J. Physiol. (1970), **207**, *pp.* 581–609
With 7 text-figures
Printed in Great Britain

EFFECT OF SOME MONOVALENT ANIONS ON CHLORIDE AND SULPHATE PERMEABILITY OF HUMAN RED CELLS

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(*Received 25 August 1969*)

SUMMARY

1. The permeability of human red cells to $^{36}Cl^-$ and to $[^{35}S]SO_4^{2-}$ was studied in the presence of various monovalent anions.

2. A maximum decrease of anion permeability was found in a study of the steady-state exchange of ^{36}Cl in a medium containing 120 mM salicylate. The exchange had a half-time of 3 hr at 0° C, a reduction of normal chloride permeability by a factor of 10^5. The activation energy of chloride exchange decreased from a value of 45 to 22 kcal/mole in the interval between 0 and 10° C. Simultaneous determination of the permeability to potassium and chloride proved that salicylate induced a reversal of the normal selectivity of red cells at 0° C (permeability coefficient P_K of $3{\cdot}5 \times 10^{-9}$ cm/sec to be compared with a P_{Cl} of 2×10^{-9} cm/sec).

3. In contradistinction to the slow movement of ^{36}Cl, the exchange of [^{14}C]salicylate was completed within 4 min, when red cells were suspended at 0° C in the salicylate medium.

4. A study of the sulphate permeability at 38° C showed that the rate of steady-state exchange decreased, when chloride was replaced by lyotropic anions other than bromide. The sequence of the permeability decrease was: $Cl^- = Br^- < I^- < NO_3 < SCN^-$ < salicylate, the same sequence which previously has been shown to increase the permeability to sodium and potassium. The activation energies of sulphate exchange were 32 kcal/mole (chloride medium), and 38 kcal/mole (thiocyanate medium).

5. Sufficient data were obtained during the study to demonstrate that when equilibrium has been obtained, there is a good agreement between the values of ^{36}Cl (cell water)/^{36}Cl (extracellular water) and of $\{[^{35}S]SO_4$ (cell water)/$[^{35}S]SO_4$ (extracellular water)$\}^{\frac{1}{2}}$.

6. It is concluded that the anion-induced changes of permeability are due to binding of anions to fixed cationic charges in the red cell membrane.

INTRODUCTION

The present study was carried out to examine whether red cell permeability to sulphate and chloride is influenced by the presence of those monovalent anions, which have been shown to modify red cell permeability to sodium and potassium (Funder & Wieth, 1967*b*; Wieth, 1970*a*). It is demonstrated that the anions of the lyotropic series which cause an increased permeability to cations: ($Cl^- = Br^- < NO_3^- < I^- < SCN^-$ < salicylate) produce a graded decrease of the permeability towards sulphate. An extreme reduction of chloride permeability was brought about by salicylate. In a medium containing 120 mM salicylate the permeability to potassium exceeded the permeability to chloride by a factor of almost two at 0° C, in fact causing a reversal of the permselectivity of human red cells, which prefer chloride to potassium by a factor of 10^6 under normal conditions (Tosteson, 1959). In the preceding article (Wieth, 1970*a*) it was suggested that changes of permselectivity are due to binding of adsorbable anions to fixed cationic charges in the membrane, a process which is increased at low temperatures. The results of the present investigation of anion permeability are compatible with the adsorption hypothesis. In another study (Wieth, 1970*b*) it is shown that a lyotropic series of monovalent cations do not exert effects on the ion permeability of red cells comparable to those caused by the anions. Therefore it is considered likely that the interaction between anions and membrane components is of an electrostatic nature. The experimental results will be discussed in relation to the evidence suggesting that positive fixed charges (most likely to be amino groups) control the ion permeability of the red cell (Passow, 1965). It is concluded that the lipoprotein membrane model of Korn (1968) possesses the properties required for the interpretation of the anion effects.

METHODS

All experiments with incubated red cells were performed under close control of temperature and pH by the techniques described by Funder & Wieth (1967*a*) and by Wieth (1970*a*).

Chemicals

The *electrolyte media* employed for washing and incubation of the cells had the following composition: Na 142 mM, K 3·7 mM, Ca 1·5 mM, Mg 1 mM, HCO_3 22 mM, Cl 6·5 mM, phosphate 1·1 mM, and X^- 120 mM, X^- representing one of the following monovalent anions: Cl^-, Br^-, NO_3^-, I^-, SCN^- or $C_6H_5(OH)COO^-$. For the study of sulphate exchange the media additionally contained 1 mM-Na_2SO_4, in order to provide carrier sulphate for the isotope $[^{35}S]SO_4$. All media contained 5 mM glucose. pH was adjusted to 7·4 at the appropriate temperature by titration with CO_2. Reference to the various media is made as, e.g. 'chloride medium', meaning the medium in which 120 mM of X^- is made up by chloride.

The following modifications were introduced in the preparation of cells for incubation in salicylate medium. Washing of cells was performed at 25° C, and after each

resuspension of cells the suspension was left for 10–20 min in order to provide time for the salicylate/chloride exchange. The cells were washed 4 times before the final resuspension. Chemical analyses of chloride in cells and medium were made by electrometric titration with 0·001 M-$AgNO_3$ (Funder & Wieth, 1966). The results (viz. Table 1) showed that equilibration of chloride was achieved before the start of experiments.

The cell suspensions employed for the determination of sulphate exchange at 38° C were pre-equilibrated at 38° C for 1–3 hr before starting the experiment by addition of [^{35}S]SO_4. An equilibrium distribution of sulphate was thereby obtained before the addition of the carrier-free tracer. When the slow sulphate exchange was followed at temperatures lower than 38° C, it was not possible to pre-equilibrate the cells. However, the distribution of ^{36}Cl turned out to be stable during all the experiments. Further the kinetics of tracer sulphate exchange (Tables 5, 6 and 7) provided no basis for assuming that sulphate distribution was not stationary during the experiments, presumably because the sulphate concentration of the medium was close to the concentration in normal plasma (1 mM).

Isotopes. ^{36}Cl was obtained as HCl with a specific activity of 0·018 mc/m-mole (Philip-Duphar, Holland). The amount of activity employed for the determination of ^{36}Cl influx into red cells was 0·02 μc/ml. cell suspension, corresponding to the addition of approximately 1 m-equiv chloride/l. cell suspension. In the sulphate exchange expts. 0·006 μc ^{36}Cl was added per ml. cell suspension, in order to enable us to record the chloride distribution between cells and medium.

[$^{14}C_7$]salicylic acid was delivered by Philips-Duphar, Holland. The specific activity was 35·7 mc/m-mole. Before use the acid was dissolved in 5 mM-NaOH. The activity employed was 0·08 μc/ml. cell suspension.

^{35}S was obtained from AEK, Risø Denmark as carrier-free [^{35}S]SO_4^{2-} in 0·01 M-HCl. The amount of radioactivity employed for experiments was 0·1–0·2 μc/ml. cell suspension.

^{42}K (AEK, Risø) had a specific activity of 1 mc/m-mole K on delivery, and was used within 24 hr. The amount of activity employed in the two experiments of Table 4 was 0·3 and 1 μc/ml. cell suspension.

Determination of radioactivity. Separation of the activity derived from ^{42}K and ^{36}Cl respectively followed the procedure of Wieth (1969). The same reference described the precipitation of cells and medium for β-spectroscopy. ^{36}Cl could be counted without interference from the weaker radiation of ^{35}S (Tricarb liquid scintillation spectrometer model 3324), and, because the relative amounts of radioactivity employed were favourable, the β-radiation of ^{35}S, counted in the energy range of 0·05–0·17 MeV could easily be corrected for the radiation of ^{36}Cl in that range. The recovery of [^{35}S]SO_4 added to red cell lysate together with ^{36}Cl was 99·6 % (S.E. of mean 0·9, n = 6).

[7-^{14}C]salicylic acid was prepared for counting in the following way: 10 ml. concentrated HCl was dissolved in 90 ml. ethanol (99 %). 1 ml. HCl–ethanol reagent was added to the weighed sample containing 100–200 mg of medium, cell suspension, or red cells. After mixing the samples were centrifuged and 100 μl. of the supernatant was transferred to a counting vial. The recovery of [7-^{14}C]salicylate added to lysed red cells was almost complete (98·5 %, S.E. of mean 0·6, n = 8).

Calculations

Rate of anion exchange. As demonstrated in the Results section there was reasonable basis for treating the fluxes of both chloride and sulphate as a steady-state exchange in a two-compartment system. The time course of the specific activity of the cells was well described by the equation

$$a = a_\infty(1 - e^{-bt}), \qquad (1)$$

where a is the specific activity of chloride or sulphate at the time of sampling, a_∞ is the specific activity in cells and medium at isotopic equilibrium, and the rate constant b is related to k, the rate constant of cellular chloride or sulphate exchange, by the equation

$$b = \left(\frac{S_1+S_2}{S_1}\right) k, \tag{2}$$

where S_1 and S_2 represent the total amount of chloride (or sulphate) in the extracellular and intracellular compartments. Following calculation of the regression line

$$\ln\left(1-\frac{a}{a_\infty}\right) = -bt+A \tag{3}$$

by the method of least squares the rate constant of anion exchange could be determined by means of eqn. (2) (viz. Tables 3, 5, and 6). Ideally A, the interception with the ordinate, should be zero at $t = 0$, and in fact none of the values of A reported in Tables 3, 5 and 6 differed significantly from zero. To be able to calculate the specific activity of cell sulphate in those experiments in which the study of sulphate exchange was not continued until isotopic equilibrium had been achieved, the intracellular sulphate concentration was calculated from the chloride distribution. The experimental results showed that the following relation applied to the equilibrium distribution of the anions (Table 8):

$$\begin{aligned} r_{Cl} &= \text{Cl (cell water)/Cl (extracellular water)} \\ &= (\text{SO}_4 \text{ (cell water)/SO}_4 \text{ (extracellular water)})^{\frac{1}{2}}. \end{aligned}$$

Because the extracellular sulphate concentration was 1 mM, sulphate concentration in cell water was equal to $(r_{Cl})^2$.

Activation energy of anion exchange. The temperature dependence of anion fluxes of red cells suspended in various media was evaluated by means of Arrhenius plots as shown in Figs. 3 and 7. Because of the curvature of the graph of ln k vs. $1/T$ in Fig. 3, the activation energy could not be calculated by linear regression analysis, and instead the following equation was employed (Exner, 1964).

$$E = \frac{RT_1T_2}{T_1-T_2}(\ln k_1 - \ln k_2), \tag{4}$$

where E is the activation energy at the mean temperature $\frac{1}{2}(T_1+T_2)$. T_1 and T_2 are the upper and lower absolute temperatures, R is the gas constant, and k_1 and k_2 are the rate constants of chloride exchange at the temperatures T_1 and T_2. The activation energies of sulphate exchange were calculated by linear regression analysis of the Arrhenius plots shown in Fig. 7.

Membrane permeability to Cl^- and K^+. The permeability coefficients of chloride and potassium were calculated from the data presented in Tables 3 and 4, making the simplifying assumption that the electrical field through the membrane is constant (Goldman, 1943). According to Katz (1966)

$$P_x = \frac{M_{12}}{X_1 \cdot f_\psi} \text{ (cm/sec)}, \tag{5}$$

where P_x is the permeability coefficient of the monovalent ion species X, M_{12} is the unidirectional flux from compartment 1 to 2 of the ion (mole/cm^2.sec), X_1 is the concentration of the ion in the water phase of compartment 1 (mole/cm^3), and f_ψ is the factor representing the effect of the electrical field in lowering or raising the chances

of permeation of individual ions. The value of f_ψ was calculated according to Katz (1966)

$$f_\psi = \frac{EF/RT}{1-\exp(-EF/RT)} \quad (6)$$

where E is the potential difference across the membrane, its sign being taken as positive when the ion movement is assisted, and negative when the movement is opposed by the force of the electrical field, F is Faraday's number and RT the product of the gas constant and the absolute temperature.

RESULTS

1. *The effect of salicylate on chloride fluxes at 0° C*

The results illustrated by Fig. 1 show that chloride permeability was very low when red cells were suspended in the salicylate medium at 0° C. The influx of radioactive chloride was followed for almost 11 hr, and the Figure shows the increasing activity found in the water phase of the cells, accompanied by a decrease of extracellular radioactivity. Chemical analysis of chloride in cells and medium was performed on all samples (Table 1). The mean value of the distribution ratio of chloride between intra- and extracellular water was 0·68 (S.E. 0·01), and the data proved that there was a steady-state distribution of chloride during the determination of ^{36}Cl exchange.

The rate constant of cellular chloride exchange was 0·24 hr^{-1} in the experiment shown in Fig. 1, and values of 0·228 and 0·229 hr^{-1} were found in experiments with cells from another donor (cf. Table 3). This means that the half-time of chloride exchange is 3 hr, to be compared with values of 0·1–0·2 sec measured at 21° C on cells suspended in plasma (Luckner, 1939) or in phosphate buffered saline solution (Tosteson, 1959).

Another point, illustrated by Table 2, is the fact that the cations sodium and potassium exchange across the cell membrane at an appreciable rate in the salicylate medium (Wieth, 1970*a*). During the first 4 hr of the experiment the net fluxes of potassium and sodium were of almost identical magnitudes, and the water content of the cells did not change. In the last 4·5 hr there was a net flux of water into the cells, due to the fact that the accumulation of sodium continued, at a time when the potassium loss had almost ceased. With the swelling of the cells haemolysis also made its appearance, as evidenced by the haemoglobin liberated from the cells to the medium.

In conclusion, it appears that the salicylate ion possesses the ability to change the properties of the red cell membrane so that the cation permeability is increased (Wieth, 1970*a*), whereas the chloride permeability is drastically reduced. A quantitative comparison of chloride and potassium permeability is presented in section 3 of the present work.

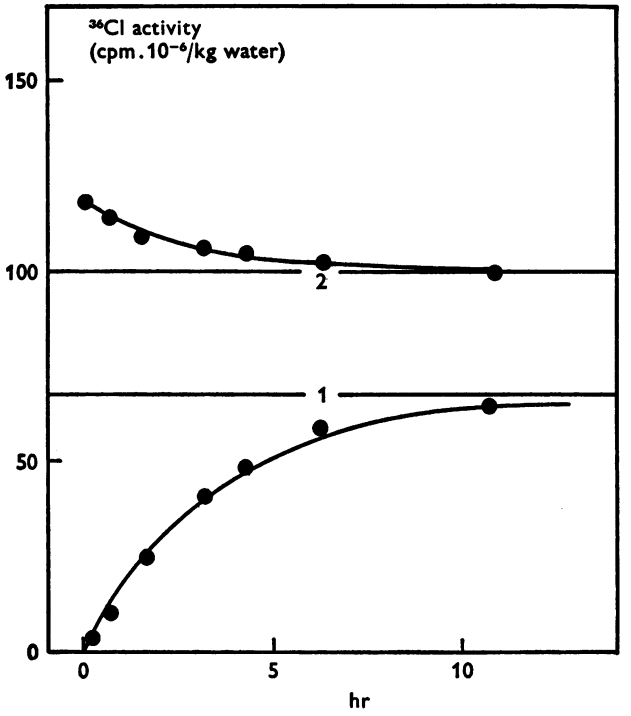

Fig. 1. The steady-state exchange of radioactive chloride between salicylate medium (upper graph) and human red cells (lower graph) at 0° C, pH 7·40. The lines 1 and 2 indicate the equilibrium levels of ^{36}Cl activity in cell water, and in the extracellular water phase calculated from the results of chemical analyses (shown in Table 1).

TABLE 1. Chemical analyses of chloride in the water phases of cells and medium during the exchange of radioactive chloride (^{36}Cl) illustrated in Fig. 1. The cells were suspended at 0° C in the salicylate medium described in the Methods section. Donor J.T., pH 7·40. Data concerning net fluxes of water, potassium and sodium are presented in Table 2

Time (hr)	Chloride (m-equiv/kg water)		Chloride distribution r_{Cl} Cl (cell water) / Cl (extracellular water)
	Medium	Cells	
0·07	9·83	6·99	0·711
0·60	9·74	6·96	0·715
1·58	9·88	6·85	0·693
3·07	10·15	6·64	0·654
4·22	9·95	6·59	0·662
6·13	9·89	6·25	0·632
10·7	10·03	6·60	0·668
Mean	9·92	6·70	0·676
S.E. of mean	0·06	0·10	0·012

TABLE 2. Net movements of potassium, sodium and water during incubation in the salicylate medium at 0° C. (Results from the experiment shown in Fig. 1)

Time (hr)	Haemoglobin (g/100 ml. suspension)	Haematocrit (relative cell volume)	Haemoglobin in extracellular phase (g/100 ml. medium)	Cell water content (g H_2O/kg solids)	Cell K (m-equiv/kg solids)	Cell Na (m-equiv/kg solids)	Cell [K]+[Na] (m-equiv/kg solids)
0·07	10·7	0·273	0·060	1710	222·7	37·0	259·7
0·60	10·4	0·279	0·054	1653	204·1	49·3	253·4
1·58	10·4	0·273	0·057	1653	166·9	92·1	259·0
3·07	10·5	0·273	0·067	1696	115·4	142·0	257·4
4·22	10·4	0·276	0·075	1725	95·3	161·9	257·2
6·13	10·4	0·285	0·105	1817	79·3	191·7	271·0
10·7	10·0	0·290	0·142	2021	74·4	229·6	304·0

An attempt was made to determine the red cell permeability to [$^{14}C_7$]-salicylate at 0° C. However, equilibrium was obtained within 4 min in the salicylate medium, and the distribution of salicylate between cell water and medium remained constant in the following 4 hr:

$$^{14}C_{\text{cell water}}/^{14}C_{\text{medium}} = 1{\cdot}25; \quad \text{S.E. of mean } 0{\cdot}01, \quad n = 6,$$

indicating that a considerable amount of salicylate was bound to intracellular proteins. In the chloride medium the intracellular salicylate concentration was 91 % of the equilibrium value in the sample isolated 3·5 min after the addition of isotope. After 30 min the distribution of salicylate between cell water and medium was stable during the following 3 hr:

$$^{14}C_{\text{cell water}}/^{14}C_{\text{medium}} = 2{\cdot}36; \quad \text{S.E. of mean } 0{\cdot}06, \quad n = 4.$$

The results clearly indicate that salicylate does not cause a universal reduction of anion permeability. As discussed later these findings are believed to indicate that chloride and salicylate ions compete for the same path during their penetration of the cell membrane.

2. *The temperature dependence of chloride exchange*

It has previously been shown that sodium and potassium permeabilities exhibit a paradoxical temperature dependence in the presence of thiocyanate or salicylate. An increase of temperature from 0° C caused a pronounced decrease of both potassium and sodium fluxes through the membranes of red cells suspended in the aforementioned media (Wieth, 1970*a*). It was therefore of interest to learn how chloride exchange is affected by temperature. Fig. 2 shows the time course of the approach to isotopic equilibrium in a series of experiments carried out on red cells in salicylate media between 0 and 10° C. The calculated regression lines are shown in Table 3 together with the derived rate constant of chloride exchange, and the mean value and S.E. of the chloride distribution ratio. The rate of chloride exchange increased by a factor of almost 10 in the interval between 0 and 10° C. It appears from Fig. 3 that the Arrhenius activation energy of chloride exchange decreased with increasing temperature. Calculated as indicated in the Methods section, it was 44·8 kcal/mole in the temperature interval between 0 and 4° C to be compared with 34·5 and 22·4 kcal/mole in the temperature intervals between 4 and 6·5 and between 6·5 and 10° C.

The experimental technique employed was not suited for determining a more rapid exchange than that found at 10° C. The exchange between cells and extracellular phase was completed in the very first sample, 3 min after the addition of the isotope, when ^{36}Cl was added to the salicylate medium at 28° C. During the following 3 hr the chloride distribution ratio was

constant (range 0·51–0·54). As mentioned in the Methods section this finding provides the explanation of the fact that a complete exchange of chloride and salicylate can be obtained by washing the cells at room temperature before incubation in a cold medium.

Although the effect of thiocyanate on fluxes of sodium and potassium is considerably smaller than that of salicylate, the qualitative effects of the two anions are very similar (Wieth, 1970*a*) cf. Fig. 5. It was therefore

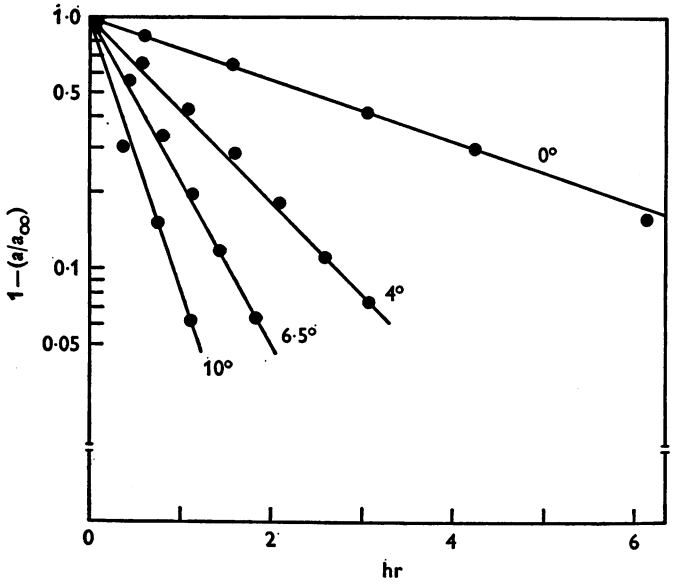

Fig. 2. The rate of steady-state exchange of ^{36}Cl between salicylate medium and human red cells in the interval between 0 and 10° C. Details about the calculation of the straight lines from the specific activity at the time of sampling (a) and the specific activity at isotopic equilibrium (a_∞) are given in Table 3 (expts. 1, 4, 5, 6).

examined whether the rate of ^{36}Cl exchange could be determined with the present 'slow' technique, when red cells were incubated at 0° C in the thiocyanate medium. Again a complete equilibration of ^{36}Cl between cells and medium was found in the first sample isolated 4 min after the addition of ^{36}Cl. The chloride distribution ratio of the first sample was 0·614, to be compared with a mean value of 0·627 (S.E. of mean 0·01, $n = 5$) during the first 20 hr of incubation. Therefore a more slowly permeating anion (sulphate) was employed to study the effect of thiocyanate and other monovalent anions on the anion permeability of red cells as described in sections 4 and 5.

TABLE 3. The dependence of ^{36}Cl exchange on temperature.

The rate constants of chloride exchange were determined by calculating the least square regression lines of the relation

$$\ln (1-a/a_\infty) = -bt+A,*$$

where a is the specific activity of cell chloride at the time of sampling, a_∞ is the specific activity of chloride at isotopic equilibrium, and t is the time (hr). The interception (A) was not significantly different from zero in any of the experiments. The relation between the regression coefficient (b) and the rate constant (k) is described in the Methods section. The mean value of six to nine determinations of chloride in extra- and intracellular water, and of the distribution ratio (r_{Cl}) of each experiment is presented in the right-hand columns of the Table. S.E. of mean is in parentheses

			Statistics of straight line*				Rate constant of cellular chloride exchange (hr^{-1})	Chloride (m-equiv/kg water)		
Expt. no.	°C	Duration (hr)	A Interception with ordinate at $t = 0$	b Regression coefficient (hr^{-1})	S.D. of regression coefficient	r Correlation coefficient		Medium	Cells	r_{Cl}
1	0	10·7	−0·002	0·285	0·006	−0·999	0·244	9·92 (0·06)	6·70 (0·10)	0·68 (0·01)
2	0	6·5	−0·002	0·298	0·007	−0·998	0·228	—	—	—
3	0	9·8	−0·031	0·268	0·004	−0·999	0·229	8·95 (0·04)	6·87 (0·06)	0·77 (0·01)
4	4	3·1	0·019	0·859	0·02	−0·998	0·77	8·77 (0·08)	5·71 (0·09)	0·65 (0·01)
5	6·5	3·1	−0·007	1·53	0·06	−0·995	1·35	7·98 (0·07)	6·49 (0·17)	0·81 (0·02)
6	10	3·1	−0·028	2·58	0·19	−0·994	2·22	8·60 (0·06)	5·23 (0·06)	0·61 (0·01)

3. *Potassium and chloride permeability in salicylate medium*

One of the most remarkable properties of the normal red cell membrane is its high permeability to small anions as compared with cations. This permselectivity is profoundly changed, when erythrocytes are incubated in the salicylate medium at 0° C. Table 4 shows the determinations of unidirectional potassium fluxes, calculated from potassium net fluxes and the influx of ^{42}K. Fluxes in m-mole/kg solids × hr were converted to mole/cm^2 × sec by multiplication with $0{\cdot}57 \times 10^{-14}$ (Funder & Wieth, 1967*a*).

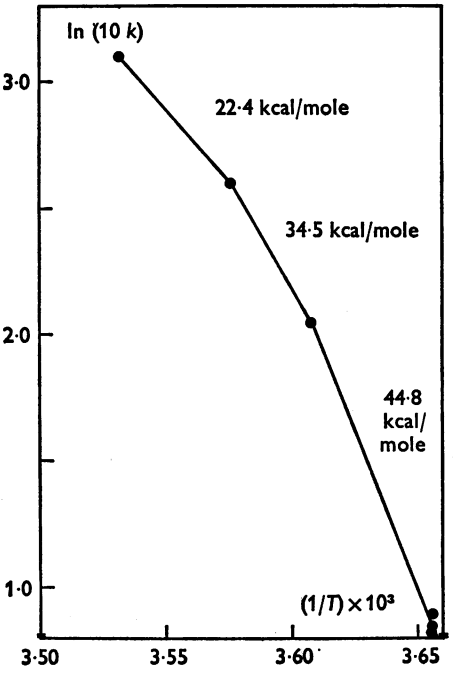

Fig. 3. Arrhenius diagram of the natural logarithm of the rate constant (k) *vs.* the reciprocal absolute temperature ($1/T$) employed for calculating the activation energies indicated on the graph, as described in the Methods section (the results were derived from the experiments shown in Table 3).

The mean value of the rate constant of potassium efflux, determined during six periods in experiment 3 was $0{\cdot}32\,hr^{-1}$ (S.E. of mean 0·01) and during three periods in expt. 7, $0{\cdot}29\,hr^{-1}$ (S.E. of mean 0·02), to be compared with a rate constant of chloride exchange of $0{\cdot}23\,hr^{-1}$ (Table 3). The rate constants of potassium and of chloride efflux are of the same magnitude, but also the driving force of the potential difference must be considered, if the permeabilities of the ions are to be compared. The membrane potential ($\psi_i - \psi_o$) was calculated by means of the Nernst equation from the mean

TABLE 4. Unidirectional potassium fluxes of human red cells suspended in the salicylate medium at 0° C, pH 7·40, donor J. W. The chloride permeability of the red cells was also determined in experiment 3 (cf. Table 3).

M_o = efflux, M_{in} = influx, r_{Cl} = chloride (cell water)/chloride (extracellular water).

The r_{Cl} values stated are those found at the beginning of each period. f_ψ is the factor employed to correct P_K for the effect of the membrane potential, assuming a constant electrical field opposing or assisting the movement of a charged particle through the membrane, cf. Methods section

Period (hr)	Mean potassium concentration (m-equiv/kg H_2O)		Potassium fluxes (mole/cm².sec) × 10^{13}				Permeability coefficient $P_K \times 10^9$ (cm/sec)	
	Intracellular	Extracellular	M_o	M_{in}	r_{Cl}	f_ψ	From M_o	From M_{in}
Expt. 3								
0·08–0·53	132·2	8·65	4·10	0·40	0·810		3·56	2·98
0·53–1·07	114·6	11·85	3·54	0·43	0·759		3·55	3·18
1·07–2·07	94·3	16·3	3·00	0·61	0·747		3·66	3·26
2·07–3·07	73·0	21·5	2·55	0·91	0·762		4·02	3·69
3·07–4·07	58·7	25·4	2·07	0·96	0·766		4·05	3·28
4·07–5·50	48·1	28·3	1·39	0·90	0·761		3·32	3·26
					0·772	0·87		
Expt. 8								
0·08–0·57	134·1	6·8	3·52	0·21	0·762		3·26	2·70
0·57–1·07	116·2	8·85	3·79	0·41	0·714		3·84	3·98
1·07–1·83	95·9	12·3	3·31	0·60	0·721		4·06	4·27
					0·777	0·85		
						Mean	3·70	3·40
						s.E. of mean	0·10	0·16

values of the chloride distribution ratios shown in Table 4, yielding the results of $-6{\cdot}1$ and $-7{\cdot}4$ mV in the two experiments. It may seem illogical to calculate the membrane potential from the chloride distribution in a situation where the chloride permeability is very low. However, the distribution of chloride between cells and medium must represent an equilibrium distribution, because there was no net flux of chloride for periods corresponding to 2–5 half-times of chloride exchange, as shown by the results of Tables 1 and 3. Therefore it was a reasonable assumption that the membrane potential was equal to the equilibrium potential of the chloride ions. The permeability coefficient of potassium was calculated as indicated in the Methods section, assuming a constant electrical field through the membrane. From the efflux of potassium a mean value of $3{\cdot}7 \times 10^{-9}$ cm/sec was found in the nine periods of the two experiments. The value calculated from potassium influx was slightly smaller ($3{\cdot}4 \times 10^{-9}$ cm/sec), but the difference was not statistically significant ($0{\cdot}20 > P > 0{\cdot}10$). The results suggest that there was no interaction between the unidirectional potassium fluxes. The chloride permeability was calculated from the results of expts. 1, 2 and 3 of Table 3, yielding permeability coefficients of 1·99, 1·93 and $2{\cdot}02 \times 10^{-9}$ cm/sec. Thus, the permeability to potassium ions was almost twice the permeability to chloride, when red cells were incubated in the salicylate medium at 0° C.

4. *Effects of monovalent anions on sulphate permeability*

In order to investigate the anion permeability of human red cells at 38° C, it was necessary to choose a more slowly permeating anion than chloride. It was therefore decided to examine the rate of $[^{35}S]SO_4$ exchange in the presence of various monovalent anions. The results are presented in Table 5 and Fig. 4. The Figure shows the rate of approach to isotopic equilibrium during steady-state exchange of tracer sulphate, when red cells were incubated in the various electrolyte media, which all contained 1 mM sulphate. The predominant monovalent anion of the medium turned out to be a decisive factor for the rate of $[^{35}S]SO_4$ exchange. The rate constants (Table 5) are almost identical in chloride and in bromide media, but decrease gradually, if chloride is replaced by iodide, nitrate, thiocyanate or salicylate. In the presence of salicylate the rate of $^{35}SO_4$ exchange is reduced to about 5 % of the rate found in a chloride medium.

The experiments reported in Table 5 were all performed with cells from one single donor, but control experiments indicated that interindividual variations were small, the mean value of the rate constants in chloride medium was $2{\cdot}11\,hr^{-1}$ (S.E. 0·07, $n = 4$), and in the thiocyanate medium $0{\cdot}65\,hr^{-1}$ (S.E. 0·05, $n = 4$).

In the left part of Fig. 5 the rate of sulphate exchange in the various

media has been compared with the rate found in the presence of chloride, which has been set to an arbitrary value of 1·0. The relative rates of sulphate exchange in the other media are indicated by the heights of the columns. The relative rates of the passive fluxes of sodium and potassium are similarly illustrated in the right-hand part of the Figure, in order to demonstrate the fact that the ability to cause a decrease of anion permeability accompanied by an increase of cation permeability is also a characteristic effect of the lyotropic series of anions at 38° C.

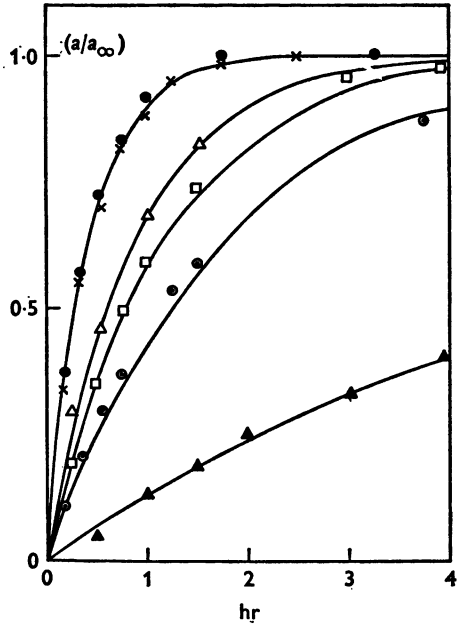

Fig. 4. The approach of $[^{35}S]SO_4$ exchange towards isotopic equilibrium between cells and electrolyte media containing various monovalent anions. The media are described in the Methods section, and the symbols employed are Cl ×, Br ●, I △, NO_3 □, SCN ⊙ and salicylate ▲. From top to bottom the sequence is: Cl, Br, I, NO_3, SCN, salicylate. All the experiments were carried out with cells from donor J.W. at 38° C, pH 7·40. The fraction a/a_∞ is the specific activity at the time of sampling divided by the specific activity at isotopic equilibrium.

5. *The temperature dependence of sulphate permeability*

The above results showed that the effect of monovalent anions on the permselectivity of red cells is present at 38° C, although the effect seems to be smaller by several orders of magnitude than the one seen at 0° C in a salicylate medium. Red cell permeability to $[^{35}S]SO_4$ was examined in the temperature range between 38 and 0° C in chloride and in thiocyanate media. ^{36}Cl distribution between cells and medium was followed in all

TABLE 5. The effect of monovalent anions on the rate of sulphate exchange (donor J.W., 38° C, pH 7·40). The electrolyte media are described in the Methods section. The rate constant was calculated in the way described in the legend of Table 3. The approach to isotopic equilibrium in the present six experiments is illustrated in Fig. 4. $r_{^{36}Cl}$ is the mean value of six to ten determinations of the ratio between radioactive chloride in the water phase of cells and medium

		Statistics of straight line				k		
Medium	Duration (hr)	A Interception with ordinate at $t = 0$	b Regression coefficient (hr^{-1})	S.D. of regression coefficient	r Correlation coefficient	Rate constant of cellular sulphate exchange (hr^{-1})	$r_{^{36}Cl}$	$r_{^{36}Cl}$ S.E. of mean
Chloride	2·5	−0·006	2·168	0·04	−0·999	2·027	0·593	0·003
Bromide	3·3	−0·0003	2·301	0·08	−0·998	2·112	0·635	0·005
Iodide	4·5	−0·023	1·150	0·03	−0·999	1·040	—	
Nitrate	4	0·008	0·896	0·01	−1·0	0·835	0·591	0·005
Thiocyanate	3·75	−0·027	0·568	0·01	−0·998	0·541	0·585	0·007
Salicylate	4	−0·001	0·132	0·005	−0·997	0·128	0·440	0·012

experiments, and the ratio between chloride activity in cell water and medium remained stable during the experiments, indicating that the sulphate fluxes were not subjected to variations of electrical driving forces (Table 6). Fig. 6 shows the approach to isotopic equilibrium in chloride media (upper part of the Figure) and in thiocyanate media (lower part of the Figure). The results obtained in chloride media below 9° C and in thiocyanate media below 18° C are not shown in the Figure, but the statistical treatment of all the experiments is shown in Table 6. The exchange

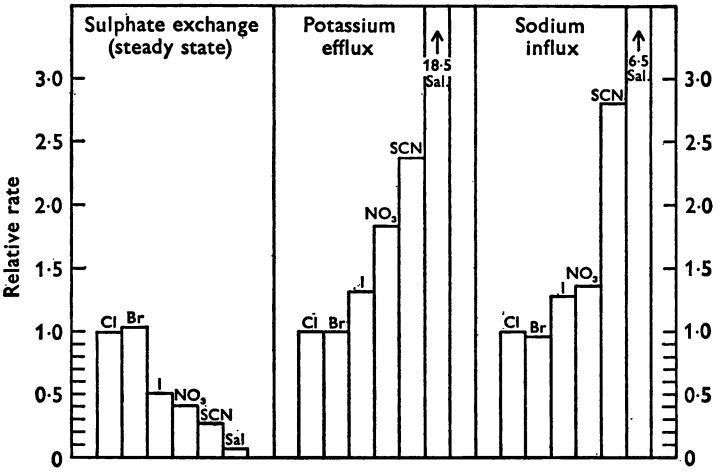

Fig. 5. Comparison of the relative effect of the lyotropic series of anions on the permeability of human red cells to sulphate, potassium and sodium (donor J. W., pH 7·40, 38° C). The rates of sulphate exchange, potassium efflux and of sodium influx in the chloride medium were chosen as reference (1·0) and fluxes in other media were compared to these values of reference. The data about sodium and potassium fluxes were taken from Funder & Wieth (1967*b*), with exception of the salicylate results (Wieth, 1970*a*). Note that the anions that caused a decrease in anion permeability produced an increased cation permeability.

could be determined down to 0° C in the chloride medium, but the rate constant decreased so much in a thiocyanate medium below 9° C that it was only possible to establish that the cells become virtually impermeable to sulphate in a cold thiocyanate medium, as no uptake was demonstrable over periods of 2–3 days. The rate constant of sulphate exchange at 9° C in the thiocyanate medium was 0·001 hr^{-1}, corresponding to a half-time of sulphate exchange of approximately 1 month.

Fig. 7 shows the relation between the reciprocal of the absolute temperature and the natural logarithm of the exchange constants. It appears that the relations were linear for the results obtained in chloride media between 38 and 0° C, and in thiocyanate media between 38 and 9° C. The Arrhenius

TABLE 6. The temperature dependence of $[^{35}S]SO_4$ exchange. Human red cells suspended in chloride and thiocyanate media, pH 7·40, donor J.W. The calculation of the rate constant of exchange is described in the legend of Table 3. The approach to isotopic equilibrium is illustrated in Fig. 6. $r_{^{36}Cl}$ is the mean value of six to ten determinations of the ratio between radioactive chloride in the water phases of cells and medium

			Statistics of straight line						
Medium	°C	Duration (hr)	*A* Interception with ordinate at $t = 0$	*b* Regression coefficient (hr^{-1})	s.d. of regression coefficient	*r* Correlation coefficient	*k* Rate constant of cellular sulphate exchange (hr^{-1})	$r_{^{36}Cl}$	$r_{^{36}Cl}$ s.e. of mean
Chloride	38	2·5	−0·006	2·168	0·04	−0·999	2·03	0·593	0·003
	28	5	0·039	0·473	0·01	−0·998	0·441	0·640	0·002
	18	23	−0·005	0·062	0·001	−0·999	0·057	0·692	0·02
	18	35·9	−0·021	0·054	0·001	−0·997	0·050	0·678	0·01
	9	143	−0·055	0·0077	0·0001	−0·995	0·0071	0·772	0·02
	0	215	−0·001	0·0019	0·0001	−0·997	0·0018	0·800	0·01
	38	3·75	−0·027	0·568	0·01	−0·998	0·541	0·585	0·01
Thiocyanate	28	9·3	0·009	0·105	0·003	−0·996	0·097	0·584	0·01
	18	24	−0·001	0·010	0·001	−0·998	0·0094	0·614	0·004
	9	116	−0·001	0·0012	0·0001	−0·997	0·0011	0·684	0·003

activation energies calculated from the slopes of the lines were: chloride medium 32·0 kcal/mole (S.D. 1·0), thiocyanate medium 37·7 kcal/mole (S.D. 1·1). These values differ significantly (Student's test: $0{\cdot}01 > P > 0{\cdot}005$). The difference between the sulphate permeabilities of cells suspended in thiocyanate and in chloride medium increases when the temperature of incubation is lowered. At temperatures below 9° C the sulphate permeability becomes extremely low, and the working hypothesis is that this further

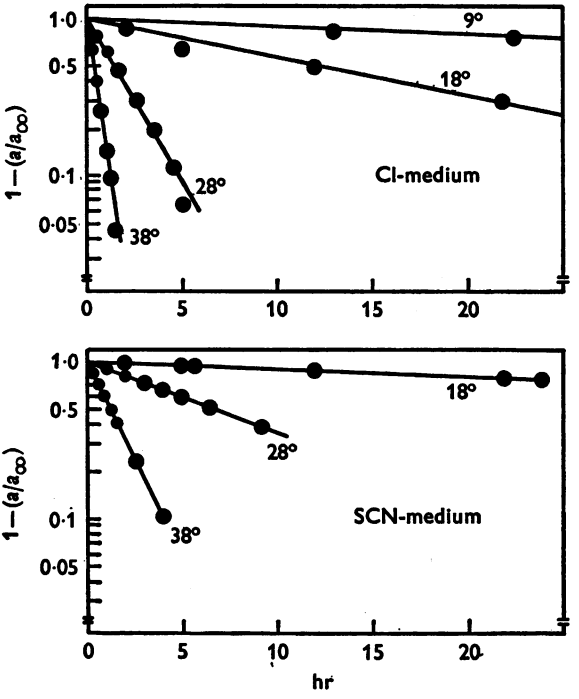

Fig. 6. Temperature dependence of the rate of [^{35}S]SO_4 exchange in chloride medium (upper graph) and in thiocyanate medium (lower graph). a is the specific activity at the time of sampling, a_∞ the specific activity at isotopic equilibrium. Details about the experiments and about the calculation of the straight lines are given in Table 6.

reduction of sulphate permeability, and the reduction of chloride permeability, described in section 2, are caused by closely related processes changing the permselectivity of the membrane.

6. *The Donnan equilibrium of chloride and of sulphate*

During the study of sulphate exchange an appreciable amount of data was accumulated, which demonstrates the relation between the equilibrium distributions of chloride and sulphate in the water phases of cells and

medium. Table 7 shows the approach of $[^{35}S]SO_4$ towards isotopic equilibrium during a sulphate influx experiment performed in the chloride medium at 38° C. The distribution of ^{36}Cl remained stable during all 4 hr, and it appears that r_{SO_4} reached a stable value of 0·37–0·38, exactly the value to be expected, if both chloride and sulphate are distributed according to a Donnan equilibrium, in which $r_{Cl} = \sqrt{r_{SO_4}}$.

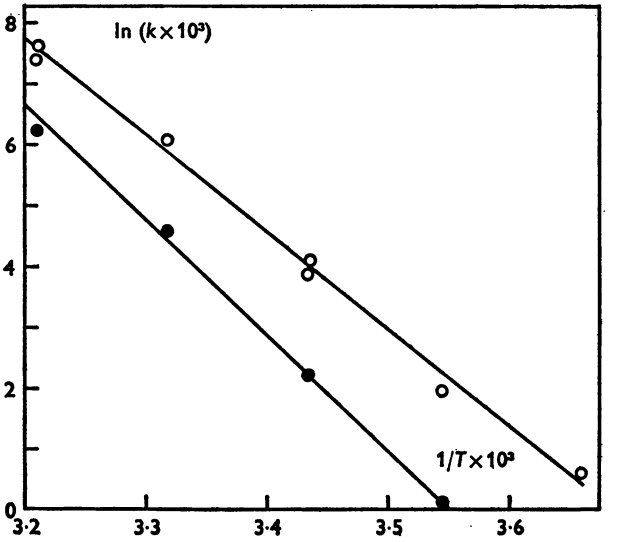

Fig. 7. Arrhenius diagram of the natural logarithm of the rate constant of sulphate exchange (k) vs. the reciprocal of the absolute temperature ($1/T$) in chloride (○) and thiocyanate (●) media with a pH of 7·40. The activation energies (calculated as described in the Methods section) were: sulphate exchange in the presence of chloride: 32·0 kcal/mole (S.D. 1·0); in the presence of thiocyanate: 37·7 kcal/mole (S.D. 1·1).

Similar results from nine other experiments have been listed in Table 8. Besides showing the time of sampling after addition of $[^{35}S]SO_4$, and the medium employed, the Table also shows the degree of completion of sulphate exchange, calculated as described in the Methods section. The mean value of r_{Cl} in the sixteen samples listed in Table 8 was 0·61 (S.E. 0·01) and of $\sqrt{r_{SO_4}}$ 0·62 (S.E. 0·004).

The mean value of the chloride distribution ratio between cells and electrolyte media at a pH of 7·40 and at a temperature of 38° C is 0·04 lower than the value of 0·66 previously found in normal blood (Funder & Wieth, 1966). The difference is presumably due to the absence of plasma proteins from the electrolyte media employed for the present study. The r_{SO_4} of blood has been reported to be lower than the ratio which is predicted by a Gibbs–Donnan equilibrium (Richmond & Hastings, 1960). The smaller

TABLE 7. Distribution of radioactive sulphate and chloride between cells and medium during a sulphate influx experiment (chloride medium 38° C, pH 7·40, donor J.T.) ^{36}Cl was added to the cell suspension 1 min before the start of the experiment, $[^{35}S]SO_4$ at $t = 0$. r_{Cl} and r_{SO_4} are the ratios between the radioactivities measured in the water phases of cells and medium. When the equilibrium had been attained $\sqrt{r_{SO_4}} = r_{Cl}$

Time (hr)	$[^{35}S]SO_4$ (cpm/kg H_2O) $\times 10^{-6}$				^{36}Cl (cpm/kg H_2O) $\times 10^{-6}$		
	Extracellular water	Intracellular water	r_{SO_4}	$\sqrt{r_{SO_4}}$	Extracellular water	Intracellular water	r_{Cl}
0·02	225·6	4·97	0·022	0·148	27·96	17·85	0·638
0·22	217·1	33·85	0·156	0·395	27·64	17·34	0·627
0·37	210·7	46·63	0·221	0·470	27·76	16·94	0·610
0·52	211·3	55·61	0·263	0·513	27·93	17·09	0·613
1·27	208·7	77·24	0·372	0·610	27·84	17·36	0·624
2·0	208·4	77·20	0·370	0·608	28·02	16·85	0·601
3·0	207·0	79·41	0·384	0·620	28·14	17·18	0·611
4·0	209·9	78·42	0·374	0·612	27·88	17·13	0·614

ratio for sulphate relative to chloride was ascribed to a binding of sulphate to plasma proteins, a point of view which is supported by the present findings.

TABLE 8. The equilibrium distribution of ^{36}Cl and of $[^{35}S]SO_4$ between cells and medium. r_{Cl} and r_{SO_4} are the ratios between the radioactivities measured in the water phases of cells and medium. The values of $(1-e^{-bt})$indicate the degree of approach to the equilibrium distribution of $[^{35}S]SO_4^{2-}$, complete exchange being 1·0 (cf. eqn. (1) of Methods section). Values > 0·9995 were listed as 1·0. The results are derived from nine expts. performed at 38° C, pH 7·40. The mean value of r_{Cl} was 0·61 (S.E. of mean 0·01) and of $\sqrt{r_{SO_4}}$ 0·62 (S.E. of mean 0·004)

Time (hr)	Medium	Donor	Approach to equilibrium $(1-e^{-bt})$	r_{Cl}	r_{SO_4}	$\sqrt{r_{SO_4}}$
2·58	Chloride	J.W.	0·992	0·604	0·399	0·632
2·5		—	0·996	0·597	0·397	0·630
2·02		A.J.	0·985	0·633	0·408	0·639
3·0		—	0·998	0·623	0·402	0·634
2·0		J.T.	0·990	0·601	0·370	0·608
3·0		—	0·999	0·611	0·384	0·620
4·0		—	1·0	0·614	9·374	0·612
2·02		E.S.	0·992	0·573	0·380	0·616
3·1		—	0·999	0·563	0·381	0·617
4·02		—	1·0	0·573	0·386	0·621
2·0	Bromide	J.W.	0·990	0·639	0·426	0·653
3·28		—	1·0	0·626	0·415	0·644
4·0	Nitrate	J.W.	0·972	0·610	0·370	0·608
5·25	Thiocyanate	J.T.	0·985	0·593	0·344	0·587
6·5		A.J.	0·985	0·622	0·356	0·597
7·5		—	0·999	0·640	0·375	0·612

DISCUSSION

The effect of lyotropic ions on red cell permeability

The present work provides new information about the permselective properties of the human red cell membrane. Before discussing the possible mechanisms of interaction between anions and membrane components, it will be useful to summarize the principal effects of the lyotropic ions on the permeability of the red cell membrane.

(i) *The effect of lyotropic anions*

Cation permeability. Human erythrocytes exhibit an increased permeability to sodium and potassium when chloride or bromide in the incubation medium is replaced by other monovalent anions of the lyotropic series (Fig. 5). The effect of anions on cation permeability increases throughout the series: $NO_3 < I < SCN <$ salicylate (Funder & Wieth, 1967*b*; Wieth,

1970*a*). In the latter work it was shown that the cation permeabilities vary with temperature in a complex way, when the incubation media contain 120 mM thiocyanate or salicylate. Between 0 and 18° C the cation fluxes showed a negative activation energy with a decreasing rate of permeation following an increase of temperature. Above 18° C the rate of sodium influx and of potassium efflux increased with temperature, exhibiting the positive activation energies characteristic for passive fluxes of sodium and potassium through the human red cell membrane.

Anion permeability. The present investigation shows that the anion-induced increase of cation permeability is accompanied by a comparable decrease of the permeability to the sulphate anion (Fig. 5). The quantitative effects of the anions on the rate of sulphate exchange were identical with the effects of anions on phosphate permeability reported by Deuticke (1967), nourishing the suspicion that the ability of the lyotropic anions to either increase or decrease red cell permeability depends on the electrical charge of the permeating ion. The activation energies of sulphate influx were constant in the temperature intervals studied both in the chloride medium and in the presence of thiocyanate (Fig. 7), but the activation energy was significantly higher in the thiocyanate medium (38 kcal/mole) than in the presence of chloride (32 kcal/mole). Unfortunately, the effect of SCN^- on sulphate exchange could not be studied in the temperature interval where sodium and potassium exhibit a paradoxical temperature dependence, because the cells were almost impermeable to sulphate at temperatures below 9° C (cf. Fig. 6 and Table 6). However, the results of Fig. 2 and Table 3 show that red cell permeability to chloride could be studied in the presence of salicylate in the interesting temperature range between 0 and 10° C. Net fluxes of sodium and potassium were increased by a factor of 10, when the temperature was lowered from 10 to 0° C (Wieth, 1970*a*; Table 5). In contradistinction to this increase, a similar change of temperature caused a reduction of the rate of chloride exchange by a factor of 10 (Table 3). The fact that the activation energy increased simultaneously from 20 to 45 kcal/mole (Fig. 3) shows that the impediment to chloride permeation increased with decreasing temperature.

(ii) *The effect of lyotropic cations on ion permeability*

In order to determine if the effects of anions on red cell permeability are mimicked by a lyotropic series of cations, Wieth (1970*b*) examined the sodium permeability of red cells suspended in electrolyte media containing lithium, sodium, potassium, rubidium, caesium, or choline as predominant cation. A moderate increase of sodium permeability was found, when NaCl or LiCl in the medium were replaced by the chlorides of the above mentioned cations, whereas the cations had no effect on sodium permeability in

bicarbonate media. None of the cations had the ability to induce a high sodium permeability at 0° C, and the rate of [^{35}S]SO_4 exchange was not affected by the substitution of NaCl by KCl, the salt that caused the maximum increase of sodium permeability at 38° C. Therefore, the anion-induced permeability changes of the red cell membrane do not appear to be caused by properties of the anions, which are shared by members of a lyotropic cation series. The possible existence of specific electrostatic interactions between adsorbable anions and fixed cations in the membrane is considered in the following sections.

The role of fixed charges

To be compatible with the present findings, a model of the red cell membrane must account for the fact that the foreign anions simultaneously increase the permeability to cations, and reduce the permeability to anions. Therefore, it seems worthwhile to consider the possibility that an anion exchange mechanism located in the cell membrane is responsible for the remarkable ability of the normal red cell to distinguish between anions and cations. A long established view is that the ionic permeability of the erythrocyte is controlled by immobile cations, but the evidence is indirect, and the responsible positive group has not been identified. Solomon (1960) calculated that a fixed charge density of 106 mole/l. was required to explain the anion selectivity of the red cell by the ion-exchange theory of Meyer & Sievers (1936). However, Solomon (1960) also stressed that a single positive charge suffices to block the hypothetical anion permeable pore to positive ions in the limiting case, when the channels are very narrow. An equivalent pore radius of approximately 4 Å has been determined by mutually independent methods (Solomon, 1968). The hydrated radii of small ions as K^+, Na^+ and Cl^- are of the same order of magnitude, so it is warranted to consider seriously that fixed charges may play an important role in determining red cell permselectivity. Passow (1965) studied the exchange of tracer sulphate between cells and media of varied ionic strength, and concluded that the effect of pH on sulphate permeability was compatible with pH-dependent variations of the concentration of charged amino groups in the membrane. The calculated maximum concentration of fixed charges was 3 mole/l. membrane water. LaCelle & Rothstein (1966) studied the cation permeability of red cells in media of low ionic strength. Above a critical chloride concentration of 0·05–2 mM (dependent on temperature) the permeation of cations could be explained by the presence of dissociable fixed positive charges. The activation energy of passive cation efflux (12 kcal/mole) was interpreted to represent the activation energy of the dissociation process causing variations in the concentration of fixed cations. The activation energy agreed reasonably with values of 9–13 kcal/mole reported for the dissociation of hydrogen ions from the amino groups of proteins.

The identity of the fixed charges

Berg, Diamond & Marfey (1965) made a more direct approach to determine the possible permeability controlling role of charged amino groups. They showed that the amino-reactive reagent: 1-fluoro-2,4-dinitrobenzene (Sangers reagent) caused a thirty-fivefold increase of sodium and potassium permeability, and presented evidence indicating that the permeabilities to erythritrol, xylose and sucrose were not increased. In addition Passow (1968) has shown that fluorodinitrobenzene reduces sulphate permeability considerably. This observation supports the proposal of Berg *et al.* (1965) that the permeability changes are due to the removal of positive, fixed charges accompanying the formation of stable dinitrophenyl compounds between the reagent and RNH_2 groups.

There are striking similarities between the anion-induced permeability changes described in the present work and those caused by fluorodinitrobenzene. However, there is not enough evidence to permit the conclusion that reactions with the same chemical groups are responsible for the occurrence of charge-specific permeability changes in the two cases. A few pieces of experimental evidence show that small adsorbable anions can be bound to the same groups, which are involved in the dinitrophenol reaction, and the thermodynamic properties of the ion-binding show a qualitative agreement with those used to explain the paradoxical temperature dependence of the cation permeability (Wieth, 1970*a*). Increasing adsorption of monovalent anions to proton-carrying groups of albumin has been demonstrated through the series: Cl, Br, NO_3, I, SCN (Scatchard & Black, 1949). The amino end-groups of protein and the free amino groups of lysine were found to be the likely location of binding sites with a high affinity for anions. Weaker binding sites were related to groups with a lower pK, supposedly imidazole groups (Scatchard & Yap, 1964). Davison, Spitzer & Smith (1959) showed that the above mentioned groups were responsible for the protein binding of salicylate. The adsorption suggested by us to be responsible for the permeability changes of red cells required two types of anion binding, one decreasing and the other increasing with rising temperature (Wieth, 1970*a*). The thermodynamic data of Scatchard & Yap (1964) are therefore of special interest. Two types of reactions were demonstrated by a thermodynamic analysis of the binding of thiocyanate to albumin (Scatchard & Yap, 1964). The decreases of free energy accompanying the binding of thiocyanate to the sites with the highest affinity were 6·40 and 6·19 kcal/mole at 0 and 25° C respectively. The enthalpy change was $-8{\cdot}8$ kcal/mole and the entropy change $-8{\cdot}75$ e.u. In contradistinction, the corresponding figures for the binding of thiocyanate to the weaker binding sites were $\Delta G°$ at 0° C: $-1{\cdot}99$ kcal/mole, at 25° C: $-2{\cdot}14$ kcal/mole; $\Delta H°$ was slightly

negative: $-0{\cdot}31$ kcal/mole, and $\Delta S°$ was positive: 6·15 e.u. The strongest type of anion binding is favoured by low temperature because it is exothermic and accompanied by a considerable decrease of entropy. The weaker binding is almost isothermic and is accompanied by a large increase of entropy. Therefore, it will be favoured at higher temperature levels, in agreement with the requirements stated above.

There is not enough evidence to prove that the control of ion permeability is carried out by protein components of the membrane, so other possibilities must also be considered. Solomon (1960) has suggested that calcium ions might function as fixed charges in the red cell membrane, but this hypothesis does not agree with the finding that calcium ions induced a marked increase of the potassium permeability, when red cells were suspended at 0° C in an isotonic medium containing 120 mM thiocyanate (Table 3; Wieth, 1970*a*). The fact that hydrated liquid crystals of phospholipids display selective permeability towards anions (Bangham, Standish & Watkins, 1965; Papahadjopoulos & Watkins, 1967) raises the question whether the lipids of the red cell membrane are responsible for the permselectivity. The choline groups of phosphatidyl choline have an affinity towards monovalent anions (Booij & Bungenberg de Jong, 1956) which corresponds to the sequence of anions found by us to exert increasing effect on red cell permeability. Booij (1966) has advanced the hypothesis that the effect of ions on the permeability of biological membranes is due to the more or less close association with the fixed charges on the hydrophilic surfaces of the phospholipids. According to Booij close 'site binding' of counter ions is supposed to change the packing of carbon chains in the membrane, or even to induce phase transitions of the lipid structure, creating pores lined by the polar groups as suggested by Lucy (1968). Both types of changes must be expected to be accompanied by an increased permeability to small neutral hydrophilic molecules. So far only the permeation of erythritrol has been examined in various anionic media (J. O. Wieth, unpublished). These experiments showed that the rate of [^{14}C]erythritrol permeation in the range between 0 and 38° C is not affected, when chloride is replaced by salicylate or thiocyanate. Before it can be concluded that the anions do not affect the penetration of neutral molecules, it is necessary to study the effect of anions on the diffusion of other hydrophilic compounds. The investigation of Bowyer & Widdas (1955) indicate that erythritrol may be transported into human red cells by means of the facilitated diffusion process responsible for the transport of hexoses. It has not been determined to which extent this mechanism contributed to the erythritrol influx in the experiments mentioned above. However, the studies of erythritrol permeation make it unlikely that ion selective pores with a radius of 3·5 Å or more, as those accompanying the phase transitions proposed by Luzzati &

Husson (1962) or by Lucy (1968), are created in the membrane, when red cells are incubated in thiocyanate or salicylate media at 0° C. The erythritrol influx, as determined by experiment, seems to be the sum of two components, one being transferred by the hexose transport system, and another diffusing through the aqueous regions of the membrane. An increased influx of erythritrol (molecular radius 3·4–3·7 Å; Solomon & Schultz, 1961) should accompany the formation of aqueous channels large enough to accommodate hydrated sodium ions (radius 2·8 Å) with the same ease as hydrated potassium ions (radius 2·3 Å). The finding that erythritrol permeability of red cells was not affected by the anions between 0 and 38° C, whether the cells were incubated in salicylate, thiocyanate or chloride media, therefore suggest that the lyotropic anions do not induce phase transitions of the above mentioned types.

Diamond & Wright (1969) concluded that it is not likely that the quaternary ammonium groups of the phospholipids are identical with fixed positive charges of the red cell membrane. Their argument was based on the electrical field strength theory of ionic selectivity (Eisenman, 1961). According to this theory the fixed charges must possess a high field strength in order to account for the sequence of halide permeability: Cl > Br > F > I found at room temperature by Tosteson (1959). In contrast to protonated amino groups ($R\text{-}NH_s^+$), quaternary ammonium groups will not be able to provide the field strength required.

The lipoprotein membrane model

The concept that amino groups play a determinant role for the ion permeability of the red cell places the functional role of the membrane proteins in a more central position than the one which appears at a first glance at the morphological 'unit membrane theory' (Robertson, 1964). Since only a minute fraction of the membrane area must be available to chloride in order to account for the normal anion permeability, it is evident that scattered strands of proteins penetrating the lipids of the membrane may easily escape detection by electron microscopy. Korn (1968) has recently suggested that lipoproteins form an integral part of the membrane matrix. According to his model hydrophilic end groups of both proteins and phospholipids are situated at the aqueous interfaces, and the bimolecular lipid layer is penetrated by α-helices and by proteins with a random coil configuration. It seems worth considering whether the lipoprotein membrane model has properties which are consistent with our observations concerning membrane permeability: scattered protein chains may provide channels for the passage of hydrophilic substances, as well as dissociable amino groups located both within and at the surfaces of the membrane. Regular pores, or thermal movements of the phospholipids, admit anions

and hydrophilic molecules with a radius below 4 Å to the hydrated regions surrounding the fixed charges. The positive charges exclude cations from the polar regions, and exhibit selectivity towards binding of anions so that e.g. chloride, bromide, nitrate, iodide, thiocyanate and salicylate have increasing power to compete with sulphate (cf. Table 5). The affinity towards salicylate is so high at 0° C that chloride is almost excluded from the anion sites, explaining the low chloride permeability found (Table 3), in spite of the fact that [^{14}C]salicylate equilibrates between cells and medium within 4 min at 0° C. The decrease in activation energy from 45 to 22 kcal/mole, which occurred when the temperature was increased to 10° C (Fig. 3), is attributed to the temperature dependent desorption of salicylate from the binding sites (Wieth, 1970*a*).

The anion exchange membrane model can also account qualitatively for the temperature dependent effect of anions on cation permeability. Ion association or even ion-pair formation between fixed ionic groups and their counter ions reduce the potential difference between external solutions and the membrane phase, thereby reducing the Donnan exclusion of cations (Helfferich, 1962, pp. 137–138). The strength of electrostatic attraction between fixed charges and anions depends on the distance of closest approach, a distance which decreases with increasing polarizability of the mobile anions (Helfferich, 1962, p. 162). Thiocyanate and salicylate are the most strongly polarizable ions of the lyotropic series. The facilitation of cation fluxes induced by thiocyanate and salicylate below 18° C (Wieth, 1970*a*) can therefore also be ascribed to an increasing adsorption of anions. The very high affinity of salicylate to the fixed charges may be related to the fact that ion exchangers selectively prefer ions with organic groups resembling the components of the matrix (Helfferich, 1962, p. 164). Accommodation of the benzene ring of salicylate between the carbon–hydrogen chains of the lipids might also account for the surprising high salicylate permeability. It is not likely that non-ionic diffusion of undissociated salicylic acid contributes importantly to the permeation of salicylate at an extracellular pH of 7·4 because the pK of the acid is 2·97 (Hodgman, 1961, p. 1755).

In conclusion, it appears that the anion induced permeability changes described may be interpreted in terms of interactions between the anions and a lipoprotein cell membrane. To supplement the indirect information obtainable from permeability studies, future investigation must also be directed towards the chemical components of the membrane. The thermodynamic properties of the anion-binding, which can be deduced from the temperature dependence of ion permeabilities, may provide useful criteria for the identification of the charges controlling the permselectivity of the red cell.

This work was supported by grants from P. Carl Petersens Foundation (B 708/66) and from the Danish State Research Foundation (N 155/66 and A 26/67). The valued technical assistance of Mrs Annie Jørgensen is gratefully acknowledged.

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