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ON THE MECHANISM OF INHIBITION OF THE SULFATE TRANSFER ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

The SO_4^{2-} flux across the erythrocyte membrane can be inhibited by various organic substances such as phenol, benzoate, phlorhizin and dipyridamol. The inhibition is noncompetitive and reversible. The SO_4^{2-} flux exhibits a strong pH dependence (maximum at pH 6.3, inflection point at pH 6.9) which can be removed almost completely by high doses of the inhibitors. The fractional inhibition of the SO_4^{2-} flux has a pH optimum at about pH 7.0. The apparent activation energy of the SO_4^{2-} transition is about 33 kcal/mole. It is independent of pH and not affected by the inhibitors salicylate, benzoate, phlorhizin and dipyridamol.

On the basis of the fixed-charge hypothesis of the erythrocyte membrane an equation is derived which enables the calculation of the sulfate concentration SO_{4m}^{2-} within the fixed-charge layer of the erythrocyte membrane.

$$2SO_{4m}^{2-} + p\sqrt{SO_{4m}^{2-}} = \frac{\bar{A} \cdot q}{K(1 + I_o/K_i)\sqrt{SO_{4m}^{2-}} + q}$$

$\bar{A} = 2.5$ M and $K = 1 \cdot 10^{-9}$ M denote the concentration of amino groups in the fixed-charge layer and their dissociation constant, respectively, I_0 the inhibitor concentration in the outside solution, and K_i the inhibition constant. $p = Cl_o^-/\sqrt{SO_{4m}^{2-}}$ and $q = H_o^+ \cdot \sqrt{SO_{4o}^{2-}}$, SO_{4o}^{2-}, Cl_o^- and H_o^+ are the concentrations of SO_4^{2-}, Cl^- and H^+ in the outside solution, respectively. The relation between the SO_4^{2-} flux J_{SO_4} and SO_{4m}^{2-} is

$$J_{SO_4} = J_o \cdot \exp a SO_{4m}^{2-}$$

At 37 °C $J_0 = 1 \cdot 10^{-8}$ moles $\cdot$ min$^{-1} \cdot$ g^{-1} cells, $a = 4.6$ M^{-1}. K_i (benzoate) $= 5 \cdot 10^{-3}$ M and K_i (dipyridamol) $= 1 \cdot 10^{-6}$ M gave the best fit to our experimental data. J_0 and a are not significantly affected by the inhibitors benzoate and dipyridamol. The results suggest that compounds which cannot react covalently with amino groups inhibit the SO_4^{2-} flux by a reduction of the effective fixed-charge density of the erythrocyte membrane and a consequent reduction of SO_{4m}^{2-}.

INTRODUCTION

The high anion permselectivity of the erythrocyte membrane is attributed to positively charged ammonium groups, which are supposed to be in a superficial layer

of the erythrocyte membrane. These positive fixed charges increase the concentration of mobile anions at the surface of the main diffusion barrier of the cell membrane and thus promote the anion transfer across the erythrocyte membrane[1,2]. The anion transfer across the erythrocyte membrane can be inhibited by amino group reagents such as 1-fluoro-2,4-dinitrobenzene, 1-methoxy-5-nitrotropone[3] and 4-acetamido-4-isothiocyanate stilbene-2,2-disulfonic acid[4]. It was suggested that these agents form covalent bonds with amino groups and, therefore, consequently lower the fixed-charge density of the erythrocyte membrane. However, most of the inhibitors of the anion permeability cannot react covalently with amino groups and their mode of action is still subject to discussion[3,5-9]. The present paper is concerned with the mechanism of inhibition of the SO_4^{2-} transfer by compounds which cannot form covalent bonds with a membrane constituent.

METHODS

Execution of the $^{35}SO_4^{2-}$ influx experiments

Erythrocytes from freshly drawn citrated human blood were washed three times in isotonic NaCl solution (165 mM). 15 g tightly packed cells were incubated in 15 ml of a solution containing 10 mM Na_2SO_4, 67.5 mM NaCl and 165 mM sucrose. If higher SO_4^{2-} concentrations were required Na_2SO_4 was added in exchange for isoosmotic amounts of NaCl. The erythrocytes were incubated for 120 min at 37 °C in order to achieve an equilibrium distribution for the SO_4^{2-} and the Cl^- present in the system. Then the inhibitors were added and the incubation was continued for another 30 min. Finally for each sample the Cl^- concentration in the supernatant, wet weight and dry weight of 1 ml suspension, hematocrit and pH were determined. The SO_4^{2-} distribution between the intracellular compartment and the outside solution was calculated from the $^{35}SO_4^{2-}$ distribution. The Cl^- concentration was measured according to the method of Lang[10]. The experiments were started by adding tracer amounts of $^{35}SO_4^{2-}$ to the suspensions and the disappearance of the radioactivity from the outside solution was observed. For this purpose portions of each sample were withdrawn at suitable time intervals and the radioactivity in the supernatant was counted.

Execution of the $^{35}SO_4^{2-}$ efflux experiments

The $^{35}SO_4^{2-}$ efflux experiments were performed with a 5 % cell suspension (w/v). The incubation solution contained 5 mM Na_2SO_4, 157.5 mM NaCl, and tracer amounts of $^{35}SO_4^{2-}$. After a 60-min preincubation at 37 °C the inhibitors were added and the incubation was continued for another 30 min. 2 ml of suspension were withdrawn in order to determine the amount of SO_4^{2-} in the intracellular compartment. The erythrocytes were washed three times in 5 ml of a 2.5 % sodium citrate solution. Since SO_4^{2-} can only leave the cells in exchange for citrate anions and since the citrate anions cannot enter the cells under these conditions, the radioactivity can be removed completely from the outside solution without loosing $^{35}SO_4^{2-}$ from the cell interior. Special precautions were taken so that no cells were lost during the washing procedure. After hemolysing the cells with saponin, the radioactivity in the cells was counted. The erythrocytes loaded with $^{35}SO_4^{2-}$ were then transferred to a $^{35}SO_4^{2-}$-free solution (5 mM Na_2SO_4, 157.5 mM NaCl, *plus* the inhibitor) and the $^{35}SO_4^{2-}$ back-exchange was studied as described above.

Calculation of the SO_4^{2-} flux from the $^{35}SO_4^{2-}$ influx

The erythrocyte suspension was treated as a closed two-compartment system. The one compartment is represented by the intracellular space, the other by the external solution. Both compartments are separated by the cell membrane. The tracer exchange at equilibrium conditions follows first-order kinetics. The radioactivity y in the outside solution at time t is given by Eqn 1:

$$y = y_\infty - (y_\infty - y_0)e^{-kt} \tag{1}$$

where y_0 and y_∞ denote the radioactivity in the outside solution at zero time or at infinite time, respectively, and k the rate constant. y_0, y_∞ and k were determined from the time course of the $^{35}SO_4^{2-}$ exchange by means of a least square fit. The SO_4^{2-} flux J_{SO_4} was calculated by means of Eqn 2

$$J_{SO_4} = k \cdot \frac{n_i \cdot n_o}{n_i + n_o} \tag{2}$$

n_i and n_0 denote the amount of SO_4^{2-} in the intracellular compartment and in the outside solution, respectively. The dimension of the flux is moles·min^{-1}·g^{-1} cells; the term g cells refers to the wet weight of tightly packed erythrocytes. For details, see Gardos *et al.*[11].

Calculation of the SO_4^{2-} flux from the $^{35}SO_4^{2-}$ efflux

The SO_4^{2-} flux from the tracer back-exchange was calculated by an essentially similar procedure. However, if the cell density is low the radioactivity at infinite time in the outside solution y_∞ approximates the total radioactivity $\bar{y}$. Thus Eqn 1 can be replaced by Eqn 3

$$y = \bar{y} - (\bar{y} - y_0)e^{-kt} \tag{3}$$

and Eqn 2 simplifies to Eqn 4

$$J_{SO_4} = k \cdot n_i \tag{4}$$

The symbols have the meaning as defined above. For a 5 % cell suspension (w/v) n_i is maximally 3 % of n_0 and the error which is introduced by neglection of this amounts to about 1%.

Determination of the benzoate distribution

The distribution of benzoate between the outside solution and the intracellular compartment was tested with [^{14}C]benzoate. The concentration of benzoate in the outside solution I_0 obeys the equation:

$$I_o = \frac{v_o \cdot I_s}{v_o + (1 + b)v_i \cdot R} \tag{5}$$

v_0 and v_i denote the volumes of the outside compartment (1—hematocrit) and of the intracellular compartment (cell water) respectively, R the Donnan ratio and I_s the inhibitor concentration in the incubation solution before equilibration. Under our experimental conditions the constant b is 0.3. Since v_i, v_0 and R were determined as a routine, I_0 can easily be calculated by means of Eqn 5.

Measurements of $^{35}SO_4^{2-}$ and of [^{14}C]benzoate

The radioactivity was determined by means of a Packard Tricarb Liquid Scintillation Counter. The supernatant and the hemolysate were deproteinized by the addition of trichloracetic acid. The final concentration of trichloracetic acid amounted to 6 % (w/v). 0.25 ml of the deproteinized supernatant or of the deproteinized hemolysate was solved in 10 ml of methanol–toluene (1:1, v/v) scintillator containing 0.025 g POPOP/l and 2 g PPO/l. Due to the methanol the quenching was high but constant, so it did not disturb the measurements.

RESULTS

The inhibition of the SO_4^{2-} flux

The SO_4^{2-} transfer across the erythrocyte membrane can be inhibited by a large number of chemically unrelated substances. Fig. 1 shows the fractional inhibition of the SO_4^{2-} flux plotted *versus* the inhibitor concentration in the incubation solution.

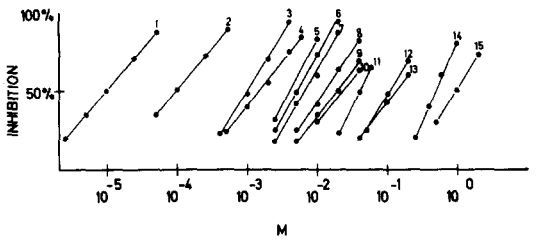

Fig. 1. Fractional inhibition of the SO_4^{2-} flux. Ordinate: fractional inhibition (%). Abscissa: concentration of the inhibitors within the incubation solution in M (logarithmic scale). Cell concentration of the suspension 50 % (w/v), pH 7.2–7.3, temperature 37 °C. Composition of the incubation solution: 10 mM Na_2SO_4, 67.5 mM NaCl, 165 mM sucrose. The inhibitors with exception of sodium benzoate and sodium salicylate were added to the incubation solution in addition to the substances listed. Sodium benzoate and sodium salicylate were added in exchange for isoosmotic amounts of NaCl. 1, dipyridamol; 2, phenylbutazone; 3, phlorhizin; 4, dinitrophenol; 5, octylamine; 6, salicylate; 7, *p*-nitrophenol; 8, benzoate; 9, phenol; 10, caprylic acid; 11, aniline; 12, butanol; 13, butylamine; 14, propanol; 15, ethanol. The fluxes were determined by the $^{35}SO_4^{2-}$ influx.

TABLE I

FRACTIONAL INHIBITION OF THE SULFATE, SUCCINATE, FUMARATE AND LACTATE FLUXES

Temperature 37 °C, lactate 25 °C; pH 7.2–7.3. Composition of the incubation solution: 10 mM Na_2SO_4, sodium succinate, sodium fumarate or sodium lactate, respectively, 67.5 mM NaCl + 165 mM sucrose. The fluxes were determined by means of the isotope influx

Inhibitor	*Concn (M)*	*Inhibition (%)*			
		Sulfate 37 °C	*Succinate 37 °C*	*Fumarate 37 °C*	*Lactate 25 °C*
Benzene	$2 \cdot 10^{-2}$	0	0	—	0
Aniline	$2 \cdot 10^{-2}$	23	27	42	10
Phenol	$2 \cdot 10^{-2}$	50	58	54	12
Benzoate	$2 \cdot 10^{-2}$	64	63	63	25
Salicylate	$2 \cdot 10^{-2}$	83	81	79	45
Dinitrophenol	$1.5 \cdot 10^{-3}$	49	57	44	35
Phlorhizin	$2.5 \cdot 10^{-3}$	56	56	65	70
Dipyridamol	$1 \cdot 10^{-5}$	42	49	62	58

The compounds differ tremendously in their inhibitory efficiency. The slope of the dose–response curves at halfmaximal inhibition, however, is almost constant and independent of the chemical nature of the inhibitor. The maximal inhibition which was reached amounts to at least 95 %.

The action of the inhibitors is not restricted to the SO_4^{2-} permeation. Most of the inhibitors also impair the phosphate anion transfer[6] and the transfer of succinate, fumarate and lactate anions (Table I). It seems, therefore, that the inhibitors cause a general inhibition of the anion permeability of the erythrocyte membrane.

The only common property of the inhibitor molecules is their amphiphilic nature. The more potent inhibitors contain systems with π-electrons. In contrast to the inhibition caused by amino group reagents such as 1-fluoro-2,4-dinitrobenzene or 4-acetamido-4-isothiocyanate stilbene-2,2-disulphonic acid, the inhibition of the sulfate flux by the inhibitors listed in Fig. 1 is reversible, and complete recovery occurs if the erythrocytes are washed in an isotonic NaCl solution. Hence the action of the inhibitors seems to be due to a simple adsorption of the inhibitor molecules to the membrane rather than to a covalent binding of the inhibitors to a membrane constituent.

The inhibition of the SO_4^{2-} flux, as indicated by the Dixon plots (Fig. 2), is

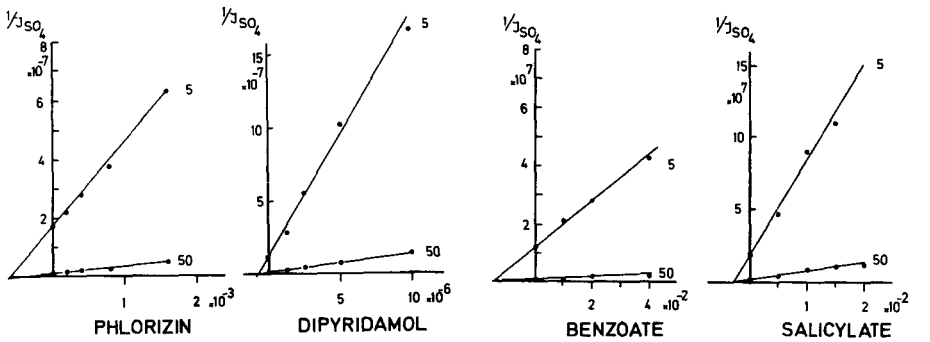

Fig. 2. Dixon plots. Ordinate: $1/J_{SO_4}$ (g cells·min·moles^{-1}). Abscissa: inhibitor concentration (M). Composition of the incubation solution: 5 mM Na_2SO_4, 157.5 mM NaCl and 50 mM Na_2SO_4, 90 mM NaCl, respectively. Phlorhizin and dipyridamol were added in addition to the substances listed. Benzoate and salicylate were added as sodium salts in exchange for isoosmotic amounts of NaCl. Fractional concentration of the cell suspension 5 % (w/v), pH 7.2–7.3, temperature 37 °C. The fluxes were determined from the $^{35}SO_4^{2-}$ efflux.

TABLE II

INHIBITION CONSTANT K_i DETERMINED BY MEANS OF THE DIXON PLOTS

Temperature 37 °C, phenol 32 °C. pH 7.2–7.3. Composition of the incubation solution: see legend to Fig. 2.

Inhibitor	K_i (*M*)
Phenol (32 °C)	$1.5 \cdot 10^{-2}$
Benzoate	$1.4 \cdot 10^{-2}$
Salicylate	$3 \cdot 10^{-3}$
2,4-Dinitrophenol	$1 \cdot 10^{-3}$
Phlorhizin	$6 \cdot 10^{-4}$
Phenylbutazone	$1 \cdot 10^{-4}$
Dipyridamol	$7 \cdot 10^{-7}$

noncompetitive irrespective of the fact whether anionic, cationic or neutral inhibitors were employed. This is somewhat surprising. At least the small aromatic anions benzoate and salicylate were expected to inhibit the SO_4^{2-} flux competitively. From the intercept of the straight lines with the abscissa, the inhibition constants K_i were obtained, a few of which are listed in Table II. The noncompetitive type of inhibition suggests that short-range forces predominate in the interaction of the inhibitors with the erythrocyte membrane. Furthermore, the applicability of the Dixon procedure shows that the interaction of the inhibitor with the membrane adheres to a simple law of mass action.

pH Dependence of the fractional inhibition

The SO_4^{2-} flux exhibits a strong pH dependence with a maximum at about pH 6.3 and an inflection point at about pH 6.9. High concentrations of benzoate (Fig. 3a), dipyridamol (Fig. 3b), phenol, aniline, salicylate, 2,4-dinitrophenol and phlorhizin reduce the pH dependence of the SO_4^{2-} flux considerably. In addition, the inflection points show a tendency to shift to lower pH with increasing inhibition of the flux. The fractional inhibition of the SO_4^{2-} flux has a pH optimum at approximately pH 7.0. The pH dependence of the fractional inhibition becomes less pronounced with increasing inhibition of the SO_4^{2-} flux (Fig. 4a and Fig. 4b). These observations are irreconcilable with the assumption that the protons and the inhibitors interact with two separate diffusion barriers in series. The fractional inhibition then should show no pH optimum and should be unaffected by variations of pH. The protons and the inhibitors, therefore, are assumed to interact with essentially the same structures of the erythrocyte membrane. Possibly the inhibitors induce a conformational change of the membrane lipoproteins which impairs the binding of protons to the amino groups. As a consequence the SO_4^{2-} concentration in front of the main diffusion barrier of the erythrocyte membrane would be reduced.

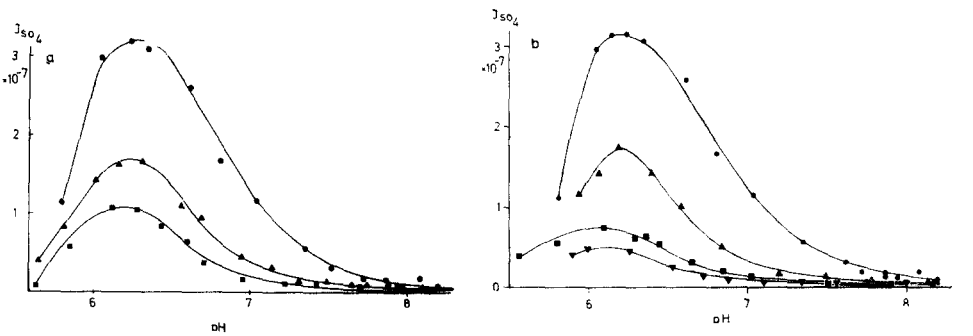

Fig. 3. (a) pH dependence of the SO_4^{2-} flux in the presence of benzoate. Ordinate: SO_4^{2-} flux J_{SO_4} (moles·min^{-1}·g^{-1} cells). Abscissa: pH. ●, controls; ▲, 10 mM benzoate; ■, 20 mM benzoate. Composition of the incubation solution: 5 mM Na_2SO_4, 157 mM NaCl; sodium benzoate was added in exchange for isoosmotic amounts of NaCl. Cell concentration 5% (w/v). Temperature 37 °C. The fluxes were calculated from the $^{35}SO_4^{2-}$ efflux. (b) pH dependence of the SO_4^{2-} flux in the presence of dipyridamol. Ordinate: SO_4^{2-} flux J_{SO_4} (moles·min^{-1}·g^{-1} cells). Abscissa: pH. ●, controls; ▲, $9 \cdot 10^{-6}$ M dipyridamol; ■, $2.5 \cdot 10^{-5}$ M dipyridamol; ▼, $5 \cdot 10^{-5}$ M dipyridamol. Composition of the incubation solution: 5 mM Na_2SO_4, 157 mM NaCl, dipyridamol was added to the solution in addition to the substances listed. Cell concentration of the suspension 5% (w/v). Temperature 37 °C. The fluxes were calculated from the $^{35}SO_4^{2-}$ efflux.

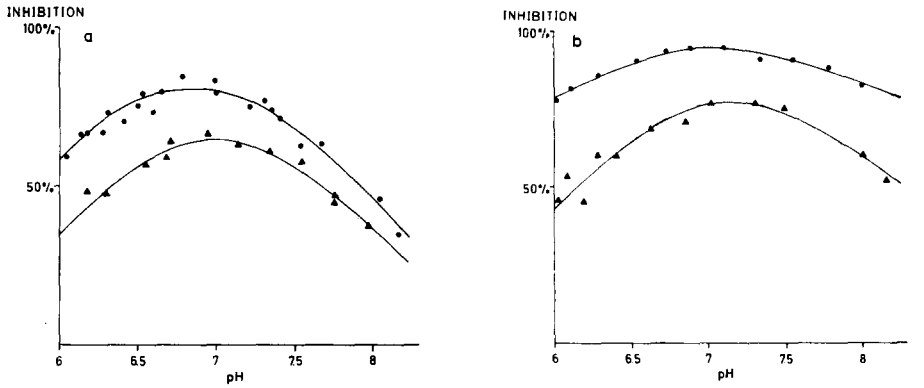

Fig. 4. (a) Fractional inhibition of the SO_4^{2-} flux by benzoate. Ordinate: fractional inhibition (%). Abscissa: pH. ●, 20 mM benzoate; ▲, 10 mM benzoate. Composition of the incubation solution: 5 mM Na_2SO_4, 157 mM NaCl, sodium benzoate was added in exchange for isoosmotic amounts of NaCl. Cell concentration of the suspension 5% (w/v). Temperature 37 °C. (b) Fractional inhibition of the SO_4^{2-} flux by dipyridamol. Ordinate: fractional inhibition (%). Abscissa: pH. ●, $5 \cdot 10^{-5}$ M dipyridamol; ▲, $1 \cdot 10^{-5}$ M dipyridamol. Composition of the incubation solution: 5 mM Na_2SO_4, 157 mM NaCl, dipyridamol was added in addition to the substances listed. Cell concentration of the suspension 5% (w/v). Temperature 37 °C.

Activation energy of the SO_4^{2-} transition

From the temperature dependence of the SO_4^{2-} flux the apparent activation energy E_A was calculated. Two Arrhenius diagrams are shown in Figs 5a and 5b. E_A is about 33 kcal/mole. It is independent of pH and not affected by the inhibitors

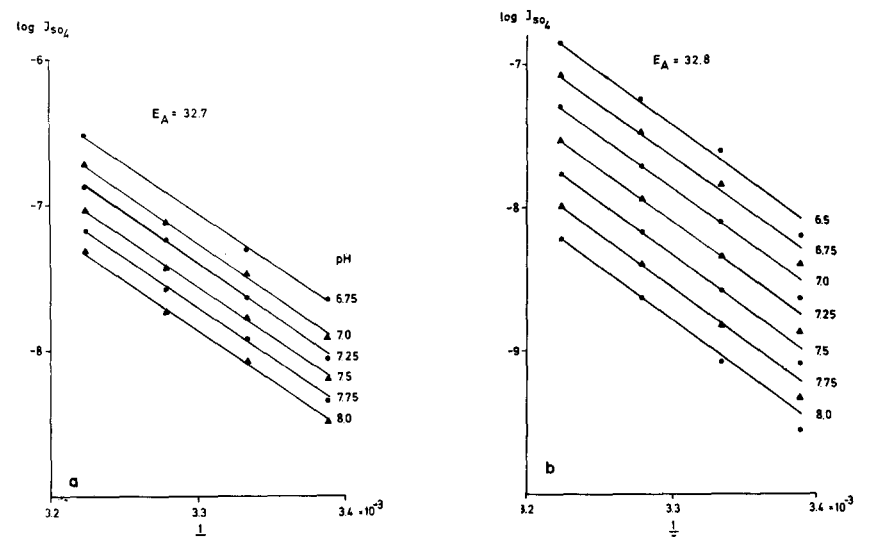

Fig. 5. (a) pH dependence of the activation enthalpy. Ordinate: log J_{SO_4}. Abscissa: $1/T$. The SO_4^{2-} fluxes were determined from the $^{35}SO_4^{2-}$ influx at 22 °C, 27 °C, 32 °C and 37 °C. Composition of the incubation solution: 10 mM Na_2SO_4, 67.5 mM NaCl, 165 mM sucrose. (b) pH dependence of the activation enthalpy in the presence of benzoate. Ordinate: log J_{SO_4}. Abscissa: $1/T$. The SO_4^{2-} fluxes were determined by the $^{35}SO_4^{2-}$ influx at 22 °C, 27 °C, 32 °C and 37 °C. Composition of the incubation solution: 10 mM Na_2SO_4, 47.5 mM NaCl, 20 mM sodium benzoate and 165 mM sucrose.

salicylate, benzoate, phlorhizin or dipyridamol. As an exception, a deviation from linearity in the presence of benzoate was found at the lowest temperature. Since, however, the SO_4^{2-} flux at low temperature is very slow, the studies are restricted to the temperature range from about 22 °C to 37 °C. Thus it cannot be tested whether this deviation is significant. The apparent activation energy of the SO_4^{2-} flux is of the magnitude expected for diffusion in solid states. It exceeds the activation energy for diffusion of comparable anions in highly cross-linked artificial ion exchangers by at least a factor of 2.5. Hence, the fixed-charge layer itself cannot account for the high activation energy but it has to be attributed to the main diffusion barrier of the erythrocyte membrane.

Calculation of the SO_4^{2-} concentration in the fixed-charge layer

According to the model outlined above, the SO_4^{2-} flux across the erythrocyte membrane is determined by the SO_4^{2-} concentration of the fixed-charge layer. For the calculation of the SO_4^{2-} concentration in the fixed-charge layer the following suppositions were made:

(1) The protons H^+ interact with the amino groups A according to a law of mass action converting them to ammonium groups A^+

$$\frac{A \cdot H^+}{A^+} = K \tag{7}$$

The subscripts m refer to magnitudes in the membrane, the subscript o to magnitudes in the outside solution.

(2) The inhibitor I_0 interacts with the immediate surroundings of the amino group forming an inactive complex AI. The adsorption of the inhibitor obeys a law of mass action.

$$\frac{A \cdot I_o}{AI} = K_i \tag{6}$$

(3) The total concentration of amino groups $\bar{A}$ is the sum of the charged form A^+, the uncharged form A *plus* the complex AI

$$\bar{A} = A^+ + A + AI \tag{8}$$

(4) The positive charges of the ammonium groups A^+ in the fixed-charge layer are counterbalanced by an equal amount of negative charges, mainly carried by SO_4^{2-} and Cl^-

$$2SO_{4m}^{2-} + Cl_m^- = A^+ \tag{9}$$

(5) Because electrostatic forces predominate in the interaction of the ions with the fixed-charge layer, the distribution of SO_4^{2-}, Cl^- and H^+ between the fixed-charge layer and the adjacent aqueous phase is supposed to obey a Donnan distribution[1,2]

$$Cl_m^- = Cl_o^- \frac{\sqrt{SO_{4m}^{2-}}}{\sqrt{SO_{4o}^{2-}}} \tag{10}$$

$$H_m^+ = H_o^+ \cdot \frac{\sqrt{SO_{4o}^{2-}}}{\sqrt{SO_{4m}^{2-}}} \tag{11}$$

Substituting A and AI in Eqn 8 by means of Eqns 6 and 7 yields the concentration of positively charged ammonium groups in the fixed-charge layer. Inserting Eqn 9 and eliminating the magnitudes in the membrane by means of Eqns 10 and 11 the SO_4^{2-} concentration in the fixed-charge layer SO_{4m}^{2-} is obtained as a function of the SO_4^{2-} concentration SO_{4o}^{2-}, the Cl^- concentration Cl_o^-, the H^+ concentration H_o^+, and of the inhibitor concentration I_0 in the outside solution (Eqn 12)

$$2SO_{4m}^{2-} + p\sqrt{SO_{4m}^{2-}} = \frac{\bar{A}q}{K(1 + I_o/K_i)\sqrt{SO_{4m}^{2-}} + q} \tag{12}$$

Here $p = Cl_o/\sqrt{SO_{4o}^{2-}}$ and $q = H_o^+ \cdot \sqrt{SO_{4o}^{2-}}$. The other symbols have the meaning defined above. Eqn 12 is a cubic equation which can be solved conveniently by a Newton approximation. For each choice of the parameters $\bar{A}$, K, K_i, I_0, p and q Eqn 12 has only one positive solution.

Correlation between SO_4^{2-} flux and SO_4^{2-} concentration in the membrane

Using the experiments shown in Fig. 6a and 6b *plus* one additional dose response curve SO_{4m}^{2-} was calculated by means of Eqn 12. For $\bar{A} = 2.5$ M and $K = 1 \cdot 10^{-9}$ M the values from Passow[2] were inserted which were determined without inhibitors under comparable experimental conditions. K_i was varied until an optimal statistical correlation between the logarithm of the sulfate flux $\log J_{SO_4}$ and SO_{4m}^{2-} was obtained.

For benzoate an optimal correlation (correlation coefficient = 0.945) was found at $K_i = 5 \cdot 10^{-3}$ M. The points scatter randomly around a straight line. Only at SO_4^{2-} concentrations below 0.2 M do deviations become apparent. The linear dependence of $\log J_{SO_4}$ on SO_{4m}^{2-} indicated an exponential relation between J_{SO_4} and SO_{4m}^{2-} which is expressible by Eqn 13

$$J_{SO_4} = J_0/\exp a SO_{4m}^{2-} \tag{13}$$

If only SO_4^{2-} concentrations above 0.2 M in the membrane are considered, J_0 can be treated as a constant. J_0 is $1 \cdot 10^{-8}$ moles$\cdot$min$^{-1}\cdot$g^{-1} cells; and a is 4.6 M^{-1}. They were determined from the intercept and slope of the extrapolated regression line. By reinserting these values in Eqn 13 the SO_4^{2-} fluxes can be calculated within the pH range from about pH 6.3 to pH 8.5 (*cf.* continuous lines in Figs 6a and 6b). Essentially similar results were obtained with dipyridamol with $K_i = 1 \cdot 10^{-6}$ M.

DISCUSSION

The present paper is concerned with the mechanism of inhibition of the SO_4^{2-} permeation. Hence it seems necessary to make a few short comments on the penetration of SO_4^{2-} across the erythrocyte membrane. As mentioned already, the anion transfer across the erythrocyte membrane is facilitated by positively charged ammonium groups which are supposed to determine the concentration of mobile anions at the surface of the main diffusion barrier of the cell membrane. The SO_4^{2-} flux across the erythrocyte membrane, therefore, should be a function of the SO_4^{2-} concentration in the fixed-charge layer and of the specific transition rate of SO_4^{2-} across the main diffusion barrier of the cellular membrane.

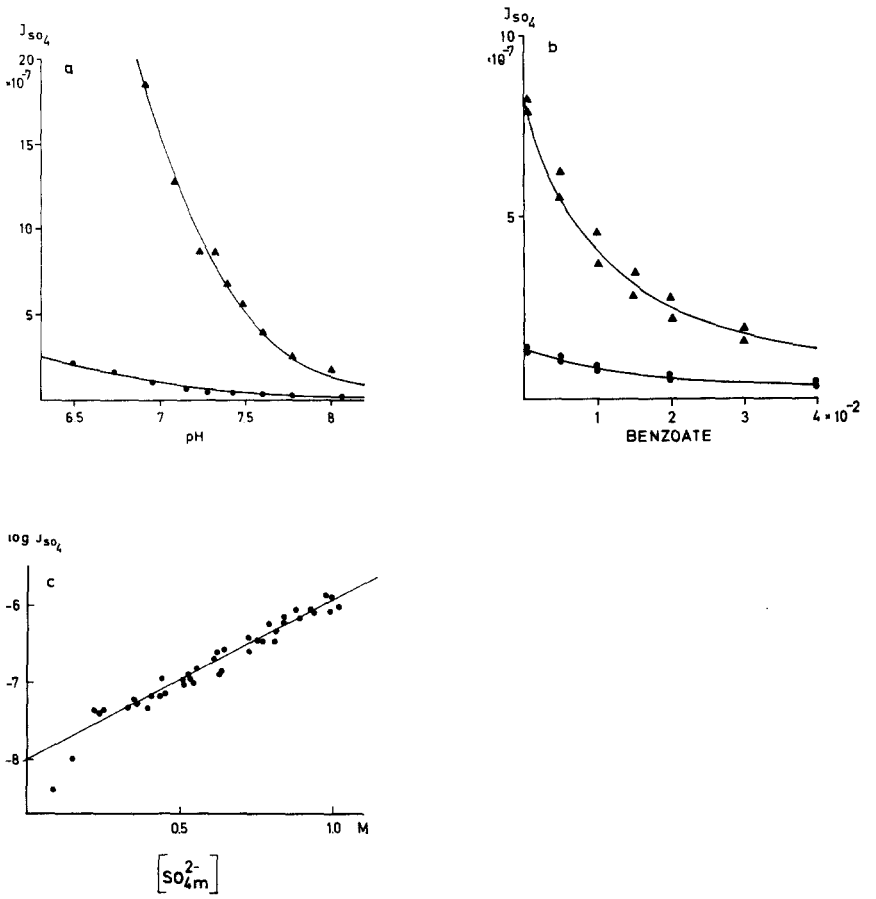

Fig. 6. (a) pH dependence of the SO_4^{2-} flux in the presence of benzoate. Ordinate: SO_4^{2-} flux J_{SO_4} (moles·min^{-1}·g^{-1} cells). Abscissa: pH. Cell concentration of the suspension 50% (w/v). Temperature 37 °C. The sulfate fluxes were determined from the $^{35}SO_4^{2-}$ influx. Composition of the incubation solution prior to equilibration: 50 mM Na_2SO_4, 20 mM sodium benzoate, 165 mM sucrose (▲); 10 mM Na_2SO_4, 47.5 mM NaCl, 20 mM sodium benzoate, 165 mM sucrose (●). The continuous lines were computed according to Eqns 12 and 13. $\bar{A} = 2.5$ M; $K = 1 \cdot 10^{-9}$ M; $K_t = 5 \cdot 10^{-3}$ M; $J_0 = 1 \cdot 10^{-8}$ moles·min^{-1}·g^{-1} cells; $a = 4.7$ M^{-1}. The equilibrium concentrations amounted to 50 mM Na_2SO_4, 10 mM sodium benzoate and 165 mM sucrose (▲) and to 4 mM Na_2SO_4, 70 mM NaCl, 10 mM sodium benzoate and 165 mM sucrose (●). (b) Dose–response curves. Ordinate: SO_4^{2-} flux J_{SO_4} (moles·min^{-1}·g^{-1} cells). Abscissa: Benzoate concentration in the incubation solution (M) prior to equilibration. pH 7.2–7.3. Temperature 37 °C. Composition of the incubation solution: 40 mM Na_2SO_4, 30 mM NaCl (sodium benzoate was added in exchange for NaCl), 165 mM sucrose (▲), 10 mM Na_2SO_4, 67.5 mM NaCl, 165 mM sucrose (●). The continuous lines were computed according to the combined Eqns 12 and 13. $\bar{A} = 2.5$ M; $K = 1 \cdot 10^{-9}$ M; $K_t = 5 \cdot 10^{-3}$ M; $J_0 = 1 \cdot 10^{-8}$ moles·min^{-1}·g^{-1} cells; $a = 4.7$ M^{-1}. The equilibrium concentrations amounted to 20 mM Na_2SO_4, 60 mM NaCl + sodium benzoate and 165 mM sucrose (▲) and to 5 mM Na_2SO_4, 75 mM NaCl + sodium benzoate and 165 mM sucrose (●). (c) Log J_{SO_4} *versus* $SO_{4\,m}^{2-}$. Ordinate: logarithm of the experimentally determined sulfate fluxes. Abscissa: SO_4^{2-} concentrations SO_{4m}^{2-} (M) in the fixed charge region of the erythrocyte membrane calculated by means of Eqn 12. $\bar{A} = 2.5$ M; $K = 1 \cdot 10^{-9}$ M; $K_t = 5 \cdot 10^{-3}$ M. The experiments shown in (a) and (b) were used *plus* one additional dose–response curve which is not shown in (b).

The SO_4^{2-} concentration in the fixed-charge layer was calculated by means of Eqn 12. The distribution of SO_4^{2-}, Cl^- and H^+ between the fixed-charge layer and the adjacent aqueous phase was assumed to adhere to a Donnan distribution, whereas the inhibitors were supposed to interact with the fixed-charge layer according to a mass law equation without taking into account their electrical charge. This is reasonable since weak electrolytes become adsorbed into artificial ion exchangers in the same manner as non-electrolytes, far in excess of the ion-exchange capacity of the ion exchanger and mainly in their uncharged form. The adsorption in most cases follows a Langmuir adsorption isotherm. This means that the interaction with the ion exchanger can be described by a mass law equation[12]. In addition, the Dixon plots showed no differences between neutral, negatively charged, and positively charged inhibitors. Since the ammonium groups within the membrane presumably are surrounded by a hydration shell and since the inhibitors are assumed to be present in the fixed-charge layer mainly in their uncharged form, they should become adsorbed preferentially into the surroundings of the uncharged amino groups, as assumed for the derivation of Eqn 12. No attemps were made to take into account other factors which possibly contribute to permeability changes such as changes in shape of the erythrocytes, changes in membrane hydration, or effects of the electrical double layer.

Eqn 12 only can be applied within the pH range from about 6.3 to 8.5. The fixed-charge concept gives no description of the descending branch of the SO_4^{2-} flux pH curves below 6.3. Above 8.5 the fixed charge density becomes too low to exclude co-ions from entering the fixed charge layer. Thus, above 8.5 the anion concentration at the surface of the main diffusion barrier should become independent of the fixed-charge concentration.

No definite statement can be made concerning the mechanism by which the inhibitors reduce the effective fixed charge density of the erythrocyte membrane. There are several possibilities which can finally lead to similar equations:

(1) The inhibitors could induce a conformational change of the membrane lipoproteins, which impairs the proton binding.

(2) Anionic and cationic inhibitors could compete with the amino group for the protons, if the inhibitor molecule becomes adsorbed to the fixed-charge layer in a close neighborhood of the amino groups.

(3) Negatively charged inhibitors could counterbalance the positive charges of the ammonium groups, if they become adsorbed into the fixed-charge layer in their dissociated form.

The exponential relationship between the SO_4^{2-} flux across the erythrocyte membrane and the SO_4^{2-} concentration in the fixed-charge layer can be expressed by Eqn 13. The inhibitors affect mainly SO_{4m}^{2-}, whereas the constants a and J_0 do not seem to be affected significantly. a has exactly the same magnitude as found without inhibitors. J_0 in our case is slightly lower; however, the difference seems to be insignificant[13].

The statement that the inhibition is caused by a reduction of SO_{4m}^{2-} is substantiated by experiments with pronase-pretreated erythrocytes. Pronase, a mixture of several proteolytic proteins from *Streptococcus griseus*, degrades proteins from the outer surface to the erythrocyte membrane. Due to the pronase pretreatment the SO_4^{2-} flux across the erythrocyte membrane is reduced considerably[14]. The inhibitory

effect of pronase on the SO_4^{2-} permeability was not additive to the inhibitory action of benzoate, salicylate, phlorhizin or dipyridamol (Passow, Gerhardt and Schnell unpublished results). This suggests that, due to the enzymatic treatment, a part of the superficial amino groups are removed. Hence the inhibitors cannot cause a further reduction of the SO_4^{2-} flux.

The results reported in the present paper give strong evidence to the contention that inhibitors which cannot react covalently with amino groups also reduce the fixed-charge density of the erythrocyte membrane, and as a consequence inhibit the SO_4^{2-} flux across the membrane. The specific transition rate of SO_4^{2-}, *i.e.* the probability of a SO_4^{2-} jumping over the main diffusion barrier if it is adsorbed to the fixed-charge layer, seems to be unaffected by the inhibitors benzoate and dipyridamol.

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