The Permeability of the Human Red Blood Cell to Sulfate Ions

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Summary. Sulfate permeability was measured at Donnan equilibrium as a function of three variables, the sulfate, chloride, and hydrogen ion concentration in the medium. The data were used for a quantitative evaluation of a number of typical predictions of a fixed charge model of the ion permeable regions of the red blood cell membrane. It could be shown that more than 1,000-fold variations of sulfate flux, J_{SO_4}, could be represented as a function of a single variable SO_{4m}^{2-}, the sulfate concentration in the membrane. SO_{4m}^{2-} was calculated from the measured values of all three variables by means of a previously published equation (Passow, *Progress in Biophysics and Molecular Biology*, vol. 19, pt. II, pp. 425–467, 1969). In this equation, two constants can be arbitrarily chosen: $\bar{A}$, the sum of the charged and uncharged forms of dissociable fixed charges, and K, the dissociation constant of the fixed charges. For the present calculations, the previously obtained values $\bar{A} = 2.5$ and $K = 1 \cdot 10^{-9}$ were used. The resulting relationship between J_{SO_4} and SO_{4m}^{2-} was found to obey the equation

$$J_{SO_4} = c_1 \cdot \frac{SO_{4m}^{2-}}{c_{II} + SO_{4m}^{2-}} e^{a\, SO_{4m}^{2-}}$$

where $c_1 = 1.62 \cdot 10^{-9}$, $c_{II} = 2.3 \cdot 10^{-2}$, $a = 4.94$ gave the best fit for data obtained at 27 °C. The exponential increase of J_{SO_4} with SO_{4m}^{2-} suggests that there exists a cooperative facilitation of sulfate flux with increasing SO_{4m}^{2-}. Measurements of the apparant activation energy of sulfate flux yielded a value of 32.7 Kcal/mole. This value was independent of the pH at which the measurements were made.

According to the fixed charge hypothesis, the anion/cation discrimination in the red blood cell is primarily due to coulombic interactions between diffusible ions and positive fixed charges within the membrane. Since the outer surface of the red blood cell membrane carries a net negative charge, it is assumed that the positive fixed charges involved in the control of ion permeability are located inside the membrane. Acid base titrations of hemoglobin-free red blood cell membrane preparations suggest that the

number of fixed groups which, upon dissociation, yield negative charges slightly exceeds the number of fixed groups which yield positive charges (Sanui, Carvalho & Pace, 1962; Passow & Vielhauer, ref. in Passow, 1969 a). Hence, the permeability controlling positive charges cannot be equally distributed over the whole membrane area. They are stipulated to form clusters around the anion permeable regions. The experiments presented in this and a series of subsequent papers were designed to test if the kinetics of passive ion movements can be understood in terms of ion diffusion through such positively charged regions within the red blood cell membrane.

Much of our work has been performed with sulfate ions whose behavior seems to be representative for that of divalent anions in general (LaCelle & Passow, 1966; c. f. Passow, 1969 a, p. 445). SO_4-flux across the red blood cell membrane was measured by means of $^{35}SO_4$ at Donnan equilibrium, i.e. under conditions where no net movements of ions and water occur and where diffusion potentials are negligibly small. Under these conditions, the labeled sulfate ions are moving only under the influence of their own activity gradient. If migration within the membrane rather than penetration across the membrane surface is the rate limiting process, then the ion flux at Donnan equilibrium should be primarily determined by the sulfate concentration within the membrane. The sulfate concentration in a charged membrane is, under most experimental conditions, different from that of the adjacent media. It is dependent on the concentration of fixed charges in the membrane and on the concentrations of all diffusible anion species present in the system. In our experiments, these anion species include Cl^- and OH^- in addition to sulfate ions. The sulfate concentration in the membrane, SO_{4m}^{2-}, is inaccessible to measurement but can be calculated from the known concentrations of SO_4^{2-}, Cl^-, and OH^- in the suspension media by means of the equation (Passow, 1965, 1969 a)[1]:

$$2 SO_{4m}^{2-} + p \cdot f \cdot \sqrt{SO_{4m}^{2-}} = \bar{A} \frac{q}{K \sqrt{f_{SO_{4m}} \cdot SO_{4m}^{2-}} + q}. \tag{1}$$

This equation was derived on the simplifying assumptions that: (1) the distribution of each diffusible anion species between membrane and medium obeys Donnan's law; (2) electroneutrality exists within the membrane;

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1 Explanation of symbols: SO_{4m}^{2-} = Sulfate concentration within the fixed charge region of the membrane; $f_{SO_{4m}}$, f_{Cl_m} = activity coefficients of sulfate and chloride ions in the membrane, respectively; $f = \sqrt{f_{SO_{4m}}}/f_{Cl_m}$; $p = (Cl^-)/\sqrt{(SO_4^{2-})}$; $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$ [(Cl^-), (H^+), and (SO_4^{2-}) represent activities in the external medium or in the cell water (see Passow, 1969 a)]; $\bar{A}$ = dissociable groups in the membrane, sum of charged and uncharged forms; K = apparent dissociation constant of fixed charges.

(3) the concentration of penetrating cations within the anion permeable channels is negligibly small as compared to the concentration of fixed charges; and (4) the fixed charges are dissociable; their dissociation can be described by an equation which is formally identical with the mass law equation as applied to a reaction of the type: $A^+ = A + H^+$.

Experimentally, it was observed that the sulfate flux across the membrane depends on three variables, the SO_4^{2-}, Cl^-, and OH^- concentrations in the media. These three variables were found to be interrelated according to Eq. (1). Hence, the sulfate flux across the membrane could be expressed as a function of a single variable, SO_{4m}^{2-}, which was calculated from the analytically determined anion composition of the medium.

Although some of the consequences of Eq. (1) were related to experimental results in previous papers (Passow, 1969*a*, *b*; Passow & Schnell, 1969), a thorough quantitative description of its implications and a more rigorous test of its applicability to our experimental data are still lacking. The present paper is devoted to a detailed analysis of the mathematical consequences of Eq. (1), a presentation of our original data on the kinetics of sulfate permeability, and some further investigations about the relationship between the measured sulfate flux across the membrane and the sulfate concentration within the membrane as calculated by means of Eq. (1).

Materials and Methods

All experiments were performed with freshly drawn human blood. Sodium citrate was used as an anticoagulant: 20 ml of a 3.8% solution were added to each 80 ml of blood. After centrifugation at 1,600 g, the buffy coat was removed and the cells were washed three times. The composition of the washing medium varied depending on the experimental conditions under which the flux measurements were to be performed. Isotonic saline (0.166 moles/liter) or isotonic sulfate (0.133 moles/liter) were used when the fluxes were to be measured in high chloride or high sulfate solutions, respectively. For measurements at low ionic strength, a mixture of 50% isotonic saline and 50% isotonic (0.332 moles/liter) sucrose was employed. The final sediment of the washed cells was mixed in a 1:1 ratio (w/v) with the desired sulfate containing suspension media and incubated at 37 °C for two hours. Subsequently, the cell suspension was transferred to that temperature at which the flux measurements were to be performed (usually 27 °C) and equilibration was continued for another 30 minutes. Trial runs showed that at the end of the described incubation procedure all anion species present in the system had attained the Donnan equilibrium. The flux measurements were started by the addition of a few drops of carrier-free $^{35}SO_4$ solution. The disappearance of the radioactivity from the medium was followed by centrifuging samples at suitable time intervals and measuring the radioactivity in the supernatant. The determination of $^{35}SO_4$ was performed in a Packard Tri Carb liquid-scintillation counter after deproteinization with trichloracetic acid (final concentration: 7.5%). 0.1 ml of the trichloracetic acid filtrate was mixed with 10 ml of a toluene-methanol scintillator containing 1.4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) and 2.5-diphenyloxazole (PPO).
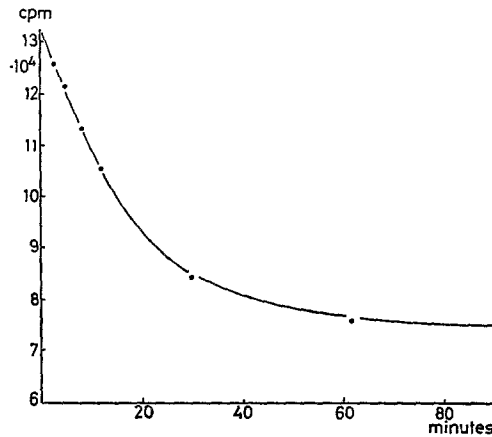
Fig. 1. Time course of disappearance of radioactivity as measured at Donnan equilibrium. • experimental points; the drawn line represents an exponential which was fitted to the data by means of the method of least squares. Concentrations in the supernatant at equilibrium: Cl^- 66.1 mmoles/liter; SO_4^{2-} 8 mmoles/liter, pH 7.31; isotonicity was maintained by the addition of sucrose; hematocrit: 46%. Temperature: 37 °C. Ordinate: $^{35}SO_4$ in the supernatant in cpm. Abscissa: time in minutes

Immediately before the start of the flux measurements, samples were withdrawn for determination of hematocrit, dry and wet weight of 1.0 ml suspension, pH, chloride and sulfate concentrations. Chloride and sulfate were estimated by the methods of Lang (1937) and of Morgulis and Hemphill (1932), respectively.

The suspension media contained Na_2SO_4, NaCl, and sucrose in varying proportions. pH was adjusted by the addition of NaOH or HCl. No attempt was made to prepare media of equal vapor pressure. Instead, regardless of the composition of the media, a total (ideal) osmolarity of 332 mosmoles/liter was established. Solutions of that osmolarity will hence be referred to as "isotonic". (For further details of the experimental procedure *see* Gardos, Hoffman, and Passow, 1969.)

The decrease of the radioactivity in the supernatant followed a single exponential (Fig. 1):

$$y = (y_0 - y_\infty) \cdot e^{-kt} + y_\infty .$$

y_0 and y_∞ represent the radioactivity at zero and at infinite time, respectively; k is the rate constant for the disappearance of the radioactivity from the medium. The three constants, y_0, y_∞, and k were determined by fitting the data to an exponential by the method of least squares. The computer program was designed by Dr. Martin Pring of the University of Pennsylvania.

According to the theory of tracer exchange in a steady state, two compartment system (*see* Gardos *et al.*, 1969),

$$k = J_{SO_4} \frac{SO_{4i}^{2-} \cdot v_i + SO_{4O}^{2-} \cdot v_0}{SO_{4i}^{2-} \cdot v_i \cdot SO_{4O}^{2-} \cdot v_0}$$

where SO_{4i}^{2-}, SO_{4O}^{2-} = sulfate concentrations in cell water and medium, respectively; and v_i, v_0 = volume of cell water and medium, respectively.

The sulfate flux, J_{SO_4}, is related to the sulfate concentration by the following expression:

$$J_{SO_4} = P_0\, SO_{4O}^{2-} = P_i\, SO_{4i}^{2-}$$

where P represents the permeability constant. The indices i and 0 refer to fluxes from the medium into the cell and vice versa, respectively.

The flux measurements are expressed in moles $\cdot$ (g cells)$^{-1}$ $\cdot$ min^{-1} and the permeability constant in min^{-1}. No attempt was made to relate the experimental data to the surface area of the cells.

The calculated rate constants are subject to considerable error. A computation of this error poses certain mathematical problems, but it is probably a good guess to assume that the overall error is of the order of magnitude of $\pm 10\%$. This is largely due to the fact that the total change of radioactivity between the start and the end of each experiment amounts at best to about 80% of the original value. In many instances, especially at high chloride and sulfate concentrations in the medium, the total change of radioactivity in one single run may be as small as 30% of the initial value. It is obvious that the precise determination of the time course of such a small change requires an extraordinary accuracy in the determination of the radioactivity of the individual samples. Here, the main limiting factor is the reproducibility of the geometry of the counting arrangement. Furthermore, five additional and independent measurements (hematocrit, wet weight of 1 ml suspension, Cl, SO$_4$, and hydrogen ion concentration in the medium), each of which is subject to some error, enter into the calculation of flux, permeability constant, and SO_{4m}^{2-} of Eq. (1).

For the calculation of p and q in Eq. (1), it is necessary to know the individual ion activity coefficients of Cl$^-$ and SO$_4^{2-}$ in the medium. In our experiments, the electrolyte concentrations are beyond the range of the applicability of the Debye-Hückel theory. Hence, a semi-empirical method was adopted for an estimate of the activity coefficients. The mean activity coefficients $f_\pm$ of Cl$^-$ and SO$_4^{2-}$ were plotted against the ionic strength of pure NaCl or Na$_2$SO$_4$ solutions, respectively. Since the equilibrium concentrations of SO$_4^{2-}$ and Cl$^-$ in the medium of the erythrocyte suspension were analytically determined, it was possible to calculate the ionic strength which existed under our experimental conditions. This calculated value was used for reading off in the graph the mean activity coefficient which would have been obtained in pure NaCl or Na$_2$SO$_4$ solutions of the same ionic strength as in the supernatant of the cell suspension. Using the customary assumption $f_+ = f_-$, the individual activity coefficients were calculated by means of the equations $f_\pm = \sqrt{f_+ \cdot f_-}$ for NaCl, and $f_\pm = \sqrt{f_+ \cdot f_-^2}$ for Na$_2$SO$_4$.

Results

Theoretical Predictions of the Sulfate Ion Concentration within the Membrane by Means of Eq. (1)

According to Eq. (1), the sulfate concentration in the membrane, SO_{4m}^{2-}, is completely determined by the two variables $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$ and $p = (Cl^-)/\sqrt{(SO_4^{2-})}$. In a system where the solutions surrounding the two surfaces of the membrane are at Donnan equilibrium, p and q have the same numerical value, regardless of whether one inserts the sulfate, chloride, and hydroxyl ion concentrations on one side or the other of the membrane.
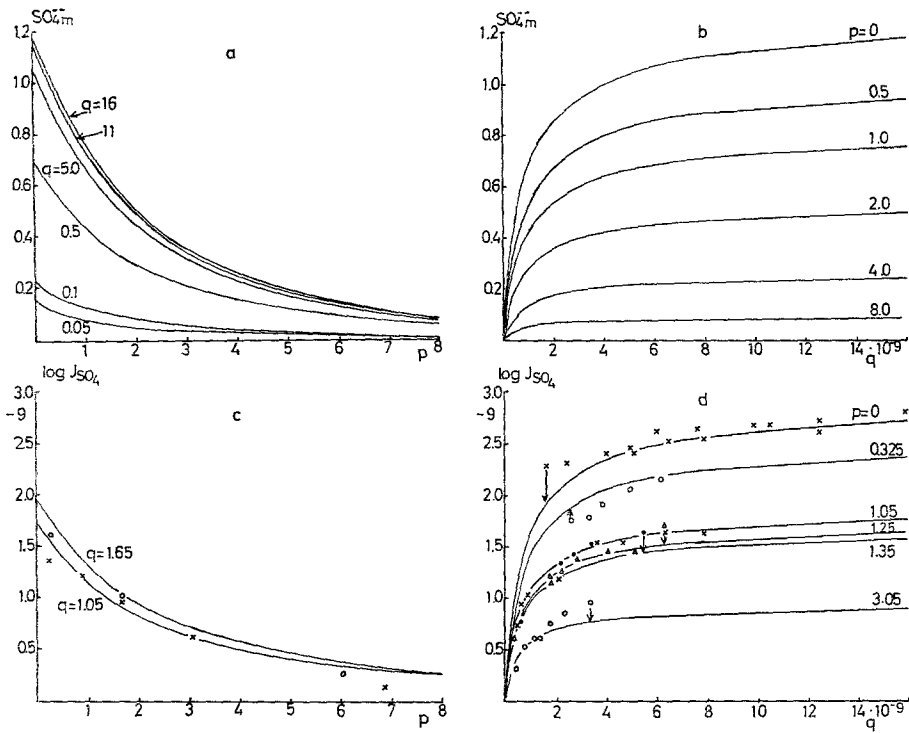
Fig. 2. (a) and (b) computed sulfate concentrations in the membrane and (c) and (d) corresponding computed sulfate fluxes as function of $p = (Cl^-)/\sqrt{(SO_4^{2-})}$ and $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$. The points represent actual measurements taken from the data presented in Table 1. The values for p and q were calculated from H^+, Cl^-, and SO_4^{2-} concentrations in the medium, using the method described on page 162 for the determination of activity coefficients. Eq. (1) was used for the determination of SO_{4m}^{2-} and Eq. (4) for an estimate of J_{SO_4}. At sufficiently high values of SO_{4m}^{2-}, $\log J_{SO_4}$ is nearly proportional to SO_{4m}^{2-}, as may be verified by taking the log of the left and the right hand sides of Eq. (4). Temperature: 27 °C. Ordinate: sulfate concentration within the membrane, SO_{4m}^{2-}, in moles/liter (Fig. 2a and b) and sulfate flux across the membrane J_{SO_4}, in moles/g/min. Abscissa: Fig. 2a and c: p; Fig. 2b and d: q. Parameters: Fig. 2a and c: $q \cdot 10^9$; Fig. 2b and d: p

Thus, SO_{4m}^{2-} can be calculated even if SO_4^{2-}, Cl^-, and OH^- concentrations are known in only one of the two solutions surrounding the membrane (Passow, 1965, 1969 a).

Eq. (1) is of the third order in $\sqrt{SO_{4m}^{2-}}$. It can be solved by approximation methods. We used an iteration procedure which was carried out by a programmable desk calculator.

Fig. 2 represents SO_{4m}^{2-} as a function of p at constant q's (Fig. 2a) and of q at constant p's (Fig. 2b). For the numerical computations, $\bar{A} = 2.5$ moles/liter and $K = 1.0 \cdot 10^{-9}$ moles/liter were inserted.

Fig. 2a shows that, at any given $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$, the sulfate concentration within the membrane decreases as $p = (Cl^-)/\sqrt{(SO_4^{2-})}$ increases. This means, for example, that at any given pH and sulfate concentration in the medium, an increase of the chloride concentration reduces SO_{4m}^{2-}, the sulfate concentration within the membrane. In other words, Cl^- ions compete with SO_4^{2-} ions for A^+, the fixed charges available at the given pH value. Furthermore, increasing q and consequently the proportion $A^+/\bar{A}$ leads to a limiting curve for the relationship between SO_{4m}^{2-} and p. At very high values of q, all potentially dissociable groups are protonated; $\bar{A} = A^+$. Under these conditions, the number of fixed charges for which the diffusible SO_4^{2-} and Cl^- ions can compete is virtually independent of pH. SO_{4m}^{2-} is solely determined by Cl^-/SO_4^{2-} competition for the maximal number of fixed charges.

The effects of pH on SO_{4m}^{2-} can be inferred from Fig. 2b. At constant p, with increasing q (e.g. with increasing H^+ at a constant SO_4^{2-} concentration in the medium) SO_{4m}^{2-} also increases. However, the curves tend to approach asymptotes that have positions which depend on p (e.g., at constant SO_4^{2-} concentration, the position of the asymptotes is a function of the Cl^- concentration in the medium).

At zero chloride concentration in the medium (i.e. at $p = 0$), SO_{4m}^{2-} assumes the value $1/2\, A^+$. Mobile sulfate ions provide all the counterions
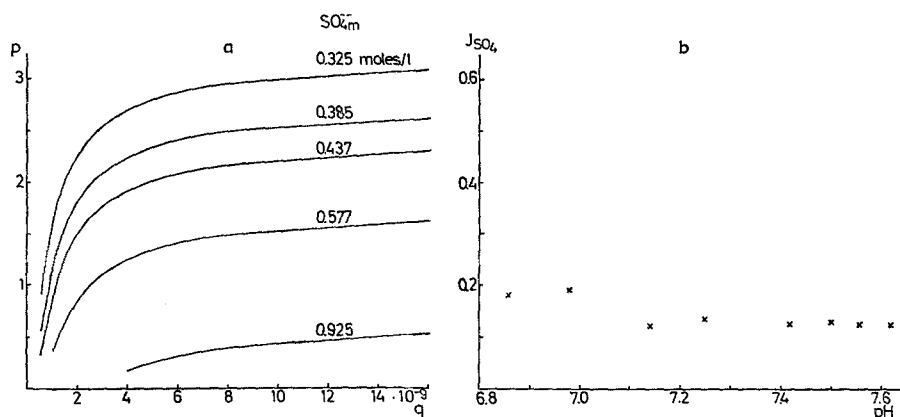

Fig. 3. (a) Pairs of $p = (Cl^-)/\sqrt{(SO_4^{2-})}$ and $q = (H^+)/\sqrt{(SO_4^{2-})}$ for which the sulfate concentration in the membrane, SO_{4m}^{2-}, assumes constant values. (b) Sulfate flux, J_{SO_4}, as a function of pH in the medium. Pairs of p and q were chosen such that SO_{4m}^{2-} and hence the fluxes were nearly constant in spite of relatively wide variations of pH in the medium and of the charge density in the membrane. $SO_{4m}^{2-} = 0.437$ moles/liter. Temperature: 32 °C. Beef erythrocytes. (Data taken from Lohmann, Passow & Privat, ref. in Lohmann, 1956.) Ordinate: sulfate flux in moles/cm²/min. Abscissa: pH in the medium

for the positive fixed charges within the membrane. A limiting case is reached when $A^+ = \bar{A}$, i.e. when the pH in the membrane is low enough to charge all the dissociable groups. Then, $SO_{4m}^{2-} = 1/2\ \bar{A}$.

Finally, (Fig. 3a), it is possible to calculate pairs of p's and q's which lead to the establishment of constant values of SO_{4m}^{2-}. In other words, various combinations of pH, SO_4^{2-} and Cl^- concentrations in the medium may lead to the same SO_4^{2-} concentrations in the membrane. This is due to the fact that, at a given sulfate concentration in the medium, SO_{4m}^{2-} may be adjusted in two ways: either (1) by the addition or removal of competing Cl^- ions at a given charge density A^+ in the membrane or, (2) at a given Cl^- concentration in the medium, by adding or removing H^+ ions and thus varying A^+. A superposition of the two effects may result in constant values of SO_{4m}^{2-}.

Eq. (1) is derived from the assumption that the charge density of the membrane is high enough to prevent coions, such as Na^+ and K^+, from entering the membrane. Hence, the equation may be used down to very low values of SO_{4m}^{2-} if SO_{4m}^{2-} is reduced by the addition of competing chloride ions. If, however, SO_{4m}^{2-} is lowered by shifting the equilibrium $A^+ = A + H^+$ far to the right, Eq. (1) loses its validity. Nevertheless, at electrolyte concentrations in the medium around 0.15 moles/liter, for $K = 10^{-9}$ the equation may still be employable up to pH 9.5 or so.

Experimental Results

Flux Measurements at 27 °C

The fixed charge hypothesis as stated in the introduction requires that J_{SO_4}, the sulfate flux across the membrane, is a function of SO_{4m}^{2-}, the calculated sulfate concentration within the membrane. SO_{4m}^{2-} in turn is, under our experimental conditions, a function of three variables, viz. the concentrations of Cl^-, OH^-, and SO_4^{2-} in the medium. Table 1 represents the results of flux measurements at arbitrarily chosen combinations of Cl^-, SO_4^{2-}, and hydrogen ion concentrations in the medium. It contains all necessary information for the computation of SO_{4m}^{2-}, Cl_m, and $A^+/\bar{A}$ by means of Eq. (1). In Fig. 4, the measured fluxes are plotted against the chloride (4c), sulfate (4b), and hydrogen (4a) ion concentrations in the respective media. It is immediately apparent that no single variable determines the penetration rate. However, if the fluxes are replotted against the calculated sulfate concentration in the membrane, all points tend to scatter

Table 1. *Sulfate fluxes (J_{SO_4}) as measured at Donnan equilibrium. pH_0, SO_{40}^{2-}, $Cl_0^- = pH$, sulfate and chloride concentrations in the supernatant of the cell suspensions in moles/liter* [a]

pH_0	SO_{40}^{2-} $\cdot 10^{-3}$	Cl_0^- $\cdot 10^{-3}$	r	SO_{4m}^{2-}	Cl_m^-	$\dfrac{A^+}{A}$	k	J_{SO_4}	$J_{SO_4}^*$
6.85	9.19	73.8	1.34	0.704	0.860	0.909	0.0234	$4.15 \cdot 10^{-8}$	$5.04 \cdot 10^{-8}$
6.91	7.80	71.4	1.93	0.670	0.876	0.886	0.0212	$4.13 \cdot 10^{-8}$	$4.30 \cdot 10^{-8}$
7.03	7.25	71.4	2.20	0.633	0.880	0.598	0.0173	$3.39 \cdot 10^{-8}$	$3.54 \cdot 10^{-8}$
7.15	7.77	70.2	1.94	0.614	0.824	0.820	0.0144	$2.82 \cdot 10^{-8}$	$3.23 \cdot 10^{-8}$
7.28	8.25	70.2	1.64	0.572	0.775	0.766	0.0117	$2.24 \cdot 10^{-8}$	$2.62 \cdot 10^{-8}$
7.41	8.53	69.6	1.60	0.564	0.737	0.731	0.0078	$1.47 \cdot 10^{-8}$	$2.32 \cdot 10^{-8}$
7.69	9.00	65.6	1.35	0.460	0.620	0.616	0.0048	$0.87 \cdot 10^{-8}$	$1.50 \cdot 10^{-8}$
7.92	7.95	64.2	1.94	0.357	0.563	0.510	0.0025	$0.49 \cdot 10^{-8}$	$0.89 \cdot 10^{-8}$
6.90	7.34	81.2	2.13	0.617	0.993	0.891	0.0262	$5.15 \cdot 10^{-8}$	$3.31 \cdot 10^{-8}$
6.99	7.35	77.2	2.13	0.614	0.981	0.885	0.0174	$3.42 \cdot 10^{-8}$	$3.23 \cdot 10^{-8}$
7.11	8.26	86.2	1.71	0.571	0.966	0.844	0.0147	$2.82 \cdot 10^{-8}$	$2.62 \cdot 10^{-8}$
7.28	8.65	86.2	1.51	0.518	0.834	0.748	0.0124	$2.34 \cdot 10^{-8}$	$2.01 \cdot 10^{-8}$
7.38	8.80	85.6	1.50	0.493	0.805	0.716	0.0096	$1.76 \cdot 10^{-8}$	$1.76 \cdot 10^{-8}$
7.49	9.58	86.2	1.45	0.474	0.825	0.709	0.0085	$1.44 \cdot 10^{-8}$	$1.60 \cdot 10^{-8}$
7.78	9.85	81.6	1.04	0.382	0.685	0.579	0.0079	$1.22 \cdot 10^{-8}$	$1.01 \cdot 10^{-8}$
8.01	9.89	79.0	1.09	0.318	0.601	0.494	0.0035	$1.54 \cdot 10^{-8}$	$0.73 \cdot 10^{-8}$
6.95	29.5	—	1.31	—	—	—	0.0314	$1.73 \cdot 10^{-7}$	—
7.02	26.5	39.7	1.61	0.963	0.326	0.901	0.0359	$2.05 \cdot 10^{-7}$	$1.83 \cdot 10^{-7}$
7.17	26.3	39.1	1.65	0.923	0.314	0.865	0.0241	$1.40 \cdot 10^{-7}$	$1.51 \cdot 10^{-7}$
7.28	28.0	40.0	1.41	0.891	0.311	0.837	0.0197	$1.13 \cdot 10^{-7}$	$1.30 \cdot 10^{-7}$
7.39	29.2	40.0	1.15	0.840	0.293	0.789	0.0156	$0.81 \cdot 10^{-7}$	$1.00 \cdot 10^{-7}$
7.51	26.3	40.6	1.12	0.781	0.301	0.746	0.0209	$0.93 \cdot 10^{-7}$	$0.75 \cdot 10^{-7}$
7.78	22.5	42.4	1.12	0.635	0.303	0.630	0.0065	$0.25 \cdot 10^{-7}$	$0.36 \cdot 10^{-7}$
8.01	19.5	44.0	0.74	0.500	0.298	0.518	0.0047	$0.11 \cdot 10^{-7}$	$0.18 \cdot 10^{-7}$
7.02	4.49	146.0	0.64	0.275	1.629	0.872	0.0133	$0.92 \cdot 10^{-8}$	$0.58 \cdot 10^{-8}$
7.19	4.72	148.8	0.53	0.253	1.550	0.821	0.0113	$0.70 \cdot 10^{-8}$	$0.52 \cdot 10^{-8}$
7.31	4.91	151.8	0.45	0.226	1.469	0.769	0.0100	$0.56 \cdot 10^{-8}$	$0.45 \cdot 10^{-8}$
7.42	4.99	154.6	0.41	0.208	1.431	0.738	0.0076	$0.41 \cdot 10^{-8}$	$0.41 \cdot 10^{-8}$
7.52	5.27	156.2	0.29	0.198	1.380	0.710	0.0096	$0.40 \cdot 10^{-8}$	$0.39 \cdot 10^{-8}$
7.69	5.43	152.8	0.22	0.178	1.251	0.642	0.0099	$0.34 \cdot 10^{-8}$	$0.35 \cdot 10^{-8}$
7.92	5.12	149.0	0.32	0.133	1.081	0.538	0.0047	$0.21 \cdot 10^{-8}$	$0.27 \cdot 10^{-8}$
8.16	5.23	145.0	0.30	0.104	0.921	0.451	0.0028	$0.11 \cdot 10^{-8}$	$0.22 \cdot 10^{-8}$
7.03	33.5	54.6	1.22	0.927	0.417	0.909	0.0392	$2.46 \cdot 10^{-7}$	$1.54 \cdot 10^{-7}$
7.11	32.2	52.0	1.36	0.924	0.391	0.895	0.0204	$1.34 \cdot 10^{-7}$	$1.48 \cdot 10^{-7}$

[a] These concentrations have been determined after establishment of Donnan equilibria between cell interior and medium, and prior to the isotopic flux measurements. $r =$ Donnan ratio for anion distribution between medium and cell water. SO_{4m}^{2-}, $Cl_m^- =$ computed sulfate and chloride concentrations in the cell membrane, respectively, in moles/liter [Eq. (1)], $\bar{A} = 2.5$, $K = 1 \cdot 10^{-9}$. $A^+/\bar{A} =$ degree of association of fixed charges. $k =$ rate constant (min^{-1}) for sulfate exchange (c.f. page 161). $J_{SO_4} =$ measured sulfate flux (moles/g/minute). $J_{SO_4}^* =$ computed sulfate flux [Eq. (4)]. In the figures presented in this Table, the last decimal is not significant. In order to avoid a cumulation of rounding off errors, they were, nevertheless, carried through the calculations.

Table 1 (continued)

| pH_0	SO_{40}^{2-} $\cdot 10^{-3}$	Cl_0^- $\cdot 10^{-3}$	r	SO_{4m}^{2-}	Cl_m^-	$\dfrac{A^+}{A}$	k	J_{SO_4}	$J_{SO_4}^*$
7.27	33.1	47.2	1.24	0.893	0.346	0.851	0.0131	$0.86 \cdot 10^{-7}$	$1.30 \cdot 10^{-7}$
7.37	32.9	47.8	1.26	0.852	0.342	0.819	0.0111	$0.72 \cdot 10^{-7}$	$1.07 \cdot 10^{-7}$
7.48	35.0	43.6	1.06	0.817	0.296	0.772	0.0096	$0.60 \cdot 10^{-7}$	$0.89 \cdot 10^{-7}$
7.59	36.1	44.8	0.93	0.780	0.294	0.741	0.0096	$0.56 \cdot 10^{-7}$	$0.74 \cdot 10^{-7}$
7.81	38.3	38.8	0.74	0.699	0.234	0.654	0.0081	$0.41 \cdot 10^{-7}$	$0.49 \cdot 10^{-7}$
8.01	35.8	35.8	0.96	0.584	0.203	0.594	0.0039	$0.23 \cdot 10^{-7}$	$0.28 \cdot 10^{-7}$
6.87	4.52	70.2	2.52	0.586	1.040	0.885	0.0325	$4.24 \cdot 10^{-8}$	$2.84 \cdot 10^{-8}$
6.92	4.16	70.2	2.59	0.556	1.060	0.869	0.0297	$3.78 \cdot 10^{-8}$	$2.44 \cdot 10^{-8}$
7.07	4.43	70.8	2.54	0.529	1.010	0.826	0.0253	$3.30 \cdot 10^{-8}$	$2.12 \cdot 10^{-8}$
7.18	4.67	71.4	2.29	0.490	0.960	0.776	0.0202	$2.64 \cdot 10^{-8}$	$1.75 \cdot 10^{-8}$
7.29	5.20	73.8	1.92	0.475	0.925	0.750	0.0164	$2.14 \cdot 10^{-8}$	$1.62 \cdot 10^{-8}$
7.41	5.76	73.8	1.52	0.460	0.865	0.714	0.0128	$1.63 \cdot 10^{-8}$	$1.50 \cdot 10^{-8}$
7.69	6.54	71.4	1.07	0.388	0.721	0.598	0.0125	$1.41 \cdot 10^{-8}$	$1.04 \cdot 10^{-8}$
7.90	6.97	69.2	0.86	0.330	0.625	0.514	0.0082	$0.83 \cdot 10^{-8}$	$0.77 \cdot 10^{-8}$
6.89	58.5	0.0	1.54	1.172	0.0	0.937	0.0493	$6.26 \cdot 10^{-7}$	$5.80 \cdot 10^{-7}$
7.00	59.5	0.0	1.50	1.153	0.0	0.922	0.0399	$5.13 \cdot 10^{-7}$	$4.76 \cdot 10^{-7}$
7.11	64.5	0.0	1.38	1.137	0.0	0.909	0.0352	$4.66 \cdot 10^{-7}$	$4.31 \cdot 10^{-7}$
7.20	61.0	0.0	1.16	1.110	0.0	0.886	0.0301	$3.36 \cdot 10^{-7}$	$3.81 \cdot 10^{-7}$
7.29	64.2	0.0	1.20	1.090	0.0	0.871	0.0286	$3.27 \cdot 10^{-7}$	$3.47 \cdot 10^{-7}$
7.39	62.1	0.0	1.11	1.058	0.0	0.845	0.0224	$2.45 \cdot 10^{-7}$	$2.92 \cdot 10^{-7}$
7.60	57.5	0.0	—	0.944	0.0	0.754	—	—	$1.67 \cdot 10^{-7}$
7.77	57.0	0.0	—	0.866	0.0	0.691	—	—	$1.13 \cdot 10^{-7}$
7.02	1.07	142.8	0.72	0.098	1.895	0.836	0.0097	$1.72 \cdot 10^{-9}$	$2.13 \cdot 10^{-9}$
7.20	1.04	155.6	0.78	0.072	—	—	0.0069	$1.24 \cdot 10^{-9}$	$1.76 \cdot 10^{-9}$
7.38	1.23	160.2	0.43	0.069	1.711	0.739	0.0104	$1.42 \cdot 10^{-9}$	$1.71 \cdot 10^{-9}$
7.49	1.25	159.2	0.37	0.063	1.620	0.698	0.0101	$1.25 \cdot 10^{-9}$	$1.62 \cdot 10^{-9}$
7.59	1.09	161.2	0.66	0.050	—	—	0.0038	$0.61 \cdot 10^{-9}$	$1.42 \cdot 10^{-9}$
7.72	1.31	159.4	0.27	0.040	1.261	0.537	0.0072	$0.68 \cdot 10^{-9}$	$1.26 \cdot 10^{-9}$
7.91	1.27	160.0	0.29	0.036	1.212	0.514	0.0046	$0.45 \cdot 10^{-9}$	$1.18 \cdot 10^{-9}$
8.25	1.41	151.8	0.09	0.027	0.939	0.397	0.0185	$0.67 \cdot 10^{-9}$	$1.00 \cdot 10^{-9}$
6.98	48.5	0.0	1.36	1.153	0.0	0.923	0.0413	$3.97 \cdot 10^{-7}$	$4.78 \cdot 10^{-7}$
7.05	47.5	0.0	1.34	1.144	0.0	0.915	0.0490	$4.68 \cdot 10^{-7}$	$4.52 \cdot 10^{-7}$
7.19	47.7	0.0	1.40	1.107	0.0	0.885	0.0454	$4.39 \cdot 10^{-7}$	$3.70 \cdot 10^{-7}$
7.30	50.0	0.0	1.36	1.070	0.0	0.856	0.0432	$4.21 \cdot 10^{-7}$	$3.12 \cdot 10^{-7}$
7.39	55.0	0.0	1.21	1.043	0.0	0.835	0.0268	$2.69 \cdot 10^{-7}$	$2.75 \cdot 10^{-7}$
7.48	54.1	0.0	1.09	0.984	0.0	0.786	0.0270	$2.48 \cdot 10^{-7}$	$2.02 \cdot 10^{-7}$
7.68	49.0	0.0	1.05	0.898	0.0	0.718	0.0255	$2.06 \cdot 10^{-7}$	$1.33 \cdot 10^{-7}$
7.85	45.2	0.0	0.80	0.798	0.0	0.638	0.0311	$1.88 \cdot 10^{-7}$	$0.81 \cdot 10^{-7}$
6.96	1.21	50.8	5.02	0.401	1.161	0.786	0.0357	$1.62 \cdot 10^{-8}$	$1.11 \cdot 10^{-9}$
7.04	1.26	49.2	4.66	0.400	1.091	0.759	0.0289	$1.35 \cdot 10^{-8}$	$1.11 \cdot 10^{-9}$
7.19	1.51	51.3	3.63	0.382	1.030	0.719	0.0202	$1.02 \cdot 10^{-8}$	$1.01 \cdot 10^{-9}$
7.38	1.92	53.2	2.34	0.322	0.867	0.606	0.0172	$0.91 \cdot 10^{-8}$	$0.74 \cdot 10^{-9}$
7.69	2.46	51.6	1.32	0.302	0.721	0.530	0.0133	$0.61 \cdot 10^{-8}$	$0.67 \cdot 10^{-9}$
7.99	2.82	50.4	0.78	0.236	0.581	0.422	0.0112	$0.39 \cdot 10^{-8}$	$0.47 \cdot 10^{-9}$
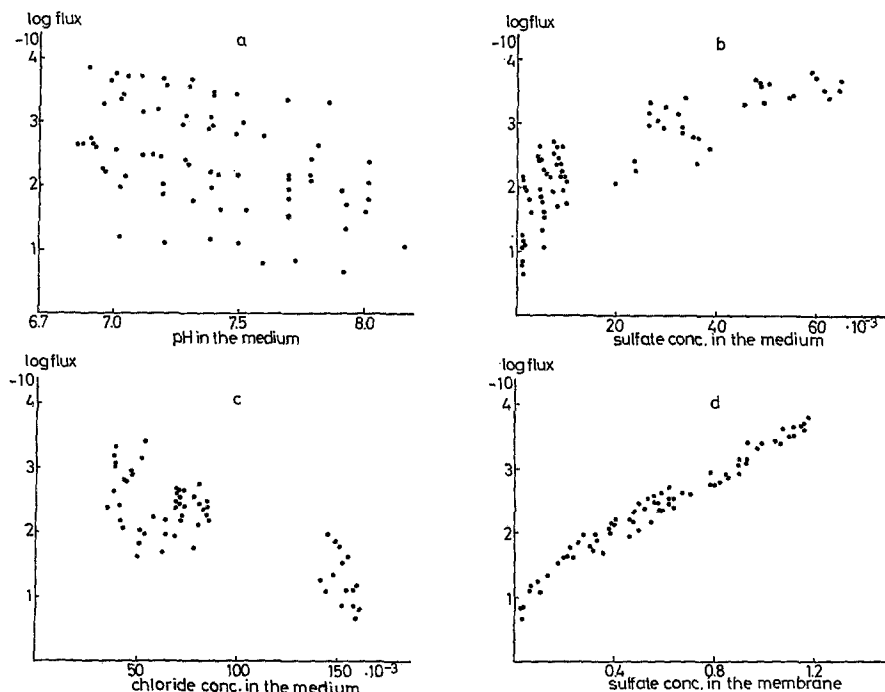
Fig. 4. The red cells were equilibrated in isotonic mixtures of NaCl, Na_2SO_4, sucrose, and either HCl or NaOH until the Donnan equilibrium was established. Subsequently, flux measurements were made by adding $^{35}SO_4$. Temperature: 27 °C. The fluxes are plotted against the various variables: pH, SO_4^{2-}, Cl^- in the medium. In Fig. 4(d), they are plotted against SO_{4m}^{2-}, the calculated sulfate concentration in the membrane [Eq. (1); $\bar{A}=2.5$, $K=10^{-9}$]. Ordinates: log J_{SO_4}, in moles/g/min. Abscissa: as indicated on the curves; concentrations in moles/liter

around a single curve for which $\bar{A}=2.5$ and $K=10^{-9}$ give the best fit[2]. This result suggests that, in accordance with the fixed charge hypothesis, SO_{4m}^{2-} is the true variable on which J_{SO_4} depends.

2 These values were obtained by trial and error: $\bar{A}$ and K were varied until a plot of J_{SO_4} against calculated values of SO_{4m} showed minimal scatter around a single curve. The scatter was estimated by eye. This qualitative procedure was adopted for the following reason: If experimental points scatter around an exponential curve, $y=a_0 \cdot e^{a_1 t}+a_2$, various combinations of the constants a_0, a_1, a_2 may yield equally good fits. If calculated by the method of least squares, the three constants are interdependent. Hence, it is not possible to assign unique values to them (Dennis, Wood & Sredni, 1969). In our case, an additional difficulty is introduced by the fact that the theory does not predict the exact mathematical relationship between J_{SO_4} and SO_{4m}. Eq. (4) is purely empirical and could possibly be replaced by others, e. g., by Eq. (8). If a statistical method based on the least square criterion would be employed for determining $\bar{A}$ and K then, in addition to the inherent ambiguities of the method, the result would also depend on the choice of the empirical equation to which the data were fitted. In spite of the described problems,

In Fig. 4d, the flux J_{SO_4} is plotted against SO_{4m}^{2-} on a logarithmic scale. Hence, the straight line relationship obtained at SO_{4m}^{2-} values greater than 0.2 moles/liter indicates that the data can be represented by the equation

$$J_{SO_4} = J_0 \cdot e^{a \, SO_{4m}^{2-}}. \tag{2}$$

At low SO_{4m}^{2-}'s deviations from this expression become apparent. Such deviations are to be expected since, as SO_{4m}^{2-} approaches zero, J_{SO_4} should also tend to zero, instead of to J_0, as implied by Eq. (2). It has been suggested therefore, that J_0 is a function of SO_{4m}^{2-} which, at SO_{4m}^{2-}'s above 0.2 moles/liter assumes a constant value. The expression

$$J_0 = c_I \cdot \frac{SO_{4m}^{2-}}{c_{II} + SO_{4m}^{2-}} \tag{3}$$

has been proposed as a tentative description of this behavior (c_I and c_{II} are empirical constants; Passow & Schnell, 1969). From the data represented in Fig. 4d it is possible to derive numerical values for the various constants involved. Using the method of least squares for the calculation of the slope of the linear portion of the curve relating log J_{SO_4} to SO_{4m}^{2-}, $a = 4.94$ and $c_I = 1.62 \cdot 10^{-9}$ were obtained. c_{II} cannot be determined with any degree of accuracy. A value of about 0.023 may serve as a very crude approximation.

In the last column of Table 1 are listed sulfate fluxes calculated from the equation:

$$J_{SO_4} = 1.62 \cdot 10^{-9} \frac{SO_{4m}^{2-}}{0.023 + SO_{4m}^{2-}} \cdot e^{4.94 \cdot SO_{4m}^{2-}}. \tag{4}$$

A comparison of the calculated values with the measured values, presented in the adjacent column of Table 1, provides a better indication of the accuracy of the predictions of J_{SO_4} by means of Eq. (4) than does Fig. 4 where the logarithmic scale tends to reduce the scatter of the flux data. It is apparent that in the whole range of anion concentrations and pH values covered in these experiments ($0 \leq Cl^- \leq 160$ mmoles/liter; $1.1 \leq SO_4^{2-} \leq$ 65 mmoles/liter; $6.85 \leq pH \leq 8.25$) more than 1,000-fold variation of sulfate flux can be roughly described by Eq. (4). Thereby, four (or, at low SO_{4m}^{2-}'s five) constants are necessary to represent the functional relationship between the flux as the dependent variable and the three independent variables which describe the composition of the medium. Two of the constants, a and c_I,

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we derived the constants J_0 and a by conventional curve fitting techniques. In these cases, the computer program yielded some information about the errors involved. Since the significance of this information is not really understood, no error estimates are included in the text. These considerations emphasize that we are only able to show the compatibility of our data with the fixed charge hypothesis. The data may also be compatible with entirely different hypotheses.

may be arbitrarily chosen since their physical meaning is unknown. The two remaining ones, $\bar{A}$ and K, must lie within a range compatible with our knowledge of the properties of ion exchangers. These constants must not be negative or unreasonably large or small. Our value of $\bar{A} = 2.5$ moles/liter for the fixed charge density is well within the limits of the charge densities achievable in ion exchange resins and $K = 1 \cdot 10^{-9}$ could refer to the dissociation of fixed amino groups.

Although Eqs. (1) and (4) represent the general pattern of the interrelationship between J_{SO_4} and the composition of the medium, the differences between calculated and measured values of J_{SO_4} may occasionally be considerable. In part, this reflects the fact that the flux measurements are not too accurate (c.f. page 162). Nevertheless, a closer inspection of the data in Table 1 reveals that perhaps not all the scatter of the experimental points represented in Fig. 4d is attributable to experimental error. At equal values of SO_{4m}^{2-}, there seems to be a tendency of the fluxes to increase with increasing Cl_m^- or A^+. In other words, the flux tends to higher values if a desired SO_{4m}^{2-} is established by competitive replacement of SO_4^{2-} ions with Cl^- ions in a highly charged membrane rather than by reducing the fixed charge density through increasing the pH. Our data shows 13 instances where two or three fluxes were measured at virtually identical SO_{4m}^{2-}'s. Only in two out of these 13 instances did J_{SO_4} decrease with increasing Cl_m^- or increasing $A^+/\bar{A}$. In all other cases, there was an increase of J_{SO_4} with increasing Cl_m^- or increasing charge density.

Fig. 4 represents the sulfate flux as a function of arbitrary combinations of three independent variables, viz. the sulfate, chloride, and hydroxyl ion concentrations in the medium. The results plotted in Fig. 4c enabled us to deduce Eq. (4) which, in conjunction with Eq. (1) allows the prediction of the sulfate flux from determinations of the ion composition of the medium. A more rigorous test of the applicability or the limitations of Eqs. (1) and (4) requires the consideration of limiting cases. The following pages will be devoted to a discussion of a number of experimental conditions under which only one or two of the three independent variables are varied while the others are kept constant, or where such combinations of the three independent variables are established that the dependent variable, J_{SO_4}, assumes a constant value. In addition, flux data will be considered which are obtained under conditions where one of the "natural" variables, $p = (Cl^-)/\sqrt{(SO_4^{2-})}$ or $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$, is varied while the other is maintained at a constant value.

J_{SO_4} as a Function of pH at Constant Cl^- or SO_4^{2-} Concentrations in the Medium. In Fig. 4a, J_{SO_4} was plotted as a function of pH. The data showed
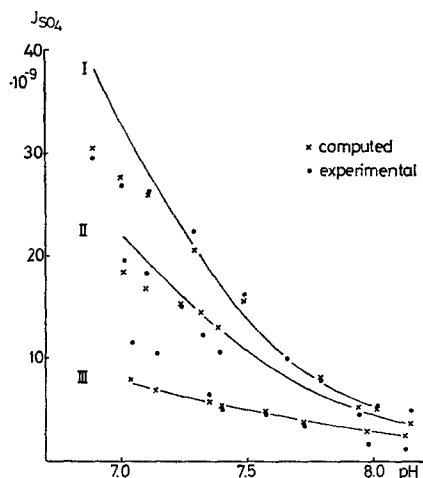
Fig. 5. Sulfate flux as a function of pH in the medium at various constant values of $p = (Cl^-)/\sqrt{(SO_4^{2-})}$. The curves were calculated for $p = 27.3$ (I), 34.4 (II), and 57.2 (III), respectively, by means of Eq. (1) and the equation

$$J_{SO_4} = 1.23 \cdot 10^{-9} \cdot e^{5.34\,SO_{4m}^{2-}}.$$

In view of the complex interrelationships between anion distribution and intracellular buffering, we were unable to establish equilibrium distributions with exactly predetermined values of p. Hence, in our actual measurements, p was not strictly constant. The curves represent only approximations to the actual experimental conditions. They were calculated from the respective mean values of the Cl^- and SO_4^{2-} concentrations established in each of the three sets of experiments. The crosses represent flux measurements calculated on the basis of the pertaining exact analytical data on SO_4^{2-}, Cl^-, and H^+ concentrations in the media. The points represent measured fluxes which correspond to the calculated values. Temperature: 25.2 °C. Ordinate: sulfate flux in moles/g/min.

Abscissa: pH

a random scatter. In these experiments, all possible combinations of pH, SO_4^{2-}, and Cl^- concentrations in the medium were allowed. However, if the pH is varied at fixed concentrations of SO_4^{2-} and Cl^- in the medium, Eqs. (1) and (4) predict an increase of J_{SO_4} with decreasing pH. The experimental results are in qualitative agreement with this prediction. In terms of the fixed charge hypothesis, this would mean that the concentration of the fixed charges and hence of SO_{4m}^{2-} increases as the medium is acidified. Increasing the chloride concentration reduces J_{SO_4} at all pH values. This is again in agreement with the hypothesis which stipulates a Cl^-/SO_4^{2-} competition for the fixed charges (Fig. 5).

J_{SO_4} *as a Function of the Ratio* SO_4^{2-}/Cl^- *at Constant pH.* An essential feature of kinetic studies is the investigation of the relationship between flux and concentration. One way of varying the sulfate concentration in the
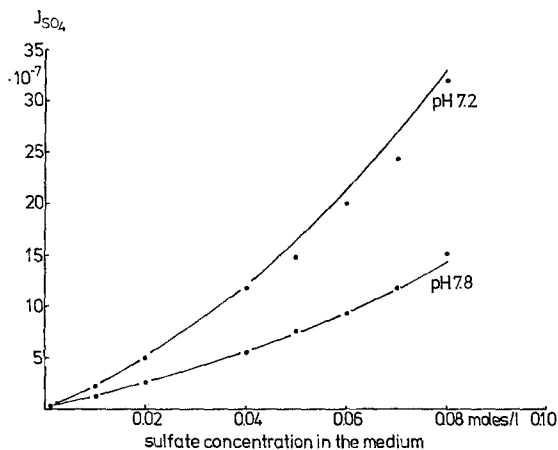
Fig. 6. Sulfate flux, J_{SO_4}, as a function of sulfate concentration in the medium at two pH values. In contrast to all other measurements presented in this paper, J_{SO_4} was determined by following the back exchange of radiosulfate from $^{35}SO_4$ preloaded red blood cells. Details of the technique are described by Passow (1971). The media in which the back exchange was measured contained a fixed concentration of Tris-Cl (66 mmoles/liter) and sucrose (20 mmoles/liter) and varying proportions of Na_2SO_4 and NaCl. The sulfate concentration was varied by replacing each 166 mmoles/liter NaCl with 132 mmoles/liter Na_2SO_4. The equilibrium concentrations which were established in the final suspensions were determined by chemical analysis of the supernatant. The cell density was 2.5 vol%. Temperature: 37 °C. The continuous curves are calculated by means of Eqs. (1)–(3) using $\bar{A}=2.5$ moles/liter, $K=1 \cdot 10^{-9}$, $c_I=4.17 \cdot 10^{-8}$, $c_{II}=0.023$, $a=4.70$. The points represent measured values. Ordinate: sulfate flux in moles/g/min. Abscissa: sulfate concentration in the medium in moles/liter

medium consists of replacing NaCl by osmotically equivalent amounts of Na_2SO_4. Under this condition, the competition between SO_4^{2-} and Cl^- for the fixed charges in the membrane varies considerably. Since the divalent sulfate ions are more effective in competing for the fixed charges than the monovalent chloride ions, one would expect a more than linear increase of J_{SO_4} with decreasing Cl^-. In addition, the exponential relationship between J_{SO_4} and SO_{4m}^{2-} should amplify this effect. Therefore one would anticipate a more than proportional increase of J_{SO_4} if the SO_4^{2-} concentration is raised at the expense of Cl^- in the medium. This is actually observed (Fig. 6). If the charge density in the membrane is lowered by an increase of the pH (i.e. by shifting the equilibrium $A^+ = A + H^+$ to the right), J_{SO_4} decreases at any given SO_4^{2-}/Cl^- ratio in the medium and the curvature of the relationship between flux and concentration is diminished. Again, this prediction is confirmed by the experiment depicted in Fig. 6.

The continuous lines in Fig. 6 represent values calculated by the equation

$$J_{SO_4} = 4.17 \cdot 10^{-8} \cdot \frac{SO_{4m}^{2-}}{0.023 + SO_4^{2-}} \cdot e^{4.7 \cdot SO_{4m}^{2-}}. \tag{5}$$

The numerical values of the constants apply to a temperature of 37 °C. They are different from those used for the representation of the data in Fig. 4 and Table 1. The value $c_1 = 4.17 \cdot 10^{-8}$ is significantly higher than the value $1.62 \cdot 10^{-9}$ observed at 27 °C. It is questionable, however, whether the difference between the measured values of a (4.94 at 27 °C, 4.70 at 37 °C) is significant. A more detailed discussion of the temperature dependence of J_{SO_4} follows on page 174.

Combinations of the Three Independent Variables (SO_4^{2-}, Cl^-, and OH^-) Which Yield Constant Values of the Dependent Variable, J_{SO_4}. Eq. (4) predicts that at constant SO_{4m}^{2-} the sulfate flux should also be constant. This is verified by the experiment presented in Fig. 3 b. In this experiment, pH and chloride concentration in the medium were varied in such a manner that SO_{4m}^{2-} remained constant in spite of large variations of A^+ and of the Cl^-/SO_{4m}^{2-} competition for the electrical equivalents of A^+. Typically, the two points at the lowest pH values tend to be higher than the others. These two measurements were performed at a high charge density where the concentration of chloride ions within the membrane is greater than at high pH values where the charge density is lower. Thus, this experiment seems to represent another example for the slight facilitation of sulfate penetration by the presence of monovalent anions within the membrane. In contrast to all other experiments discussed in the present paper, these data were obtained with beef red blood cells.

J_{SO_4} *as a Function of p at constant q or of q at constant p.* J_{SO_4} is a function of three variables, the sulfate, chloride, and hydroxyl ion concentration in the medium. SO_{4m}^{2-} can only be calculated by means of Eq. (1) from the numerical values of these variables if the system is at Donnan equilibrium. Hence, there exists a constraint which restricts the choice of combinations of the three variables. If any two of them are adjusted to arbitrarily chosen values, then the third assumes that value which is compatible with the establishment of the Donnan equilibrium for the anions. For this reason, it is possible to transform the relationship between SO_{4m}^{2-} and the concentrations of the anion species in the medium into an expression in which only two independent variables appear explicitly. The limiting relationships between SO_{4m}^{2-} and $p = (Cl^-)/\sqrt{(SO_4^{2-})}$ at constant $q = (H^+) \cdot \sqrt{(SO_4^{2-})}$, and of q at constant p, have already been represented in Fig. 2a and b. Fig. 2c and d

show that the measured values of J_{SO_4} follow the predicted pattern. However, it is also apparent that the scatter of the measured points is relatively large. These figures show more clearly than the others the limitations of the quantitative description of our data by Eqs. (1) and (4).

Temperature Dependence of SO_4^{2-} Flux

The apparent activation enthalpy, E_A, of SO_4^{2-} movements at Donnan equilibrium was measured at various pH values and ionic strengths. E_A seems to be nearly constant over the limited temperature range of 22 to 37 °C. There was a tendency of the point obtained at the lowest temperature to fall below the straight line anticipated for constant E_A in an Arrhenius plot; however, it is not clear if this deviation is really significant. The temperature coefficient was independent of ionic composition and pH of the medium (Figs. 7 and 8).
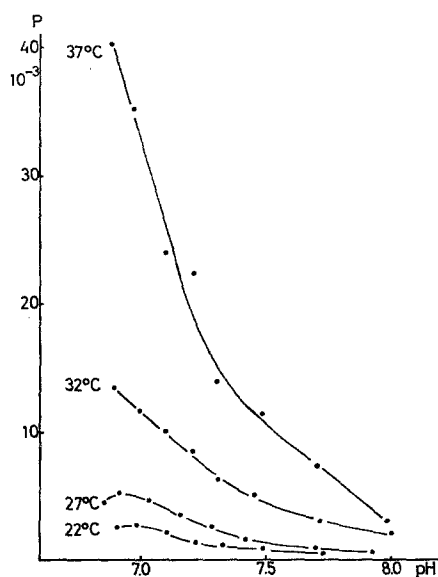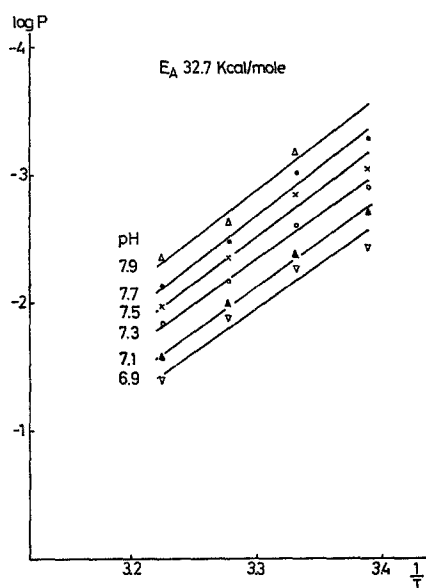

Fig. 7   Fig. 8

Fig. 7. Effect of temperature on pH-dependence of sulfate permeability. Ordinate: permeability constant (min^{-1}) measured with $^{35}SO_4$ at Donnan equilibrium. Human red blood cells. SO_4^{2-} concentration: 5.2 mmoles; Cl^- concentration: 55 mmoles. Sucrose was added to maintain isotonicity. Abscissa: pH

Fig. 8. Arrhenius plot of data taken from Fig. 7. The points were obtained by interpolation from the curves plotted in that figure. The slopes of the straight lines represent an apparent activation energy of 32.7 kcal/mole. Ordinate: log of permeability constant. Abscissa: inverse of absolute temperature

The question arises as to how the five constants, a, c_I, c_{II}, $\bar{A}$ and K, occuring in Eqs. (1)–(3) are affected by temperature changes. The data are not accurate enough to allow a conclusive answer; however, a few tentative inferences can be drawn.

In view of the exponential increase of J_{SO_4} with SO_{4m}^{2-}, small changes of SO_{4m}^{2-} should express themselves in drastic alterations of J_{SO_4}. At fixed sulfate, chloride, and hydroxyl ion concentrations in the medium, SO_{4m}^{2-} is determined by the concentration of fixed charges in the membrane which in turn is a function of temperature. If the fixed charges are NH_3^+ groups as stipulated above, an increase of temperature should bring about a shift of the equilibrium $A^+ = A + H^+$ to the right which is associated with an enthalpy change ΔH of about 9,000 to 12,000 cal/mole. Using Eq. (1) for the same type of analysis of our data as described in the previous section, one would anticipate that it should be possible to determine changes of K, the dissociation constant of the fixed charges, with temperature. However, such changes could not be detected. This puzzling fact made us doubt the validity of the inference that protonated amino groups provide the fixed charges which participate in the control of SO_4^{2-} permeability. Yet, a quantitative assessment of the situation suggests that it would be premature to base such doubts on this fact only.

In Eq. (1), the equilibrium constant K appears on the right hand side only

$$2\,SO_{4m}^{2-} + p \cdot f \cdot \sqrt{SO_{4m}^{2-}} = \bar{A} \frac{q}{K\sqrt{f_{SO_{4m}}\,SO_{4m}^{2-}} + q}. \tag{1}$$

Since $K \cdot \sqrt{f_{SO_{4m}}\,SO_{4m}^{2-}}$ represents an additive term besides q, variations of K by a certain percentage do not necessarily lead to a change of the left hand term of Eq. (1) by the same percentage. In particular, if $K\sqrt{f_{SO_{4m}}} \cdot SO_{4m}^{2-}$ is smaller than q, considerable changes of K may have relatively little effect on the right hand term of Eq. (1) and hence on $SO_{4\,m}$. This is true for our experimental conditions.

Assuming $K = 1 \cdot 10^{-9}$ for 27 °C and $\Delta H = 12,000$ cal/mole, it is possible to calculate K for 22 and 37 °C, the respective limits of our temperature range. At 22 °C, $K = 1.62 \cdot 10^{-9}$; at 37 °C, $K = 0.52 \cdot 10^{-9}$. In the bulk of our experiments, at all temperatures the q values exceed the corresponding values of $K\sqrt{f_{SO_{4m}}\,SO_{4m}^{2-}}$. Hence, the threefold variation of K with temperature has comparatively little effect on the calculated SO_{4m}^{2-}.

The values for $\bar{A}$ and K are determined by trial and error. The criterion for the right choice of the two constants is the minimalization of the scatter of the experimental points in plots of $\log J_{SO_4}$ against SO_{4m}^{2-} as represented

in Fig. 4. This procedure is rather inaccurate and thus the relatively minor effects of large variations of K on SO_{4m}^{2-} may have escaped detection. Moreover, variations of temperature may affect the amount of water within the membrane and thus have some influence on $\bar{A}$. This would further complicate the interpretation of the effects of temperature. In summary, under our experimental conditions, the measured E_A is probably little affected by temperature dependent variations of the distribution coefficient membrane/medium. Thus, the E_A mainly reflects the temperature dependence of a or c_I in Eqs. (2) and (3). A tentative evaluation of our data suggests that c_I is more dependent on temperature than a (c.f. page 173).

Discussion

The experiments described in this paper were designed with the limited aim of testing to what extent the kinetics of passive anion permeability could be explained on the basis of an anion exchange model of the red blood cell membrane. The tacit assumption was made that the rate of penetration is limited by "gel kinetics" (diffusion within the membrane) rather than "film kinetics" (diffusion across an adhering layer of unstirred water molecules). The justification of this assumption was derived from the finding that (1) the temperature coefficient for the penetration across the membrane is several times higher than the temperature coefficient for ion diffusion in aqueous solution, and (2) varying rates of stirring have no detectable effect on anion flux (*unpublished results*). Thus, the anion flux was considered to be a function of the concentration of diffusible ions within the ion exchange region of the membrane.

Assuming that an ion exchange equilibrium between membrane and medium was established, Eq. (1) was derived for calculating the sulfate ion concentration within the anion exchange region of the membrane from the known anion composition and pH of the medium. Experimentally, it was found that in suspension media of widely differing composition more than 1,000-fold variations of SO_4^{2-} flux are uniquely related to the calculated sulfate concentration in the membrane. Thus, the fixed charge hypothesis yields one possible explanation for the kinetics of anion penetration across the erythrocyte membrane.

In spite of the enormous range of its applicability, Eq. (1) is likely to oversimplify a much more complicated situation. Some of the more obvious shortcomings of Eq. (1) have already been mentioned in a previous review on this subject (Passow, 1969 *a*). Nevertheless, it seems appropriate to list

them again in the present context and to emphasize additional ones as follows:

1. The distribution of anions between membrane and adjacent media is calculated on the assumption that Donnan equilibria are established at the interfaces and that electrical neutrality occurs within the membrane. Donnan equilibria and electrical neutrality can only be expected to exist in macroscopic phases, i.e. in phases whose dimensions are large as compared to the Debye length. In thin membranes, the Poisson equation should replace the equation of electroneutrality ($A^+ = SO_{4m}^{2-} + Cl^-$) and suitable adaptations of double layer theory should be employed for the calculation of ion distribution between membrane and medium.

2. In the derivation of Eq. (1) the thermodynamically ambiguous individual ion activity coefficients have been used instead of the more rigorously defined mean ion activity coefficients (for a discussion of the applicability of the individual ion activities to the calculation of ion exchange equilibria *see* Reichenberg, 1966). In the absence of a better method, a rather questionable one has been employed for the calculation of individual ion activities in the external suspension medium (*see* page 162 for a description of this method). In Eq. (1), the activity coefficients inside the membrane appear in two places: on the left hand side in the form of the ratio $f = f_{Cl_m}/\sqrt{f_{SO_{4m}}}$ and on the right hand side in the form of the product $K \cdot \sqrt{f_{SO_{4m}}}$. Since nothing is known about the structure of the membrane, its dielectric constant, charge distribution, counter ion association with fixed charges etc., no theoretical estimates of f_{Cl_m} or $f_{SO_{4m}}$ were attempted. Instead, the *ratio f* was arbitrarily assumed to be independent of the ion composition of the membrane phase and set equal to 1.0. Moreover, by the method described on page 168 one estimates the product $K \cdot \sqrt{f_{SO_4}}$ rather than K. The square root tends to reduce concentration dependent variations of f_{SO_4} and hence, may escape detection.

Since the activity coefficients take into account special properties of individual ion species, it should be possible to test the influence of our simplifications by measuring sulfate exchange in the presence of ion species other than chloride. There is some data available in the literature on the effects of various monovalent ion species on phosphate permeation (Deuticke, 1967) which obeys similar rules as sulfate penetration (Passow, 1964; Deuticke, 1970). Deuticke's data seem to indicate that replacing chloride with nitrate and other anions has some influence on phosphate flux. However, replacing part of the sulfate in the medium by a variety of other divalent anions (including succinate, phosphate, malonate, and others) has a much

smaller or no detectable effect on the ratio sulfate flux: sulfate concentration in the membrane (*unpublished results*). Similar observations were made by Deuticke (1967) who studied the influence of replacing inorganic phosphate by sulfate on phosphate flux. However, more detailed studies on this question are clearly required before any definite conclusions can be drawn.

3. A further simplification was introduced in our theoretical treatment by the assumption that the dissociation constant, K, of the fixed charges is independent of the ionic composition of the membrane and of variations of the density of the fixed charges with pH.

4. It was tacitly assumed that the anion exchange region of the red cell membrane is homogeneous. There is no evidence to this effect. Moreover, the degree of swelling of the membrane should be a function of the pH and the Cl^-/SO_4^{2-} ratio. Consequently, $\bar{A}$ should vary with the composition of the medium. Yet, possible effects of variations of $\bar{A}$ have been completely ignored.

The above list of shortcomings of Eq. (1) is incomplete. Nevertheless, it suffices to show that a further refinement of the mathematical evaluation of our kinetic data would require a large number of very detailed assumptions about the structure and physical properties of the ion permeable channels. There is little if any evidence available which could be used as a sound basis for such assumptions. Hence, for the time being, Eq. (1) may serve as a semiquantitative formulation of the predictions of the fixed charge hypothesis about the dependence of sulfate concentration in the membrane on the concentrations of sulfate, chloride, and hydroxyl ions in the medium.

The observation of a simple functional relationship between J_{SO_4} and SO_{4m}^{2-} as calculated by means of Eq. (1) justifies the proposal of the fixed charge hypothesis as one possible explanation of the kinetics of anion permeability of the red cell membrane. However, the finding of an exponential relationship between the two variables is by no means a necessary corollary of that hypothesis and needs a separate interpretation.

The observed exponential relationship would suggest that there exists a cooperative facilitation of SO_4^{2-} penetration by sulfate ions in the membrane. If the ion exchange region were the rate-determining barrier which responds to changes of SO_{4m}^{2-} with cooperative variations of its structure, then one would anticipate a saturation of the effect as the sulfate ion concentration in the membrane approaches saturation of the fixed charges. In other words, one would expect an *s*-shaped rather than an exponential curve to relate J_{SO_4} with SO_{4m}^{2-}. Yet, the exponential increase of sulfate flux continues to increase with increasing values of SO_{4m}^{2-} up to the point where all the

chloride ions within the membrane are replaced by sulfate ions or where, in the absence of chloride, protonation of the dissociated fixed groups reaches its maximal value. Such a behavior is difficult to reconcile with the assumption that the diffusional resistance of the membrane exclusively resides in the fixed charge region of the membrane. Therefore, it would seem necessary to postulate that the anions penetrate through two layers in series. They first equilibrate with an anion exchange region and then pass through a second, rate-limiting barrier. Thereby, the anion exchange region serves as some sort of a "parking lot" which determines the concentration of diffusible sulfate ions in front of that rate-limiting barrier. This inference is in keeping with a number of other observations which were discussed elsewhere (Passow, 1969 a, b).

On the basis of the limited data presented in this paper, it would seem premature to speculate about the physical mechanisms which could possibly be responsible for the cooperative facilitation of sulfate penetration by divalent anions at the rate-limiting barrier. Nevertheless, it seems worthwhile to point out that Eqs. (2) and (3) can be rewritten in the following form:

$$J_{SO_4} = \frac{SO_{4m}^{2-}}{c_{II} + SO_{4m}^{2-}} \cdot v_{max} \tag{6}$$

where

$$v_{max} = c_I \cdot e^{a \cdot SO_{4m}^{2-}}. \tag{7}$$

According to Eq. (6), the sulfate flux follows saturation kinetics. Eq. (7) implies that not only the degree of saturation of the sulfate channels but also v_{max} is a function of SO_{4m}^{2-}. In other words, the number of sulfate channels available for sulfate penetration, or the rate at which a sulfate ion penetrates across the rate-limiting barrier, or both are functions of the sulfate concentration in the membrane. These implications lead us to adopt Eqs. (6) and (7) for a description of our experimental data. It would also be possible to use other expressions. For example, Dr. K. F. Schnell has called to our attention that instead of Eqs. (2) and (3), our data could be fitted equally well to the expression

$$J_{SO_4} = k\left(e^{a\,SO_{4m}^{2-}} - 1\right) \tag{8}$$

where k is a constant.

The facilitation of sulfate flux by sulfate ions is possibly related to the charge of the diffusible anions. It has already been pointed out above that replacing SO_4^{2-} with equivalent amounts of other bivalent anions does not affect the quotient J_{SO_4}/SO_4^{2-}, (i.e. the permeability constant for sulfate).

Under these conditions, the total concentration of bivalent anions within the membrane remains constant. If monovalent anions, such as chloride, are used as a substitute for SO_4^{2-} in the membrane, the fluxes are reduced more than proportional to the reduction of SO_{4m}^{2-}. Nevertheless, the data presented above (page 170) suggest that, in spite of this effect, monovalent ions still facilitate SO_4^{2-} movements across the membrane at constant SO_{4m}^{2-}. If this were so, one could conclude that any increase of the number of mobile negative charges within the membrane facilitates SO_4^{2-} movements, but that divalent anions are much more effective than monovalent ones.

The failure to observe the expected variation with temperature of the dissociation constant of the fixed charges, K, raises the question whether the dissociable fixed charges are amino groups as inferred from the numerical value of their pH, or if other groups with a lower heat of dissociation (e. g., imidazole groups) are involved. In view of the uncertainties associated with the fact that we are only able to determine $K \cdot \sqrt{f_{SO_{4m}}}$ instead of K alone, it would seem conceivable that K is lower than estimated above. In addition, the information necessary to calculate the intrinsic dissociation constant from K is not available. It would be possible to identify definitely the dissociable groups only if the intrinsic constants were known.

In the case of penetration across a homogeneous ion exchange membrane by gel kinetics, the E_A contains at least two contributions: (1) the heat of dissociation of the fixed charges (which participate in the determination of the distribution equilibria of the diffusible anions between membrane and medium) and (2) the activation enthalpy of the diffusion process within the ion exchanger. Since variations of temperature induce considerable changes of A^+ and hence of SO_{4m}^{2-}, one would anticipate that the heat of dissociation of the fixed charges constitutes a negative contribution to the apparent activation energy. The magnitude of this contribution should depend on pH. It should be negligible at very low or very high pH values where temperature changes produce only an insignificant change of the concentration of fixed charges. It should have a large effect at intermediate pH values. Since the heat of dissociation of amino groups (which we suppose to act as fixed charges) is about 9 to 13 kcal/mole (Greenstein & Winitz, 1961), one would anticipate that its contribution to the overall E_A should be demonstrable in experiments on the pH dependence of E_A. Yet, E_A was found to be virtually independent of pH.

It is not yet clear whether the absence of the anticipated effects is simply due to the insensitivity of the method employed in determining K or whether other factors are involved. In any event, the magnitude of the apparent activation energy — about 30 kcal/mole — far exceeds the values customarily

observed in ion exchange resins and thus suggests that the rate-determining barrier is not identical with the fixed charge region.

The experiments presented in this paper show that many features of the kinetics of sulfate permeability can be explained by the fixed charge hypothesis. If fixed charges were in fact involved in the control of anion permeability one would expect that they also govern the movements of the more rapidly penetrating halide anions. In nerve and muscle, the pH dependence of chloride permeability is inverse to that described above for sulfate and chloride concentration of the medium (for a discussion of the pertinent literature *see* Passow, 1969*b*). This finding is not compatible with the fixed charge model as employed in the present paper. It raises the question whether the essential role of fixed charges in the control of anion permeability is an exception which only applies to the red cell or if the halide anions penetrate across the red cell membrane predominantly (although not exclusively) through a channel which is parallel to the pathway of sulfate permeation and similar to the chloride channel in nerve and muscle.

References

Dennis, W. H., Wood, Ph., Sredni, J. 1969. *Abstr. Comm. III Int. Biophys. Congr.* (Cambridge, Mass.). p. 232.

Deuticke, B. 1967. Über die Kinetik der Phosphat-Permeation in den Menschen-Erythrozyten bei Variation von extrazellulärer Phosphat-Konzentration, Anionen-Milieu und Zell-Volumen. *Pflüg. Arch. Ges. Physiol.* **296**:21.

— 1970. Anion permeability of the red blood cell. *Naturwissenschaften* **57**:172.

Dodge, J. T., Mitchell, C., Hanahan, D. 1962. The preparation and chemical characterization of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119.

Gardos, G., Hoffman, J. F., Passow, H. 1969. Flux measurements in erythrocytes. *In*: Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. p. 9. Springer-Verlag, Berlin-Heidelberg-New York.

Greenstein, J. P., Winnitz, M. 1961. The Chemistry of Amino Acids. John Wiley & Sons, Inc., New York.

LaCelle, P. L., Passow, H. 1966. Effects of variations of pH and ionic strength on anion permeability of the erythrocyte membrane. *Pflüg. Arch. Ges. Physiol.* **291**:R15.

Lang, K. 1937. Eine Verbesserung der Chlorid-Bestimmung im Blut. *Biochem. Z.* **290**:289.

Lohmann, H. 1960. Möglichkeiten und Grenzen der Anwendbarkeit der 'Fixed Charge Theory' zur Erklärung des Mechanismus der Anionenpermeabilität roter Blutkörperchen. M. D. Thesis. Hamburg.

Morgulis, G., Hemphill, M. 1932. An iodometric method for the determination of sulphate in biological materials. *J. Biol. Chem.* **96**:573.

Passow, H. 1965. Passive ion permeability and the concept of fixed charges. *Proc. XXIII Int. Physiol. Sci. Congr.*, Tokyo., *Int. Congr. Ser., Excerpta Med. Amst.* **87**:555.

— 1969a. Passive ion permeability of the red blood cell. An assessment of the scope and limitations of the fixed charge concept. *Progr. Biophys. Mol. Biol.* **19**:pt. II, 423.

— 1969b. The molecular basis of ion discrimination in the red blood cell membrane. *In*: The Molecular Basis of Membrane Function. D. C. Tosteson, editor. p. 319. Prentice Hall, Inc., Englewood Cliffs, N.J.

— 1971. Effects of pronase on passive ion permeability of the human red blood cell. *J. Membrane Biol.*, b (*in press*).

— Schnell, K. F. 1969. Chemical modifiers of passive ion permeability of the erythrocyte membrane. *Experientia* **25**:460.

Reichenberg, D. 1966. Ion exchange selectivity. *In*: Ion Exchange. J. A. Marinsky, editor. p. 227. M. Dekker, Inc., New York.

Sanui, H., Carvalho, A. P., Pace, N. 1962. Relationship of hydrogen ion binding to sodium and potassium binding by rat liver cell microsomes and human erythrocyte ghosts. *J. Cell. Comp. Physiol.* **59**:241.