Biochimica et Biophysica Acta, 553 (1979) 132—141

BBA 78349

A COMPARISON OF THE INHIBITORY POTENCY OF REVERSIBLY ACTING INHIBITORS OF ANION TRANSPORT ON CHLORIDE AND SULFATE MOVEMENTS ACROSS THE HUMAN RED CELL MEMBRANE

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(Received August 22nd, 1978)

Key words: Anion transport; Transport inhibition; Cl^- transport; Sulfate transport; Band 3 protein; (Human red cell)

Summary

The effects of a variety of chemically diverse, reversibly acting inhibitors have been measured on both Cl^- and SO_4^{2-} equilibrium exchange across the human red cell membrane. The measurements were carried out under the same conditions (pH 6.3, 8°C) and in the same medium for both the Cl^- and SO_4^{2-} tracer fluxes. Under these conditions the rate constant for Cl^--Cl^- exchange is about 20 000 times larger than that for SO_4^{2-}-SO_4^{2-} exchange. Despite this large difference in the rates of transport of the two anions, eight different reversibly acting inhibitors have virtually the same effect on the Cl^- and SO_4^{2-} transport. The proteolytic enzyme papain also has the same inhibitory effect on both the Cl^- and SO_4^{2-} self-exchange. In addition, the slowly penetrating disulfonate 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic acid (APMB) is 5-fold more effective from the outer than from the inner membrane surface in inhibiting both Cl^- and SO_4^{2-} self-exchange. We interpret these results as evidence that the rapidly penetrating monovalent anion Cl^- and the slowly penetrating divalent anion SO_4^{2-} are transported by the same system.

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Abbreviations: ANS, 1-analino-8-naphthalene sulfonic acid; H_2DIDS, 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid; APMB, 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic acid; DAS, 4,4′-diacetamido stilbene-2,2′-disulfonic acid; Hoechst B, 4-chloro-*N*(2-thiofurymethyl)-5-sulfamoylanthranilic acid.

Introduction

In the human red blood cell membrane, for certain organic anion species such as lactate, there exists a specific transport system that is distinct from the transport system for inorganic anions [1,2]. It is generally assumed that all inorganic anion species are transported by one and the same transport protein, but it is also generally recognized that strict proof of this assumption is lacking.

The transport properties of all inorganic anions thus far tested share some common features: saturation, with similar half-maximal concentrations [3,4], mutual competition among different anions [4—6], and an apparent activation enthalpy of about 30 kcal/mol ($T < 15°C$) [7,8]. However, maximal transport rates of different inorganic anions vary enormously, e.g. by a factor of $\approx 10^4$ for Cl^- and SO_4^{2-} [4]. Also the pH dependences of the self-exchange of monovalent and divalent anions are quite different [3,8], and the temperature dependence of Cl^- self-exchange is much lower than that of SO_4^{2-} at $T > 15°C$ [9]. The differences of the pH dependences have been explained by the hypothesis that a change of pH may convert the binding site in a single species of anion carrier from a binding site for a monovalent anion into a binding site for a divalent anion [6,10], and the difference of temperature dependence at temperatures above 15°C can possibly be related to different rate-limiting steps with the same transport mechanism for the two ion species mentioned. However, as yet there is no explanation of the enormous differences in the V values.

In order to try to determine whether the transport system for SO_4^{2-} is distinct from that for Cl^-, we have tested the effect of a variety of reversibly acting inhibitors on Cl^- self-exchange and SO_4^{2-} self-exchange, measured under identical conditions. This has not been done in the past, because the rapid Cl^--Cl^- exchange is usually measured at 0°C, and the slow SO_4^{2-}-SO_4^{2-} exchange at a much higher temperature. For example, Fortes and Hoffman [11] found that 1-anilino-8-naphthalene sulfonic acid (ANS) is apparently a more potent inhibitor of Cl^- (0°C) than of SO_4^{2-} (37°C) transport in red cells, but these authors pointed out that because of the temperature difference, the measurements are not comparable. At pH 6.3 and 8°C, we were able to measure the self-exchange rates of Cl^- and SO_4^{2-} in the same medium. We have found that a large number of chemically unrelated inhibitors, plus the proteolytic enzyme papain, have virtually the same effect on both the Cl^- and the SO_4^{2-} transport. Thus, we find no evidence for the existence of two distinct transport systems.

Materials and Methods

Human blood (Type O, Rh^+) from apparently healthy donors was obtained from the Red Cross and stored in acid-citrate-dextrose for not more than 5 days. The ionic composition of the 'standard medium' used was 122.5 mM NaCl, 5 mM Na_2SO_4, 10 mM sodium phosphate, pH 6.3, measured at 8°C *.

* There are several obvious possibilities for the choice of the composition of the media in which SO_4^{2-} and Cl^- fluxes could be measured: all Cl^- media (containing a trace of SO_4^{2-}), all SO_4^{2-} media (containing a trace of Cl^-), or media containing Cl^- and SO_4^{2-} at the ratio of their K_m values, as derived from transport studies. Unfortunately, we were unable to think of a really convincing criterion for the choice of one or the other of the media. We stuck, therefore, to the standard medium that is usually employed in the studies of anion transport in this laboratory. We felt that only the result of the measurements could justify this choice.

Blood was first titrated with 1 M HCl to an extracellular pH of 6.3, washed three times in standard medium, and then split into two parts for loading with $^{35}SO_4^{2-}$ and $^{36}Cl^-$. For the $^{35}SO_4^{2-}$ efflux experiments, a 50% hematocrit suspension in standard medium, containing 1—2 μCi $^{35}SO_4^{2-}$/ml, was incubated 1 h at 37° C; the cells were washed twice at 0° C and resuspended at 8° C in standard medium at a 1% hematocrit. Aliquots were removed at 1-h intervals, centrifuged, and 0.5 ml of supernatant was counted with 0.5 ml 21% trichloroacetic acid in Instagel (Packard Instrument GmbH, Frankfurt am Main, F.R.G.). The radioactivity at infinite time was determined by mixing equal volumes of suspension and 21% trichloroacetic acid, centrifuging and counting 1 ml of supernatant.

An inhibitor stop method, using 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid (H_2DIDS), was developed for the measurement of the rapid Cl^--Cl^- exchange. For the $^{36}Cl^-$ loading, a 50% cell suspension in standard medium, containing 1.25 μCi $^{36}Cl^-$/ml, was incubated for 10 min at 8° C and centrifuged at 5000 rev./min for 15 min. The $^{36}Cl^-$ efflux was initiated by resuspending 0.4 ml of packed cells in 40 ml of rapidly stirred standard medium, 8.0° C. Subsequently 0.7-ml samples were taken at 2-s intervals with a repeating syringe (SMI, Emeryville, CA or Henke-Sass, Wolf GmbH, Tuttlingen, F.R.G.) and plunged into 0.5 ml of ice-cold standard medium, containing 25—50 μM H_2DIDS (Fig. 1). The H_2DIDS inhibits the $^{36}Cl^-$ efflux by at least 99%, and the transport is effectively stopped immediately upon mixing with the H_2DIDS solution. The first data point was obtained 3 s after the resuspension of the cells, following three rapid rinse strokes which were necessary to rid the syringe of air bubbles. The timing of the sampling was provided by a metronome, operating at 1-s intervals. After six samples were collected, the samples were centrifuged 30 s and 0.5 ml of supernatant counted, with 0.5 ml 21% trichloroacetic acid. The infinite time points were taken in the same manner, except into 21% trichloroacetic acid, instead of stop solution.

The inhibitors were obtained from the following sources and were used without further purification. 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′-7-disulfonic acid (APMB), gift from Prof. Petersen, Bayer AG, Leverkusen, F.R.G.; phlorizin, Roth KG, Karlsruhe, F.R.G.; 4,4′-diacetamido stilbene-2,2′-disulfonic acid (DAS), synthesized by Prof. H. Fasold as described previously [12]; sodium tetrathionate, E. Merck AG, Darmstadt, F.R.G; 1-anilino-8-naphthalene sulfonic acid (ANS) K and K Laboratories, Plainview, NY; 4-chloro-*N*(2-thiofurylmethyl)-5-sulfamoylanthranilic acid (Hoechst B), gift from Hoechst AG, Frankfurt am Main, F.R.G.; 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid (H_2DIDS), synthesized by Prof. H. Fasold as described previously [12]; persantin, gift from Prof. B. Deuticke, Rheinisch-Westfälische Technische Hochschule, Aachen, F.R.G.; 2,4-dinitrophenol, Serva Feinbiochemica GmbH, Heidelberg, F.R.G.; salicylate, E. Merck AG, Darmstadt, F.R.G.; papain, Boehringer Mannheim GmbH, Mannheim, F.R.G.

The $^{35}SO_4^{2-}$ efflux was measured without preincubation of the cells with the various inhibitors. Since in all cases the $^{35}SO_4^{2-}$ efflux followed a single exponential, the inhibitors apparently exert their full effect quite rapidly compared with the very slow time scale of the SO_4^{2-} efflux. For the $^{36}Cl^-$ efflux, the non- or very slow penetrating inhibitors phlorizin,APMB, DAS and tetrathionate,

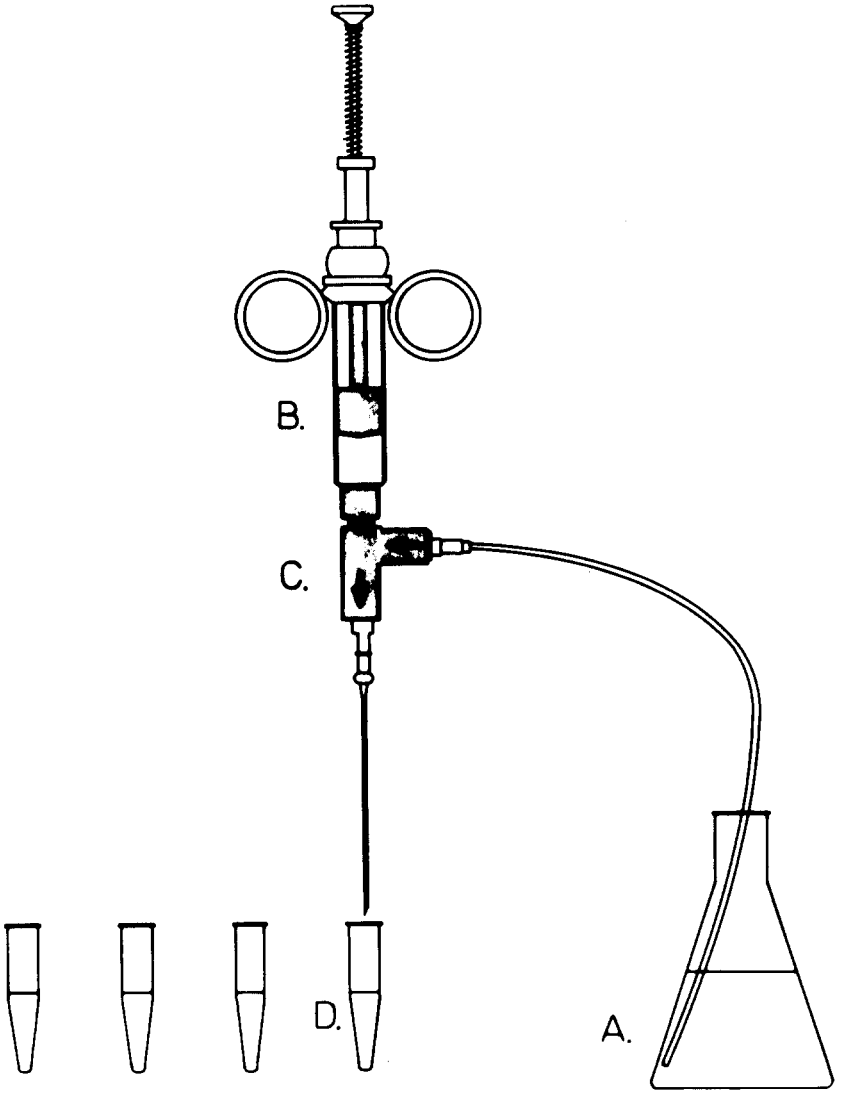

Fig. 1. Schematic diagram of inhibitor stop method used for measuring the $^{36}Cl^-$ efflux rate. Samples (0.7 ml) of the flux suspension (A) are taken by means of the self-loading syringe (B). The valve assembly (C) allows flow only in the directions indicated by the arrows, so that on the upstroke, the syringe is filled with flux suspension, and on the downstroke, the sample is ejected into the ice-cold, H_2DIDS-containing stop solution (D). Temperature of the flux suspension is 8.0° C. Ambient temperature is 6—7° C.

and the rapidly penetrating 2,4-dinitrophenol were not pre-equilibrated with the cells. The ANS, persantin, Hoechst B and salicylate were pre-equilibrated with the cells before and during the $^{36}Cl^-$ loading.

The papain treatment of the cells was carried out for 1 h at 37°C at a hematocrit of 50% in standard medium containing 0.1, 0.2 and 0.4 mg papain/ml. Before use, the papain had been dialyzed overnight against standard medium. It was activated with 0.2 mM cysteine and was inactivated at the end of the treatment by the addition of 1.0 mM iodoacetic acid and further incuba-

tion for 10 min at 37° C. The cells were then washed three times and treated as in the other experiments, except that the $^{35}SO_4^{2-}$ efflux was measured at 37° C, rather than at 8° C. The observed inhibition of SO_4^{2-} flux should, nevertheless, be comparable to the inhibition of Cl^- transport as measured at 8° C since the effect of the enzyme is irreversible and, at sufficiently high concentrations, leads to nearly complete inhibition of transport [20]. This indicates that each transporting unit is nearly completely inactivated and suggest that at partial inhibition of transport, the enzymatically modified transporting units do not contribute significantly to the observed transport rate.

The ghosts used in the APMB experiments were prepared essentially as described by Bodemann and Passow [13] by hemolysis at 0° C in 4 mM $MgSO_4$, 1.3 mM acetic acid, plus varying concentrations of APMB. After adjustment of the pH to 6.3, and adding NaCl, sodium phosphate and Na_2SO_4 to obtain the concentrations of standard medium, the ghosts were incubated 45 min at 37° C for resealing. Leaky ghosts were not separated from sealed ghosts. After resealing, fluxes were measured in the ghosts exactly as for intact red cells, except that the $^{36}Cl^-$-loaded ghosts were pelleted at 20 000 rev./min instead of at 5000 rev./min. The $^{35}SO_4^{2-}$ efflux from ghosts, at 8° C, was only about 85% inhibitable by H_2DIDS; the data in Fig. 4 refer to the percent inhibition by APMB of the H_2DIDS-sensitive SO_4^{2-} efflux.

Results

Fig. 2 shows the results of a typical experiment. Both halves of the figure are semi-log plots of the fractional completion of the tracer efflux vs. time. In both cases cells are at Donnan equilibrium with the same medium, containing various concentrations of ANS. The tracer SO_4^{2-} efflux in the absence of inhibitors has a half-time of 29 h; for Cl^-, the half-time is 4—5 s, or 20 000 times shorter than that for SO_4^{2-}.

SO_4^{2-} and Cl^- transport are over 99% inhibitable by H_2DIDS. This indicates that both anions are transported almost entirely via a specific pathway. This is also true when the transport is measured in the presence of the largest ANS concentration used; therefore ANS (like all the other inhibitors used in the present work) does not introduce a non-specific leak for SO_4^{2-} or Cl^-. For the $^{36}Cl^-$ efflux a noteworthy aspect of the figure is that the data in all experiments extrapolate to about the same point at $t = 0$. This indicates that the inhibitor stop method (Materials and Methods) does indeed stop the $^{36}Cl^-$ efflux, and that the inhibition of the transport by H_2DIDS is complete in less than 1 s.

It is clear from Fig. 2 that despite the large differences in transport rate, ANS has about the same effect on the transport of both Cl^- and SO_4^{2-}. Further comparisons are made in Fig. 3 which is a plot of fractional rate of $^{35}SO_4^{2-}$ efflux (compared with uninhibited control) vs. that for $^{36}Cl^-$, in the presence of various concentrations of different inhibitors. The inhibitors are listed on the right. Some such as APMB, DAS, and tetrathionate, have two negative charges; ANS is a monosulfate; persantin is a symmetrical molecule which is either neutral or positively charged at pH 6.3; phlorizin is a glycoside which is predominantly neutral at this pH; salicylate and 2,4-dinitrophenol have a single negative charge. All of these chemically dissimilar molecules have virtually the

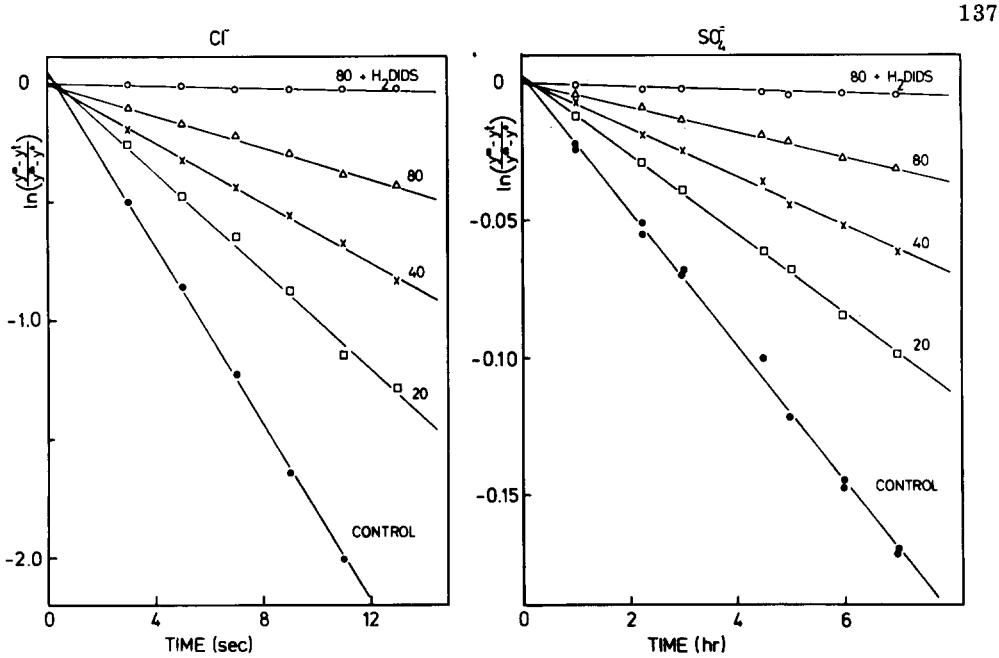

Fig. 2. Inhibition of Cl^- and of SO_4^{2-} self-exchange by ANS. pH 6.3, 8° C, standard medium, 1% hematocrit. Both halves of the figure are plots of $\ln((y^\infty - y^t)/(y^\infty - y^0))$ vs. time, where y^t represents the extracellular radioactivity ($^{36}Cl^-$ or $^{35}SO_4^{2-}$) at the time indicated on the horizontal axis, and y^0 and y^∞ are the extracellular radioactivity at $t = 0$ and $t = \infty$, respectively. For Cl^-, since no true time point at $t = 0$ could be obtained, the value for the H_2DIDS experiment was used. The number adjacent to each set of data points is the ANS concentration (μM). The H_2DIDS, when present, was at a concentration of 12.5 μM.

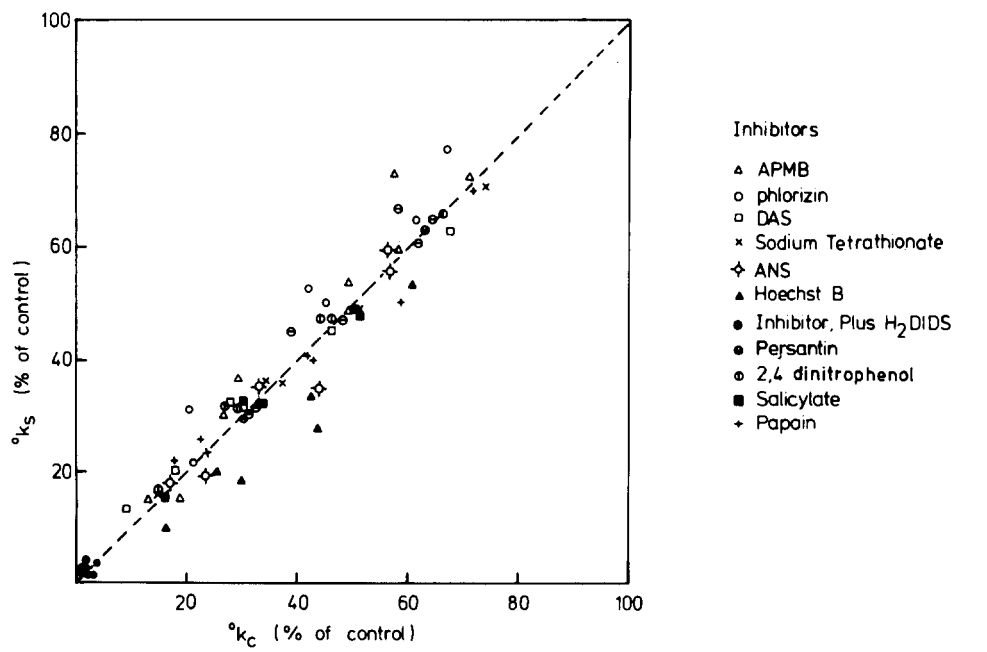

Fig. 3. Comparison of the effects of reversible inhibitors on the rate of Cl^- and SO_4^{2-} equilibrium exchange. Abscissa: rate constant k_c for $^{36}Cl^-$ efflux, as percent of uninhibited control. Ordinate: rate constant k_s for $^{35}SO_4^{2-}$ efflux, also as percent of control. Each data point (one each for Cl^- and SO_4^{2-}) obtained in the presence of some concentration of one of the inhibitors listed on the right. Uninhibited controls for both Cl^- and SO_4^{2-} were performed in duplicate.

TABLE I

INHIBITOR CONCENTRATION (mM) AT HALF-MAXIMAL INHIBITION OF Cl^- AND SO_4^{2-} SELF-EXCHANGE

Values were obtained from Dixon plots of data in Fig. 3. In all cases except APMB, the maximal inhibition was not distinguishable from 100%. For APMB, the Dixon plot was corrected for the 10% of the flux (both Cl^- and SO_4^{2-}) that appears insensitive to APMB.

Inhibitor	IC_{50}	
	Cl^-	SO_4^{2-}
1. APMB	0.140	0.170
2. Phlorizin	0.140	0.180
3. DAS	0.085	0.092
4. Tetrathionate	0.30	0.29
5. ANS	0.023	0.021
6. Hoechst B	0.038	0.023
7. Persantin	0.0050	0.0046
8. 2,4-Dinitrophenol	0.075	0.087
9. Salicylate	0.91	0.89

same effect on Cl^- and SO_4^{2-} transport when measured in the same medium at the same temperature (Table I). The proteolytic enzyme papain also inhibits Cl^- and SO_4^{2-} transport to the same extent. An exception is apparently a compound called Hoechst B, which is identical to furosemide except that a sulphur atom is substituted for the oxygen in the furan ring. Hoechst B inhibits the SO_4^{2-} flux slightly more than the Cl^- flux.

Fig. 4 shows the fractional inhibition of $^{36}Cl^-$ and $^{35}SO_4^{2-}$ equilibrium exchange produced by APMB at the outer membrane surface (cells) or the

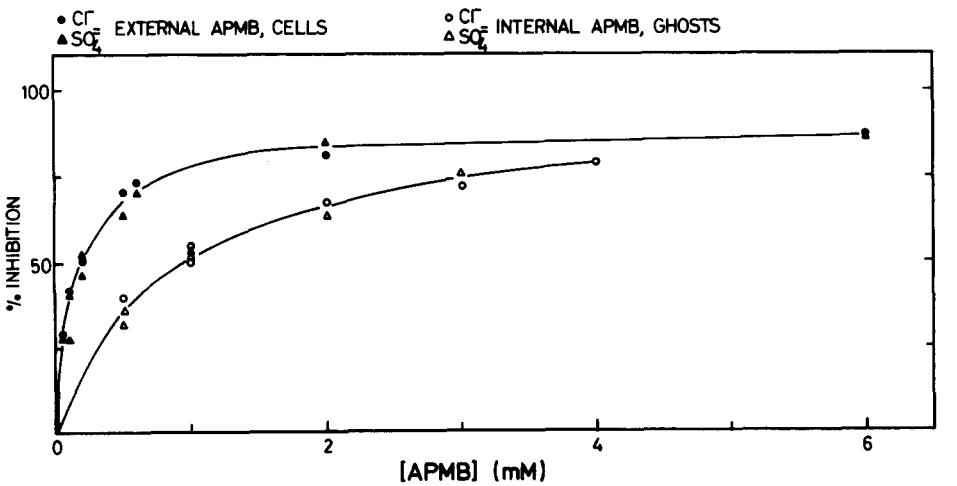

Fig. 4. Effect of APMB on Cl^- and SO_4^{2-} self-exchange in red cells and red cell ghosts, pH 6.3, 8° C, standard medium. Abscissa: APMB concentration (mM). Ordinate: percent inhibition of Cl^- (circles) and SO_4^{2-} (triangles) equilibrium exchange flux. Closed symbols refer to extracellular APMB, acting on intact red cells. Open symbols refer to intracellular APMB, acting on ghosts.

inner surface (ghosts). In accordance with previous observations [21], the maximal inhibition is only about 85–90%. At this temperature and pH the IC_{50} for the inner surface is about 5-fold larger than at the outer surface. These characteristics of the action of APMB are observed for both Cl^- and SO_4^{2-} transport when measured under the same conditions.

Discussion

The mode of action of the various inhibitors used in the experiments described here is far from being understood. A few detailed studies of the actions of APMB [14], furosemide (a compound similar to Hoechst B) [15], and ANS [11] on SO_4^{2-} or Cl^- transport have shown that at least in some cases the inhibition kinetics are quite complex and may even involve binding to several sites. Moreover, for most of the reversibly acting inhibitors it is not even established whether they affect the anion transport protein by direct combination or indirectly by a modification of the lipid bilayer. Nevertheless, it is remarkable that in all cases, over a fairly wide concentration range, all agents tested produce the same percentage inhibition of Cl^- and SO_4^{2-} equilibrium fluxes. This applies in spite of the fact that the experiments were performed in a medium consisting of 122.5 mM NaCl and 5.0 mM Na_2SO_4, i.e. under conditions where the chloride concentration suffices to nearly saturate the transport system [3] while the SO_4^{2-} concentration is considerable lower than the apparent K_m [4].

For non-competitive inhibition it is quite obvious that effects on the same transport system should produce equal fractional inhibition for the transport of all substrates of that system. However, even for competitive inhibition the same percentage inhibition of SO_4^{2-} and Cl^- transport is to be expected if the ions are transported by the same apparatus. This also applies to rather complex transport mechanisms that, like Gunn's model [6,10,16], may involve the interconversion by protonation of the binding sites on the transporter from a singly charged chloride binding site into a doubly charged sulfate binding site. In terms of this model, the fluxes for chloride and sulfate can be written in the general form:

$$j_{Cl} = \frac{a' \cdot [Cl^-]}{1 + b'[Cl^-] + c'[SO_4^{2-}] + d'[I] + e'}$$

$$j_{SO_4} = \frac{a'' \cdot [SO_4^{2-}]}{1 + b''[Cl^-] + c''[SO_4^{2-}] + d''[I] + e''}$$

where some of the coefficients a', a'', b', b'', etc. are functions of pH. According to the model all corresponding coefficients in the denominators are identical, e.g. $b' = b''$, $c' = c''$, etc., even if a given inhibitor interacts preferentially with the singly charged or doubly charged form of the carrier. These expressions are obtained by incorporating into Gunn's original treatment [16] the appropriate mass law expressions for the binding of I to the singly and doubly charged forms of the transporter. The numerators of the two expressions are, of course, different ($a' = a''$). However, the inhibitor concentration [I] will never appear in the numerator, as long as the inhibitory-bound transport systems do not con-

tribute to the measured flux. Thus the percentage change produced by a given concentration [I] should be the same, regardless of the anion species transported.

However, one way in which Cl^- and SO_4^{2-} could be transported by the same system, but affected differently by an inhibitor, is if an inhibitor acts as an allosteric modifier which, by finding to a site distinct from the transport site, changes the K_m of Cl^- and/or SO_4^{2-} binding to the transport site. This is a possible explanation of the fact that the furosemide analog Hoechst B inhibits SO_4^{2-} transport more than Cl^-. Indeed, furosemide is believed to cause an allosteric effect on K_m for Cl^- [15].

It has been pointed out above that although Cl^- and SO_4^{2-} transport in red cells is believed to be mediated by a 100 000 molecular weight protein [17,18], it is possible that some or all of the reversibly acting inhibitors used here exert their effect indirectly, e.g. by changing the surface potential or membrane fluidity. For papain, however, the effect very likely involves a direct action on a membrane protein. Rather high papain concentrations were necessary to inhibit the transport (0.1 mg/ml, 1 h, 37°C, pH 6.3, for 50% inhibition), but Cl^- and SO_4^{2-} transport were inhibited in parallel. This again suggests that the two anions are transported by the same system.

The 100 000 molecular weight protein (band 3) believed to be involved in red cell inorganic anion exchange is the most abundant integral membrane protein in the membrane [19]. Since Cl^--Cl^- and Cl^--HCO_3^- exchange are so rapid, it may be argued that a sizable fraction, if not all, of this protein is involved in the Cl^- transport. There is no need a priori to postulate that this is also true for SO_4^{2-}. A very small fraction of distinct systems could account easily for the observed maximal rate of SO_4^{2-} transport. The present results indicate that if there is a distinct SO_4^{2-} transport system, it is apparently identical in its interaction with the many different inhibitors of anion exchange used here. Although this is possible, it seems more plausible to suggest that the two anions actually are transported by the same system.

If Cl^- and SO_4^{2-} are indeed transported by the same system, then there are remarkable differences in the turnover number for the SO_4^{2-} and Cl^- transport events (a factor of 10^4). In this context, it is relevant that even between the halides Cl^- and I^-, there is a 250-fold difference in the maximal self-exchange flux at 0°C [7]. Thus, it appears to be a general feature of red cell anion exchange that even chemically similar anions are transported at very different maximal rates. Such large differences are not explained by any existing model of anion exchange.

Acknowledgments

The authors would like to thank Hoechst AG for the gift of the compound Hoechst B and Professors Deuticke and Petersen for the gifts of persantin and APMB. They would also like to thank Professor Fasold for synthesizing two of the inhibitors used. This work was supported by the Deutsche Forschungsgemeinschaft.

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