilayer both permeation rates are very similar in magnitude. Both rates become faster and reflect the phase-transition profile with increasing temperature. Applying a sudden pH-change in the external medium, the proton permeation seems to proceed in two steps. A fast overshoot component, i.e. a proton transport without cotransport of the counter-ion, is followed by a slower counter-ion limited (electro-neutral) transport. The latter process seems to be anion-specific. The actual numbers for the passive proton permeation through lipid bilayers are still a matter of controversy and values between 10^{-3} and 10^{-9} cm/s are reported (Refs. 1, 2 and references therein). Taking the wide range of these values it may be argued that the higher ones refer to the overshoot component comparable in size to the water permeation, and that the lower values correspond to the counter-ion balanced transport.

In membranes from erythrocytes the band 3

Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid; PCMBA, *p*-chloromercuribenzoic acid.

protein has been considered as a major 'pore-forming' agent mediating the water transport [4,5] in addition to its anion-exchange role [6]. In molecular models the aqueous pore is considered to be located between the two monomers of the non-covalently bound dimeric form of the band 3 protein [7]. Studies on reconstituted band 3 protein in planary lipid bilayers show association equilibria and the presence of a tetramer which is thought to form the actual 'pore' [8]. The water transport through membranes of erythrocytes and erythrocyte ghosts has been studied in great detail under a variety of experimental conditions (Refs. 5, 7, 9–14; for review see Refs. 15, 16). In the same detail the anion exchange and the proton-coupled anion exchange have been investigated [17–25]. However, so far there exist no attempts to look at both the water and proton transport at the same time. On the basis of our newly developed techniques we were able to monitor both the water and the proton transport across membranes of erythrocyte ghosts in principle under identical conditions. We have measured the water diffusional permeation rates under isoosmolar conditions in transient experiments making use of optical differences of H_2O and ^2H_2O [26,27]. The proton permeation was recorded by intracellular pH-sensitive marker molecules following a rapid pH-change in the external medium [3]. In the available time-range (ms to h) only one anion-specific permeation rate was observed, in contrast to results from vesicular phosphatidylcholine bilayers. It turns out that both processes, i.e. the water and proton permeation, are inhibited by different compounds. These results seem to rule out models where the same parts of the band 3 proteins are forming a 'water-channel' and are involved in the proton-coupled anion exchange.

Materials and Preparation of the ghosts

Chemicals

Inorganic salts at the highest purity grade available and ^2H_2O were purchased from E. Merck, Darmstadt. DIDS, PCMBS and PCMBA were purchased from Sigma Chemicals Co., St. Louis, MO, and used without further purification. 5,6-Carboxyfluorescein from Eastman Kodak Comp., Rochester, NY, was recrystallized from ethanol/water. Sephacryl S-1000 Superfine and the ion-exchanger DEAE-Sephadex A-25 were products of Pharmacia Fine Chemicals, Uppsala Sweden.

Buffers

All buffers and stock solutions were prepared in deionized water, which was subsequently purified with a Milli-Q purification system (Millipore Corp. Bedford, MA). Deuterated buffers were prepared by lyophilization of the H_2O buffers and resolution in ^2H_2O.

Buffer A (5 mM K_2HPO_4/KH_2PO_4, pH 7.4) was used for hypotonic lysis and as eluent on Sephacryl S-1000 columns. Stock solutions, washing solutions and ghost suspensions were made of buffer B (5 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4). pH-jump experiments were performed by mixing equal amounts of ghost suspension in buffer B with acidified buffer B (around pH 3.0) to reach predetermined mixing pH values between pH 6.2 and 6.0. For anion-dependent experiments Cl^- was substituted by the anions in question (NO_3^-, SO_4^{2-}). The acidified buffers were adjusted with the respective acids.

The osmolarities of all buffers (^2H_2O and H_2O) used were checked by freezing point depression and were 305 ± 5 mosM (isotonic buffers) and 25 ± 5 mosM (hypotonic buffers), respectively.

Preparation of resealed erythrocyte ghosts

All preparation steps and the storage of erythrocyte ghosts were done at 6°C; measurements were performed within 24 h after preparation. Blood, anticoagulated with heparin, was stored up to 5 days.

Haemoglobin-free erythrocyte ghosts were prepared by a method based on hypotonic lysis [28,29] and separation of the red blood cell membranes from haemoglobin by gel filtration. About 15 ml lysate were applied to a 40×5 cm Sephacryl S-1000 column and eluted with buffer A. Flow rates of 50–100 ml/h were suitable for baseline-separation of completely white ghosts from the haemoglobin fraction. Small amounts of concentrated marker (usually CF at a final concentration of 0.1–1 mM) were added to the ghost suspension, isotonicity was restored and the pH of the suspension adjusted to 7.4. The ghosts were resealed by incubation of the suspension at 37°C

for 1 h. External carboxyfluorescein (di- to trianion in the pH-range studied) was removed by ion-exchange chromatography on a DEAE A-25 column or by two or three washing steps. The carboxyfluorescein efflux from erythrocyte ghosts had a half-time of about 2.5 h at 20°C and pH 7.4.

For inhibition studies PCMBS or DIDS were added to the unsealed or sealed ghost suspensions. At early stages of this work we also used PCMBA. Excess of the external inhibitors was removed by several washing steps with buffer B or by ion-exchange chromatography. Except for time-dependent studies the reaction time was 1 h at 37°C. The addition of PCMBS to the resealed ghosts did not cause any detectable changes of the turbidity, in contrast to experiments with intact erythrocytes.

Instruments

Stopped-flow measurements were performed with a thermostatted Sigma (Biochem/Sigma, Puchheim-München/Berlin) apparatus with a 1 : 1 mixing ratio and vertically arranged drive syringes. Instead of a stop-syringe the movement of the pistons is abruptly stopped. The system is almost pressureless except during the short acceleration periods of the pistons and has a mixing-time in the millisecond range. Either the transmitted light or the emission perpendicular to the incident monochromatic light beam were recorded and stored at 12-bit resolution. Fluorescence was discriminated from stray light by cut-off filters. The transmitted light was converted into turbidities, τ, according to $\tau \cdot l = \log(I_0/I_t)$ where l is the length of the observation chamber and I_0, I_t are the intensities of the transmitted light for the solvent and the erythrocyte-ghost-containing solutions, respectively. Minimal time resolution for the A/D conversion and data storage was 0.2 ms per data point.

Stationary fluorescence and absorbance were recorded with an American Instrument Corp. SPF 500 spectral fluorometer and with a UV 5260 Beckman Instruments spectrometer, respectively. Potassium concentrations were determined with an Eppendorf K 701-A flame photometer.

Temperatures were controlled with a Lauda RC 6 thermostat and measured either inside the cuvette or close to the observation chamber by a copper/constantan thermocouple connected to a digital thermometer (Newport Laboratories) with a resolution of 0.1 K.

Buffer osmolarities were determined using a Knauer cryoscopic unit.

Methods

The K^+/Na^+ exchange of the erythrocyte ghosts were measured by incubating the ghosts (cytocrit about 5%) in K^+ medium (buffer B, but NaCl replaced by KCl) at 37°C for 1 h, washing once with Na^+ medium and suspending the ghosts in Na^+ medium (buffer B) at room temperature. Aliquots were taken at variable times and centrifuged (15000 × g for 2 min). The concentration of K^+ in the supernatant was determined by flame-photometry, normalized to 100% (by membrane rupture through the addition of Triton X-100) and plotted in Fig. 1 as function of time. From the approach to the plateau value one calculates a relaxation rate for the K^+/Na^+ exchange of $8.6 \cdot 10^{-5}\ s^{-1}$. With a volume-to-surface ratio of $0.65 \cdot 10^{-4}$ cm this corresponds to a permeability coefficient of $5.6 \cdot 10^{-9}$ cm/s (bovine erythrocyte ghosts, 20°C), which is close to values for tightly sealed pink ghosts [30] by tracer efflux measurements.

The method for measuring the water transport in the form of the $H_2O/^2H_2O$ exchange rate has been described recently [26,27]. Erythrocyte ghosts prepared in H_2O buffer were rapidly mixed with the deuterated buffer while the intensity of the transmitted light at variable wavelength was recorded. The turbidity, τ, calculated from the stored data, has a time-dependent component due to the $H_2O/^2H_2O$ equilibration across the membrane and can be approximated by

$$\tau(t) = \tau_\infty - (\tau_\infty - \tau_0)\, e^{-k_{ex}t} \qquad (1)$$

τ_0, τ_∞ are the turbidities at time $t = 0$ (just after the short mixing period) and at $t = \infty$ when the intra- and extracellular isotopic composition have become equal. From the almost single-exponential approach of the turbidity to the new stationary state the exchange relaxation rate k_{ex} or the exchange relaxation time $\tau_{ex}(= 1/k_{ex})$ was obtained by an exponential-fitting procedure. Within the time τ_{ex} the difference in the isotopic composition between the extra- and intracellular medium is

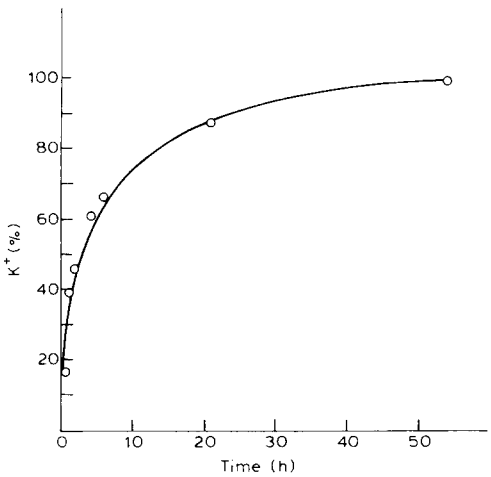

Fig. 1. Time course of the K^+/Na^+ exchange at room temperature from resealed bovine erythrocyte ghosts (cytocrit 5%). External K^+ concentration in % (normalized to the value at $t = \infty$) vs. time after transfering the K^+-loaded ghosts into a potassium-free medium. The calculated efflux rate is $(8.6 \pm 0.1) \cdot 10^{-5}\ s^{-1}$ ($P_d = (5.6 \pm 0.5) \cdot 10^{-9}$ cm/s).

reduced to 1/e of the initially induced value. At known volume-to-surface ratios (V/A), the diffusional permeability coefficient P_d is deduced according to $P_d = k_{ex}\ (V/A)$. The ratio of volume to surface used in the calculations for P_d values was assumed to be $0.65 \cdot 10^{-4}$ for human erythrocyte ghosts [30]. The same value was taken as an approximation for bovine erythrocyte ghosts (calculated from bovine erythrocyte data [31]). For small values of τI the intensity of the transmitted light can be fitted directly by a single-exponential function [32].

The proton permeation was monitored by pH-sensitive, intracellularly encapsulated probe molecules. With respect to the pH-dependent spectral responses and the low membrane permeability 5,6-carboxyfluorescein seemed most appropriate for these experiments. Carboxyfluorescein shows a pK around pH 6.4; this pK is associated with a change from an almost non-fluorescent to a highly fluorescent, deprotonated form of the molecule at higher pH. Fluorescence intensities I_f and proton concentrations are non-linearly related through sigmoid titration curves; therefore the fluorescence intensities had first to be converted into proton concentrations before the permeation rates were calculated. Following a pH-jump in the external medium the time-dependence of the fluorescence intensity I_f can be described [3] by

$$I_f = \frac{C_0 \cdot 10^{-pK} \cdot \Phi}{10^{-pK} + H_o - (H_o - H_i)e^{-k_{ex}t}} \qquad (2)$$

where Φ is the quantum yield of the fluorescent species (pH > pK), C_0 is the stoichiometric concentration of carboxyfluorescein, and H_o and H_i are the H^+ concentrations at $t = 0$ in the extracellular and intracellular medium, respectively. Due to the large excess of the external medium (usually cytocrit values of 1–2%), the low H_i^+ and carboxyfluorescein concentrations used and the pH jump into the acidic region, the pH of the external medium is supposed to stay constant during the proton-permeation periods. According to Eqn. 2 an exponential fitting of the $1/I_f$ values yielded the flux relaxation rate k_{ex} for the proton permeation.

Measurements in the absence of atmospheric CO_2 were performed by extensively bubbling N_2 through buffers and cell suspensions for at least 30 min (c.f. Ref. 33). The gas-tight syringes of the stopped-flow apparatus were flushed with N_2 and a continuous stream of N_2 was bubbled through the solutions during the measuring period. Moreover the influence of Cl^-/HCO_3^- exchange is considered to be small because of the buffer capacity of the buffering system present in external and internal solution.

Osmotic effects (shrinking or swelling) could be ruled out, because the osmolarity of all buffers used for $H_2O/^2H_2O$ exchange or proton flux measurements was adjusted to 305 ± 5 mosM. Thus, the diffusional permeability is measured in the case of the $H_2O/^2H_2O$ exchange.

Results

Resealed ghosts with a low K^+/Na^+-exchange rate have been prepared and used for studies of the water and proton permeation. The microscopic view revealed in the majority of cases intact, discoid ghosts. The completely white ghosts were separated from non-membraneous components on a Sephacryl S-1000 column. Stopped-flow methods at cytocrit values between 1 and 2% were used to measure both permeation rates across membranes

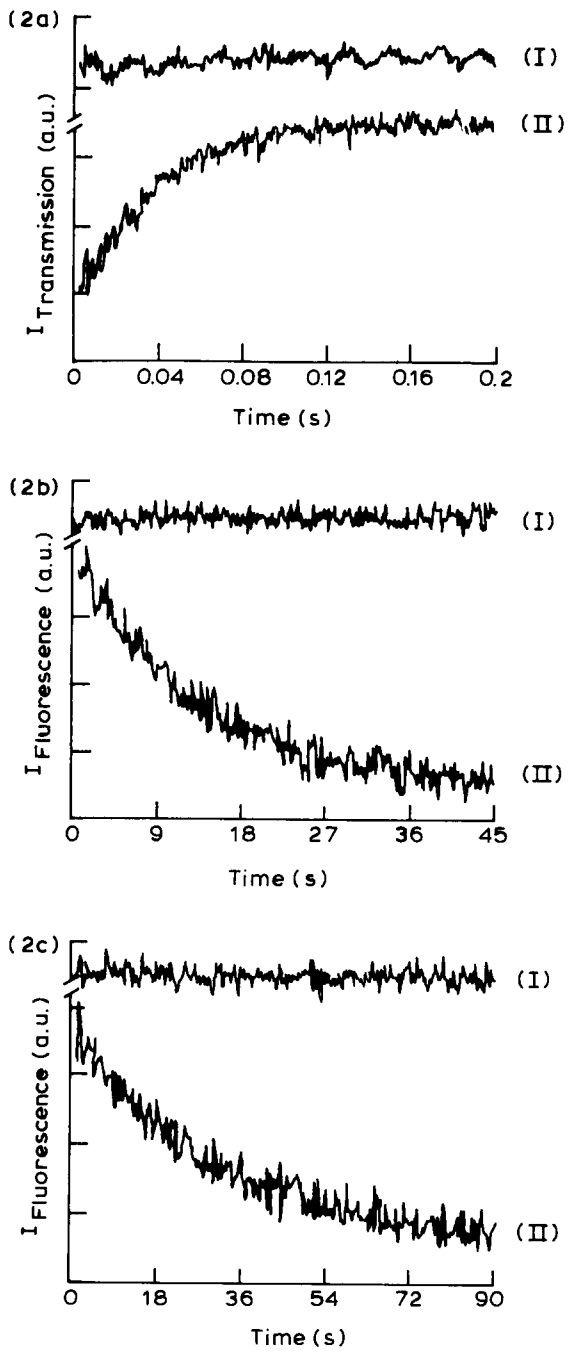

Fig. 2. Typical results from stopped-flow measurements on the $H_2O/^2H_2O$ exchange and the proton flux at 20°C. The time course (after the short mixing period) of the turbidity (A at 530 nm) or fluorescence intensity (I_f) of entrapped carboxyfluorescein (excitation at 489 nm, 495 nm cut-off filter at right angles to incident light) is shown. (a) $H_2O/^2H_2O$ exchange by the isotopic effect of light scattering. Turbidity (A at 530 nm) vs. time after the 1:1 mixing of the ghost suspension in buffer B (cytocrit 1–2%) with buffer B (I) (reference) and with deuterated buffer (II). $k_{ex} = 26.5 \pm 1.6$ s^{-1} for $H_2O/^2H_2O$ exchange determined by mono-exponential fitting. (b) Proton flux. Time-dependent decay of fluorescence intensity, I_f, of internally entrapped carboxyfluorescein (0.1 mM) after mixing the erythrocyte ghosts in buffer B with buffer B (I) and with acidified buffer B (II) to perform a pH-jump from pH 7.3 to 6.4. $k_{ex} = 0.049 \pm 0.006$ s^{-1} by mono-exponential fitting of $1/I_f$. (c) same as (b) except ghosts in SO_4^{2-}-containing medium. $k_{ex} = 0.016 \pm 0.003$ s^{-1}. The ratio $k_{ex}(Cl^-)/k_{ex}(SO_4^{2-})$ at physiological temperature (37.0°C) was 4–5.

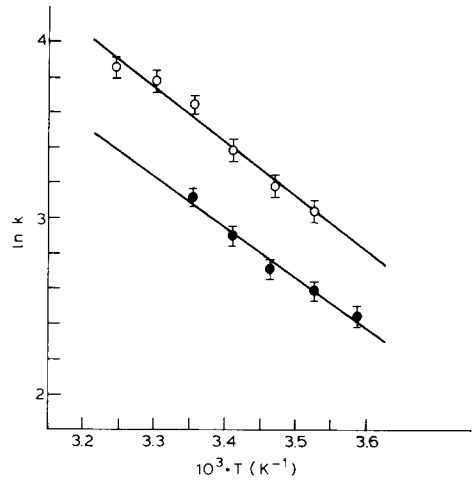

Fig. 3. Temperature dependence of the $H_2O/^2H_2O$ exchange across membranes of human (●) and bovine (○) erythrocyte ghosts in an Arrhenius-type plot. ln k_{ex} vs. $1/T$. Measurements were performed with ghost suspensions in buffer B (Cl^- as penetrating anion, cytocrit 2%, A at $\lambda = 530$ nm). Activation energies of 23.5 kJ/mol (human) and 25.4 kJ/mol (bovine) were calculated.

of the same ghost preparation by methods previously described [3,26,27,32]. Measurements of pH equilibration across membranes of red cell ghosts in N_2-degassed and non-degassed, buffered solutions led to identical k_{ex} values. The contribution of Cl^-/HCO_3^- exchange, if present, would furthermore be compensated by the buffering system used. According to Jennings [33] the measured H^+ transport therefore refers to the CO_2-independent pH equilibration.

Fig. 2 summarizes results from typical experiments on both the water exchange and the proton net flux. In principle both types of experiments can be performed at the same time and under identical conditions. However, a sequential order of experiments using the same ghost preparation

was preferred. The water transport was measured by the $H_2O/^2H_2O$ exchange at constant pH and the H^+ transport by the pH equilibration in H_2O buffers. Unless otherwise mentioned the ghost suspensions used had a cytocrit of 1–2%. The exchange curves for the water exchange, τ, and for the proton-flux, $1/I_f$, showed single-exponential behaviour. We always found $k_{ex}(H_2O/^2H_2O) > k_{ex}(H^+)$. In addition the $k_{ex}(H^+)$ values were a function of the anions present.

Water permeation

The results from temperature-dependent measurements for ghosts from human and bovine erythrocytes are plotted in Fig. 3 in an Arrhenius-type presentation. In the temperature range studied (5–35°C) no deviation from linearity was observed. Energies of activation for the water transport across membranes of human and bovine erythrocyte ghosts of 23.5 and 25.4 kJ/mol were calculated, respectively. The permeation is faster through membranes of bovine than through membranes of human erythrocyte ghosts.

The water transport could be inhibited to 30–40% of the value determined with control cells by 1–1.4 mM concentrations of the mercurial PCMBS (Fig. 4). In contrast to the mercurials, first results demonstrated that the reaction with the stilbene-derivative DIDS led to an acceleration of the water-permeation rate. The principal influence of both compounds was not affected by the time of administration during the ghost-preparation, i.e. the addition of inhibitors to sealed or unsealed ghosts showed qualitatively the same results. The inhibition experiments were usually performed 1 h after labelling at 37°C. The reaction half-time of PCMBS, indicated by the water-transport inhibition, is of the order of 20 min at 22°C [34].

Proton permeation

The permeation of protons across membranes of erythrocyte ghosts is slower than the water permeation by at least a factor of 100, and the proton permeation rate depends on the anion used (Fig. 2). Furthermore, the rate of proton permeation was a function of the magnitude of the applied pH-jump (ΔpH). For small steps from an initial pH of 7.3 k_{ex} was almost a linear function of the applied ΔpH. At larger pH-jumps a sigmoidal increase in k_{ex} values was observed, with an inflection point around pH 4.4. Our experiments were usually performed by pH-jumps from pH 7.3 ± 0.1 to pH 6.1 ± 0.1, i.e. in the linear

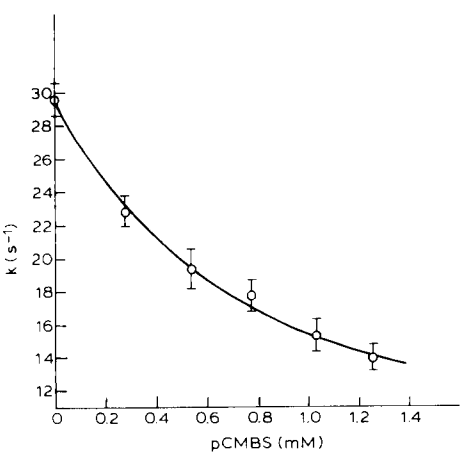

Fig. 4. Inhibition of $H_2O/^2H_2O$ exchange by the organomercurial SH-reagent PCMBS; the water-exchange relaxation values k_{ex} vs. concentration of PCMBS. Bovine erythrocyte ghosts were incubated 1 h at 37°C at different PCMBS concentrations before the measurements at 15°C were performed.

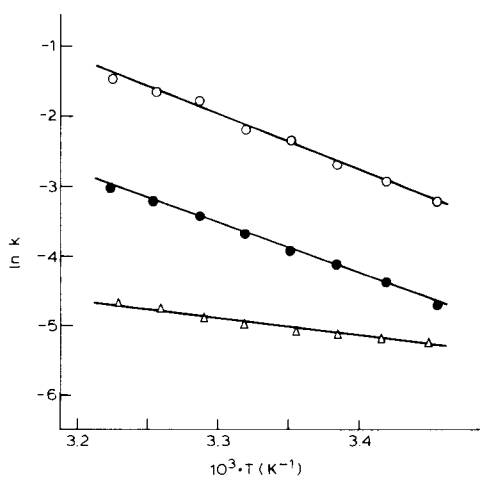

Fig. 5. Arrhenius-type presentation for the proton permeation rates across membranes of bovine erythrocyte ghosts in buffer B (Cl^-, ○), (SO_4^{2-}, ●) and in buffer B (Cl^-) treated with 20 μm DIDS (△). ln k_{ex} vs. $1/T$. In the external media pH-jumps from pH 7.3 to 6.1 were performed. Energies of activation are 64.5 kJ/mol (Cl^-/untreated), 65.5 kJ/mol (SO_4^{2-}) and 25.4 kJ/mol (Cl^-/DIDS-treated), respectively.

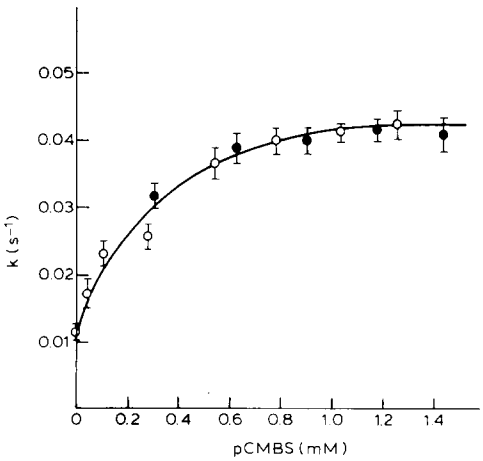

Fig. 6. The effect of the organomercurial PCMBS on the relaxation rates of the proton flux across membranes of bovine erythrocyte ghosts as function of the PCMBS concentration at 20°C. k_{ex} vs. [PCMBS]. pH-jumps were from 7.4 to 6.4. PCMBS treatment before (○) and after (●) resealing of the ghosts.

response range where the influence of the applied ΔpH on k_{ex} is small.

Similarly to the case with water permeation, the Arrhenius plots could be fitted by straight lines for ghosts from both human and bovine erythrocytes. Though the permeation rates decreased by going from Cl^- to NO_3^- and SO_4^{2-} (permeable species presumably HSO_4^- [24]) the activation energies for the proton transport were not influenced by the kind of anion and averaged 63.6 kJ/mol in the pH-region between 7.4 and 6.0 (Fig. 5).

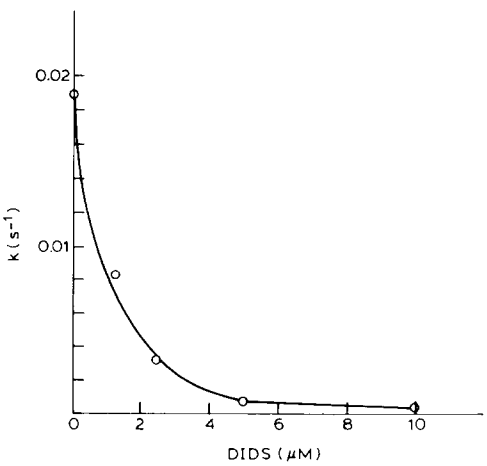

Fig. 7. Inhibition of the proton flux across the membranes of bovine erythrocyte ghosts by the stilbene-derivative DIDS. Relaxation rates k_{ex} at 20°C for pH-jumps from pH 7.3 to 6.2 as function of the DIDS concentration. DIDS was added to the resealed ghosts; the reaction time was 1 h at 37°C.

PCMBS (with lower efficiency also PCMBA) and DIDS showed opposite effects on the proton and water permeation. The addition of PCMBS increased the permeation rates for protons by a factor of 3–4 (Fig. 6) at concentrations where the water exchange was reduced to 30–40% of the original value. At concentrations above 5 μM, DIDS led to a dramatic decrease in the H^+-permeation rate to 2–3% of the control value (Fig. 7). DIDS not only lowered the actual exchange rates but also lowered the activation energy from 63.6 to 27.6 kJ/mol (Fig. 5).

Discussion

Water permeation

The measured diffusional permeability coefficient includes contributions from the true permeation across the membrane and from the diffusion through unstirred layers (for a recent report see Ref. 35). The thickness of the unstirred layer under comparable conditions is 1–2 μm [36]. The contribution of unstirred-layer effects should therefore be negligible for the data presented. The supposition that we are looking at the true membraneous barrier is further supported by the inhibition studies. The inhibition of P_d to 30–40% of its original value with PCMBS concentrations in the mM range argues in favour of the membrane barrier being the rate-limiting step. This barrier is slightly lowered or at least not increased by the addition of the stilbene-derivative, DIDS, at μmolar concentrations where the proton transport is strongly inhibited. As the turbidity of the ghost solution is not influenced by the addition of PCMBS, large volume changes as the origin of the observed reduction of the exchange relaxation rates can be ruled out.

Our P_d data, which are a mean for the permeating species H_2O, H^2HO and ^2H_2O, are at the lower limit of values recently summarized [11]. For the ^2H_2O transport Parpart had already in 1935 [37] suggested from haemolysis experiments that ^2H_2O penetrates erythrocyte membranes 44% more slowly than does normal water. Thus our

$^2H_2O/H_2O$ permeation should be smaller by a factor of 0.78 than the permeation of pure H_2O. Calculated $^2H_2O/H_2O$ permeability coefficients at 20°C with a V/A ratio of $0.65 \cdot 10^{-4}$ cm [30] are $1.7 \cdot 10^{-3}$ cm/s and $1.2 \cdot 10^{-3}$ cm/s for bovine and human erythrocyte ghosts, respectively. In agreement with Ashley and Goldstein [38], we did not observe breaks in the Arrhenius plots for ghosts from human and bovine erythrocytes, in contrast to results from other NMR studies [39,40]. The permeation rate through membranes from bovine erythrocyte ghosts is faster and shows a higher activation energy compared with the equivalent data from human erythrocyte ghosts. The activation energy E_a for the water permeation across membranes from human erythrocyte ghosts of 23.5 kJ/mol is well within the range of values reported for erythrocytes and erythrocyte ghosts (20–30 kJ/mol [9,38,41,42]) and differs from the energy of activation for the hydraulic water permeation (13.8 kJ/mol [41]).

The transport of water could be inhibited by PCMBS and first results revealed an increase by the addition of DIDS. At saturating concentrations of PCMBS the inhibition of the water transport was about 60–70% and thus similar to Brahm's value [9] and with a half-maximal inhibition around 0.5 mM, also in agreement with a value of 0.35 mM reported by Dix and Solomon [11].

Proton permeation

Band 3 catalyzes the anion exchange across membranes from erythrocytes and is involved in the H^+/Cl^- cotransport of the CO_2-independent pH equilibration [33]. Various models of the translocation mechanism have recently been reviewed in connection with Cl-NMR experiments. These data support the alternating-site model for the Cl^- transport [43]. Extensive studies with SH- and NH-blocking agents located the binding sites of the anion- and water-transport inhibitors DIDS and PCMBS at the intramembraneous regions of the band 3 protein. These results served to formulate a model where anions and water share the same channel formed by the band 3 dimer [7].

If one assumes that the proton-translocation data presented above are characterized by an anion cotransport as the rate-limiting step then our inhibition experiments on the passive water and proton transport are difficult to rationalize on the basis of one common channel. The above assumptions on the H^+/anion cotransport are in accord with Jennings's results on the pH equilibration in the absence of bicarbonate [33]. Above 13°C Jennings calculates an activation energy of 67 kJ/mol, which favourably corresponds with our data of 64–66 kJ/mol. We cannot discriminate between transport of protons in one direction or transport of hydroxyl ions in the opposite direction. However, it can be argued from the different magnitudes of the water and proton permeabilities that our proton translocation data refer to the counter-ion-balanced cotransport of protons and anions. This process leads to an equalization of both concentrations across the membrane. In addition, the dependence of the proton permeability on the kind of anion is in favour of a proton transport with anion-balanced cotransport. The interpretation of the anion-limited or balanced proton transport is further supported by the generally accepted observations that the mercurials inhibit the water transport [44] and that bifunctional stilbene-derivatives such as DIDS reduce the anion-exchange rates (e.g. Ref. 18). These observations are confirmed by our data where PCMBS leads to a 60–70% inhibition of the water transport and DIDS inhibits the proton translocation. So far these results would be in agreement with a common-channel model for both permeation pathways. However, our experiments further revealed that at concentrations where PCMBS reduces the water permeation by 60–70% the proton permeation is accelerated, and that DIDS, which blocks the proton-permeation rate to a residual value of 2% at μmolar concentrations, seems to increase the water-transport rate.

If the passive proton translocation reflects the anion transport then these mutually excluding results are rather difficult to envisage as long as both processes use the same principal transport system. It seems that the action of one inhibitor specifically closes one 'channel' and opens a leak at the same time. This induced leak might be connected with other proteins, especially as PCMBS does not exclusively react with the band 3 protein [7]. On the other hand, the leaks might also be attributed to defects induced in the lipid matrix.

The observed reduction of the activation energy

from 60–70 to about 28 kJ/mol for the proton permeation as result of the DIDS binding in the μmol range indicates that the proton/anion exchange proceeds via different mechanisms in the free and DIDS-inhibited state. It is further known that the binding of PCMBS leads to an increase in the energy of activation from 30 to 60 kJ/mol for the water permeation of ghosts treated with 1 mM PCMBS [9]. The action of PCMBS also increases the cation-permeability [45] in vesicles from red cell membranes. The activation energy for the PCMBS inhibition approaches values for the water transport through phosphatidylcholine bilayers containing high amounts (20 mol%) of cholesterol (90 kJ/mol [46]). This holds especially as at 1 mM PCMBS the saturating concentration is not reached. Effects of the cholesterol enrichment on the hydraulic water permeation have been described [47].

The steady-state fluorescence anisotropy, r, of membrane-embedded diphenylhexatrien molecules is a measure of the membrane molecular order. Our r value of 0.24 [26] at room temperature for membranes from erythrocyte ghosts corresponds in phosphatidylcholine bilayers to the 'intermediate-fluid' range between the crystalline and liquid-crystalline states [48]. It is conceivable that this 'intermediate-fluid' state of the lipid bilayer might be easily shifted to one or the other extreme so that induced defects, domains or fluctuations within the lipid bilayer part might contribute to the action of the inhibitors. In this respect it was shown that defects [49] and fluctuations [50] play a fundamental role in the permeation barriers of lipid membranes. At the present time it is too early to decide between the two possibilities. However, if our interpretation of the anion-balanced passive proton permeation can further be confirmed then it seems from our results that the common single-channel model has at least to be modified.

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References

1 Deamer, D.W. and Nichols, J.W. (1983) Proc. Natl. Acad. Sci. USA 80, 165–168
2 Elamrani, K. and Blume, A. (1983) Biochim. Biophys. Acta 727, 22–30
3 Pitterich, H., Dowidat, R., Engelbert, H.-P. and Lawaczeck, R. (1985) Proc. 3rd. Int. Conf. on Water and Ions in Biological Systems, Bucharest 1984 (Vasilescu, V. and Pullman, B., eds.), in the press
4 Brown, P.A., Feinstein, M.B. and Sha'afi, R.I. (1975) Nature 254, 523–525
5 Yoon, S.C., Toon, M.R. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 385–389
6 Jennings, M.L. (1984) J. Membrane Biol. 80, 105–117
7 Solomon, A.K., Chasan, B., Dix, J.A., Lukacovic, M.F., Toon, M.R. and Verkman, A.S. (1983) Ann. N.Y. Acad. Sci. 414, 97–124
8 Benz, R., Tosteson, M.T. and Schubert, D. (1984) Biochim. Biophys. Acta 775, 347–355
9 Brahm, J. (1982) J. Gen. Physiol. 79, 791–819
10 Osberghaus, U., Schönert, H. and Deuticke, B. (1982) J. Membrane Biol. 68, 29–35
11 Dix, J.A. and Solomon, A.K. (1984) Biochim. Biophys. Acta 773, 219–230
12 Chasan, B., Lukacovic, M.F., Toon, M.R. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 185–190
13 Lukacovic, M.F., Verkman, A.S., Dix, J.A. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 253–259
14 Craescu, C., Cassoly, R., Galacteros, F. and Prehu, C. (1985) Biochim. Biophys. Acta 812, 811–815
15 Macey, R.J. (1981) in Membrane Transport in Biology (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), pp. 1–57, Springer-Verlag, Berlin
16 Sha'afi, R.I. (1981) in Membrane Transport (Bonting, S.L. and dePont, J.J.H, eds.), pp. 29–60, Elsevier/North-Holland, Amsterdam
17 Jennings, M.L. (1976) J. Membrane Biol. 28, 187–205
18 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
19 Wieth, J.O., Brahm, J. and Funder, J. (1980) Ann. N.Y. Acad. Sci. 341, 394–418
20 Jennings, M.L. and Adams, M.F. (1981) Biochemistry 20, 7118–7123
21 Köhne, W., Deuticke, B. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 730, 139–150
22 Boron, W.F. (1983) J. Membrane Biol. 72, 1–16
23 Müller, B. and Glaser, R. (1984) Biomed. Biochim. Acta 43, 77–83
24 Passow, H., Berghout, A. and Romano, L. (1984) Information and Energy Transduction in Biological Membranes, pp. 95–102, A.R. Liss, Inc., New York
25 Peters, R. and Passow, H. (1984) Biochim. Biophys. Acta 777, 334–338
26 Lawaczeck, R. (1984) Biophys. J. 45, 491–494

27 Engelbert, H.-P. and Lawaczek, R. (1985) Ber. Bunsenges. phys. Chem. 89, 754–759
28 Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197–218
29 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31A, 172–180
30 Sze, H. and Solomon, A.K. (1979) Biochim. Biophys. Acta 550, 393–406
31 Rich, G.T., Sha'afi, R.I., Barton, T.C. and Solomon, A.K. (1967) J. Gen. Physiol. 50, 2391–2405
32 Engelbert, H.-P. and Lawaczeck, R. (1985) in Water and Ions in Biological Systems (A. Pullman et al., eds.), pp. 665–670, Plenum Publishing Corp., New York
33 Jennings, M.L. (1978) J. Membrane Biol. 40, 365–391
34 Ashley, D.L. and Goldstein, J.H. (1981) J. Membrane Biol. 61, 199–207
35 Mierle, G. (1985) Biochim. Biophys. Acta 812, 835–839
36 Brahm, J. (1983) J. Gen. Physiol. 81, 283–304
37 Parpart, A.K. (1935) J. Cell. Comp. Physiol. 7, 153–162
38 Ashley, D.L. and Goldstein, J.H. (1980) Biochem. Biophys. Res. Commun. 97, 114–120
39 Conlon, T. and Outhred, R. (1978) Biochim. Biophys. Acta 511, 408–418
40 Morariu, V.V., Pop, V.I., Popescu, O. and Benga, G. (1981) J. Membrane Biol. 62, 1–5
41 Vieira, F.L., Sha'afi, R.I. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 451–466
42 Pirkle, J.L., Ashley, D.L. and Goldstein, J.H. (1979) Biophys. J. 25, 389–406
43 Falke, J.J., Pace, R.J. and Chan, S.I. (1984) J. Biol. Chem. 259, 6481–6491
44 Benga, G., Pop, V.I., Popescu, O., Ionescu, M. and Mihele, V. (1983) J. Membrane Biol. 76, 129–137
45 Grinstein, S. and Rothstein, A. (1978) Biochim. Biophys., Acta 508, 236–245
46 Lawaczeck, R. (1979) J. Membrane Biol. 51, 229–261
47 Kutchai, H., Cooper, R.A. and Forster, R.E. (1980) Biochim. Biophys. Acta 600, 542–552
48 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393–1401
49 Lawaczeck, R., Kainosho, M., Girardet, J.-L., Chan, S.I. (1975) Nature 256, 584–586
50 Kaufmann, K. and Silman, I. (1983) Biophys. Chem. 18, 89–99