Temperature-Dependent Changes of Chloride Transport Kinetics in Human Red Cells

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ABSTRACT Chloride self-exchange in human red cells was studied between 0°C and 38°C. At higher temperatures the flow-tube method was used. Although the general features of chloride transport at 0°C and 38°C are similar, the following differences were found: (a) the maximum pH of chloride self-exchange flux was lowered 0.6 pH unit from 7.8 to 7.2 when temperature was increased from 0°C to 38°C; (b) the apparent half-saturation constant increased from 28 mM at 0°C to 65 mM at 38°C; (c) chloride transport at body temperature is slower than predicted by other investigators by extrapolation from low-temperature results. Chloride transport increased only 200 times when temperature was raised from 0°C to 38°C, because the apparent activation energy decreased from 30 kcal mol⁻¹ to 20 kcal mol⁻¹ above a temperature of 15°C; (d) a study of temperature dependence of the slower bromide self-exchange showed that a similar change of activation energy occurred around 25°C. Both in the case of Cl⁻ (15°C) and in the case of Br⁻ (25°C), critical temperature was reached when the anion self-exchange had a turnover number of about 4 · 10⁹ ions cell⁻¹ s⁻¹; (e) inhibition of chloride transport by DIDS (4,4'-diisothiocyanato-stilbene-2,2'-disulfonate) revealed that the deflection persisted at 15°C at partial inhibition (66%) presumably because DIDS inactivated 66% of the transport sites. It is suggested that a less temperature-dependent step of anion exchange becomes rate limiting at the temperature where a critical turnover number is reached.

INTRODUCTION

It is the purpose of the present work to present a study of chloride self-exchange at 38°C and to provide a link to previous studies of chloride transport performed at low temperatures (Gunn et al., 1973a; Dalmark, 1975).

It has been an open question whether the low-temperature data can be extrapolated to the physiological temperature range. For instance, it has recently been questioned (Chow et al., 1976) whether the pronounced temperature dependence of chloride exchange found between 0°C and 10°C also applies to the physiological temperature range. From studies of bicarbonate transport, Chow et al. (1976) concluded that Q₁₀ of inorganic anion exchange between 25°C and 37°C was as low as 1.7 (Eₐ ~ 8.8 kcal·mol⁻¹). In the present work chloride self-exchange has been studied in the whole range between 0°C and 38°C.
found that the transport process could not be described by a single value of the Arrhenius activation energy. The activation energy decreases from 30 kcal·mol⁻¹ in the low temperature range (0-15°C) to 20 kcal·mol⁻¹ between 15°C and 38°C. These values correspond to a decrease of Q₁₀ from 7 in the low to 3 in the high temperature range. A study of pH dependence and of the concentration dependence of chloride exchange at 38°C also suggests that the exact properties of the transport process cannot be predicted from data obtained at low temperatures, although the general features of the transport process are similar.

Bromide self-exchange was also studied at 0-38°C. A similar change of activation energy was found, but at a higher temperature (25°C). The fact that the deflection appeared at different temperatures depending on the anion studied does not support the hypothesis that a temperature-induced change of the lipids in some way may influence the function of the transport proteins. It is suggested that the activation energy of anion transport changes when a critical turnover number of about 4·10⁵ ions cell⁻¹ s⁻¹ is exceeded.

It has been shown (Cabantchik and Rothstein, 1974) that anion exchange is selectively inhibited by the potent amino reagent 4,4'-diisothiocyano-stilbene-2,2'-disulfonate (DIDS) which binds to integral membrane proteins involved in anion transport. In the present study experiments were performed after partial inhibition of chloride transport with DIDS. The results show that 66% inhibition of chloride transport did not remove the deflection at 15°C where the transfer rate was also found to be reduced to one-third of the uninhibited transport number. It is therefore likely that partial inhibition of chloride transport with DIDS inactivated two-thirds of the transport sites, whereas the turnover rate at the uninhibited transport sites was unchanged after DIDS treatment of the cells.

**MATERIALS, METHODS, AND CALCULATIONS**

**Electrolyte Media**

In the experiments performed with erythrocytes the following three media were used (mM).

**MEDIUM A** 145 NaCl, 1.5 CaCl₂, 1.0 MgCl₂, 5 D-glucose, and 27 glycyl-glycine (total Cl⁻: 150)

**MEDIUM B** 141 Na-acetate, 1.5 Ca(acetate)₂, 1.0 Mg(acetate)₂, and 27 glycyl-glycine. By adding ammonium chloride to medium B the chloride concentration was varied from 2 to 600 mM.

**MEDIUM C** 150 NaBr, 5 D-glucose, and 27 glycyl-glycine. Three media with KCl concentrations of 165, 320, and 600 mM, all buffered with 2 mM Tris, were used in the experiments performed with ghosts (viz. Tables I and II and Fig. 10).

**Labeling and Packing of Cells and Ghosts**

20-30 ml of freshly drawn, heparinized human blood was centrifuged at room temperature and the plasma and buffy coat were removed. The cells were washed once in medium A or three times in medium B or C, incubated at the temperature of the subsequent experiment, and titrated to the desired pH with either CO₂ or 150 mM NaHCO₃. The cells were treated gently by this titration and the following washes
removed the bicarbonate. At pH values below 6.0 and above 8.5 small amounts of 150 mM HCl or NaOH, respectively, were added.

After titration, the cells were washed three times in the medium of appropriate ionic strength and pH, and resuspended to a hematocrit of 60-80% before addition of either 6-10 μCi 36Cl (obtained as the sodium salt, sp act 0.3-0.5 mCi mmol⁻¹, concentration range 150-225 mM NaCl, Radiochemical Centre, Amersham, England) or 70-80 μCi 82Br (obtained as the sodium salt, sp act 4-6 mCi mmol⁻¹, concentration range 120-140 mM NaBr, AEK, Risø, Denmark). The cells were equilibrated with the tracer anions at room temperature for at least 3 min which by far exceeds six half-times of the anion exchange at this temperature.

The suspension was divided into a major portion centrifuged in 10-ml glass tubes and a minor portion centrifuged in 0.7-ml nylon tubes (ID 3 mm; Funder and Wieth, 1967) for the determination of chloride or bromide concentration, cell water, and radioactivity in cells and medium. Both portions were spun at 50,000 g for 15 min (Sorvall RC-5 centrifuge, Du Pont Instruments, Sorvall Operations, Newtown, Conn.). The supernate and the upper 2-mm layer of cells in the main portion were drawn off and the remaining cells were stored in a 5-ml syringe at 4°C until use.

In another set of experiments ghost cells were prepared with intracellular KCl concentrations of 165, 320, and 600 mM as described by Schwoch and Passow (1973). The procedures of tracer equilibration and the subsequent separation of ghosts and extracellular medium were carried out as described above.

Experiments were also performed with ghosts treated with DIDS which is a potent and selective inhibitor of anion exchange (Cabantchik and Rothstein, 1974). The DIDS was synthesized by Dr. M. Hancock, Chemical Lab. II, University of Copenhagen. Two batches of ghost cells with a chloride concentration of 165 mM were incubated with DIDS (5 μl or 50 μl 1 mM DIDS solution per milliliter of cell suspension with a hematocrit of 50%) for 45 min at 38°C before the packing procedure. At these DIDS concentrations the anion exchange was inhibited by 66% and 99.6%, respectively, in the two batches of cells used for experiments between 0°C, and 38°C.

The extracellular medium trapped between intact red cells and between ghosts determined as the [3H]inulin space was 1.6% (wt/wt) and 8.2% (wt/wt), respectively, as determined by Funder and Wieth (1976). The volume of the ghosts was calculated from the ghost counting (Coulter Counter model DN, Counter, Electronics, Inc., Hialeah, Fla.) and the cytocrit after correcting the latter for the extracellular medium trapped between the packed ghosts.

Determination of the Rate of Efflux

All experiments were performed under steady-state conditions which means that the efflux of tracer from the labeled cells is counterbalanced by an equal influx of nonradioactive ions.

In the present study two methods were used to determine the rate of tracer wash-out from the packed, labeled cells. In experiments where the half-time exceeded 1-2 s, successive cell-free filtrates were collected from a well-stirred, thermostated cell suspension by the Millipore-Swinnex filtering technique described by Dalmark and Wieth (1972). More rapid reactions were determined by means of the flow-tube technique, the principle of which is shown in Fig. 1. The internal dimensions of the mixing chamber and the flow-tube were the same as those reported by Piiper (1964). Six filtering units were connected by exchangeable lengths of constant bore tubing. Cell-free samples of extracellular medium were collected beneath the ports by inserting either Millipore filters (pore diameter 1 μm, Millipore Corp., Bedford, Mass.) or Nucleopore filters (pore diameter
THE JOURNAL OF GENERAL PHYSIOLOGY • VOLUME 70 • 1977

0.2 μm, Shandon Nucleopore Corp., Pleasanton, Calif.). The flow-tube and its accessories were thermostated with a precision of ±0.1°C. The constancy of the flow-rate (±1%) during the experiments was monitored with a strain gauge which measured the buoyancy of a perspex rod placed in the effluent cylinder (8, Fig. 1). The linear velocity always exceeded 110 cm s⁻¹ which ensured a turbulent flow as determined by the flow-pressure relationship described by Sidel and Solomon (1957). A turbulent flow was obtained at a lower linear velocity in the present study although the internal diameter was almost equal in the two flow-tubes (0.20–0.22 cm) because: (a) the filters in the flow-tube used in the present experiments bulged slightly into the bloodstream, resulting in a Bernouilli effect stimulating turbulence; (b) the suspension leaving the mixing chamber had a low viscosity because the hematocrit was only 0.3–1.0% depending on the relative amounts of packed erythrocytes or ghosts and medium used. Problems of hemolysis are greatly reduced and sufficient amounts of filtrates are easily obtained (ca. 0.5 cm³ at each port) when the hematocrit is low, offering an advantage over the concentrated cell suspensions used by other authors (Tosteson, 1959; Piiper, 1964; Hemingway et al., 1970). The total volume of filtrates collected constituted a maximum of 1–1.5% of the total volume.

In experiments performed at pH 7.4 and 9.0, the degree of hemolysis in the six filtrates and the equilibrium sample was examined spectrophotometrically at the Soret band (411 nm). At pH 7.4 the lysis expressed as the percentage of the total hemoglobin was 0.7% (range: 0.6–0.8%). At pH 9.0 where the cells are considerably more fragile the lysis was enhanced to 7.1% (range: 4.7–8.6%).

**Determination of Radioactivity and Cell Water Content**

Cell water, chloride, and activity of ³⁶Cl⁻ or ¹⁴Br⁻ in plasma, cells, whole blood, and filtrates were measured as described by Dalmark and Weith (1972).

**Calculations**

The experiments were performed under steady-state conditions and the results showed that the kinetics of the exchange was well described by a closed two-compartment system with the following relation between specific activity in the medium and time:

\[
at = a_o (1 - e^{-bt}) + a_e e^{-bt},
\]

where \(a_o\), \(a_t\), and \(a_e\) are the specific activities (cpm mmol⁻¹) at zero time, at the time \(t\), and at isotopic equilibrium, respectively. The equation can be rewritten as follows:

\[
\ln \left(1 - \frac{a_t}{a_e}\right) = -bt + \ln \left(1 - \frac{a_0}{a_e}\right).
\]

Since the exchange followed first-order kinetics, the relation between \(\ln[(1 - a_t/a_e)]\) and time was linear in a semilogarithmic plot. The slope of the line, \(-b\), is determined by linear regression analysis and is equal to the efflux rate coefficient \(k(\text{s}^{-1})\), because the extracellular volume is at least two orders of magnitude larger than the cellular volume (hematocrit below 1%). The term \(\ln[(1 - a_t/a_e)]\) expresses the interception with the ordinate at \(t = 0\) and depends ideally on the fraction of extracellular tracer trapped in the medium between the packed cells.

The unidirectional chloride flux at steady state is defined as

\[
J = k \cdot \frac{V}{A} \cdot C_t = P \cdot C_i \ (\text{mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}),
\]

where \(V\) is the intracellular solvent volume (cm³), \(A\) is the membrane area available for the exchange (cm²), \(C_t\) is the cellular anion concentration (mol·cm⁻³), and \(P\) is the apparent
FIGURE 1. Schematic diagram of the flow tube. 1-2 cm³ of packed, labeled red cells in the syringe (1) mounted in a continuous infusion pump (Perfusor IV, B. Braun Melsungen, Melsungen) were injected into the mixing chamber (2) and mixed with 350 ml of medium from the container (3). Air pressure (0.2-2.0 atm) was used as the driving force of the medium and was controlled by a precision pressure valve (4). The suspension flowed past six filtration units (6) connected by exchangeable lengths of constant bore tubing (1D 2 mm). Filters with pore diameters of 0.2-1.0 µm supported by glass fiber filters bulged slightly into the bloodstream, thereby avoiding adsorption of cells to the filters. The dimensions of the filtration slit were 10 x 2 mm. A valve (7) at the outlet directed the effluent to either a waste bucket (9) or a graduated cylinder (8) where the effluent was collected during the steady-state filtration period. The duration of this period was registered by an electrical stopwatch controlled by the valve (7). Pairs of test tubes (10), one for waste filtrate, the other for the steady-state filtrate, were placed beneath each port. The equipment was kept in a box thermostated by circulation of thermostated air (±0.2°C). The temperature was measured by three thermocouples (5).

permeability (cm·s⁻¹). Cell water and cell volume are dependent on pH. In the present study: \( d_{w,cm} \) {liter cell water·(kg cell solids)⁻¹} = 5.61(SD ±0.12) - 0.26(SD ±0.02) · pH. It is convenient to relate the anion transport to the cell solid content in order to refer to a given number of cells and thereby to a fixed membrane area. Therefore, Eq. (2a) was used in the following form
\[ M = k \cdot d \cdot C_l \left( \text{mmol} \cdot (3.1 \cdot 10^{13} \text{ cells} \cdot \text{min}^{-1}) \right), \]  

where \( d \) ensures the constant area because \( 3.1 \cdot 10^{13} \) normal erythrocytes with a mean area of \( 142 \, \mu \text{m}^2 \) contain 1 kg cell solids and have a membrane area of \( 4.4 \cdot 10^7 \, \text{cm}^2 \) (Wieth et al., 1974). Correspondingly, in the experiments with ammonium chloride-loaded cells, \( d \) was corrected for the increased content of solids.

In order to be able to compare fluxes in red cells and ghosts, chloride transport in ghosts was expressed in mmol \( (3.1 \cdot 10^{13} \text{ cells} \cdot \text{min}^{-1}) \) (cf. Funder and Wieth, 1976).

The Arrhenius activation energy of anion exchange was calculated by linear regression analysis of the relation between the natural logarithm of the flux and the reciprocal of the absolute temperature according to the equation:

\[ \ln M = - \frac{E_A}{R} \cdot \frac{1}{T} + \text{const}, \]  

where \( E_A \) is the apparent activation energy (cal \cdot \text{mol}^{-1}), \( R \) is the gas constant (1.99 cal \cdot (\text{mol} \cdot K)^{-1}) and \( T \) is the absolute temperature (K). In the experiments with cells treated with ammonium chloride-acetate, the activation energies were calculated according to Exner (1964):

\[ E_A = \frac{R \cdot T_1 \cdot T_2}{T_1 - T_2} (\ln M_1 - \ln M_2). \]  

This equation was also used to calculate the decrease of activation energy due to different degrees of saturation of the transport system.

**Results**

1. **Temperature Dependence of Chloride Exchange at \([\text{Cl}]_o = 150 \, \text{mM}\)**

By employing two methods (cf. Materials, Methods, and Calculations) it was possible to measure the chloride exchange in the whole temperature range from 0°C to body temperature, and to determine whether the pronounced temperature dependence at 0–10°C (Dalmark and Wieth, 1972) extrapolates into the physiological temperature range. Identical results were obtained at the same temperature (10°C) with the two methods, thereby checking the reliability of both techniques at their limits of performance.

In Fig. 2 the relation between unidirectional chloride flux and the reciprocal of the absolute temperature is shown in an Arrhenius diagram. At 0–10°C the results were obtained by means of the Millipore filtering technique. Between 10°C and 38°C the exchange was determined by means of the flow-tube method. Each point on the graph represents the average of the calculated chloride transport of 2–10 experiments, all performed at pH 7.2. The apparent activation energies of the transport at 0–15°C, at 15–38°C, and at 0–38°C were calculated by regression analyses. In the low temperature range an activation energy of 30 kcal \cdot \text{mol}^{-1} (SD ±0.6; \( r = 0.999 \)) was found to be contrasting with an activation energy of 20 kcal \cdot \text{mol}^{-1} (SD ±0.5; \( r = 0.999 \)) at 15–38°C. An activation energy of 24 kcal \cdot \text{mol}^{-1} (SD ±1.1; \( r = 0.993 \)) was found between 0°C and 38°C if the deflection occurring around 15°C was neglected in the regression analysis.

2. **pH Dependence of Chloride Transport at 38°C**

All the experiments of this section were performed with the flow-tube method. Fig. 3 shows the rate of tracer exchange in four experiments conducted at pH
between 6.0 and 9.0. When pH was increased from 6.0 to 9.0 the half-time of the approach of $^{36}$Cl$^-$ to isotopic equilibrium decreased from 0.2 s to 0.025 s, and the rate coefficient increased linearly with pH as shown in Fig. 4. The half-time of the exchange at pH 7.2 that gave a maximum flux (viz. Fig. 5) was 0.05 s ($k = 13.3$ s$^{-1}$).

**Figure 2.** Temperature dependence of chloride exchange at 0-38°C. The logarithm of chloride transport (ln M) is depicted vs. the reciprocal of the absolute temperature (1/T). The results were obtained by the Millipore-Swinnex filtering technique at 0-10°C and by the flow-tube method at 10-38°C. All experiments were performed at pH 7.2. Each point on the graph is an average of chloride transport calculated from more than two experiments. The ranges are shown when they exceeded the size of the point. A high apparent activation energy of 30 kcal·mol$^{-1}$ in the low-temperature interval decreases to 20 kcal·mol$^{-1}$ above 15°C. The extrapolated $Q_{10}$ of 5 in the high temperature range predicted from the data obtained at 0-10°C ($Q_{10} = 7$) is thus too high, as the present results give a value of 3.

**Figure 3.** The pH dependence of the rate of chloride exchange under steady-state conditions at 38°C. The rate of $^{36}$Cl exchange was measured as the tracer washout from labeled, packed cells into an electrolyte medium identical to the incubation medium. The flow-tube method was used in all experiments. The logarithmic ordinate is the fraction of the tracer remaining in the cells at a given time and the abscissa is time in milliseconds. The negative slopes of the graphs are equal to the efflux rate coefficients under the present conditions of a closed two-compartment system with an extracellular volume of more than 99% of the total volume.

The rate coefficients were used to calculate the fluxes as described in Materials, Methods, and Calculations. The calculated unidirectional fluxes are shown in Fig. 5 where chloride transport is depicted as a function of extracellular pH. A bell-shaped graph was found, as was the case at 0°C (Gunn et al., 1973a), but the maxima were found at different pH values. At 38°C the flux had a peaked
maximum at pH 7.2 and decreased steeply at both more acid and more alkaline pH values, whereas a broad maximum was found between pH 7.0 and 8.0 at 0°C.

The pH dependence of chloride transport was examined in resealed erythrocyte ghosts at 38°C in order to eliminate the effect of pH on intracellular chloride concentration and membrane potential. The results of Table I show that the increase of chloride self-exchange between pH 5.6 and 7.2 was similar to that found in intact cells. However, in ghosts the flux decreased much more gradu-

3. Concentration Dependence of Chloride Transport at 0-38°C

To evaluate the concentration dependence of chloride transport the cellular chloride concentration was varied by loading cells with ammonium chloride in
an acetate medium as described by Gunn et al. (1973a). The experiments were performed at 0°, 10°, 25°, and 38°C. Chloride transport was measured at pH 7.2 where the transport had a maximum at physiological ionic strength in the whole temperature interval, because the broad maximum found at 0°C became peaked by a shift only of the alkaline limb when temperature was raised. In Fig. 6 the fluxes are shown as a function of the cellular chloride concentration. The graphs demonstrate that the exchange is a saturable process at all temperatures. At 0°C the flux decreased at cellular chloride concentrations above 200 mM. This is the phenomenon termed “self-inhibition” by Dalmark (1976). This self-inhibition is not apparent at higher temperatures in the concentration range studied.

The apparent half-saturation constant, i.e. the cellular chloride concentration

| Table I |
| PH DEPENDENCE OF CHLORIDE SELF-EXCHANGE IN GHOSTS AT 38°C |
| ([Cl]i = [Cl]o = 165 mM) |
| Chloride flux |
| pH | Experimental values | Mean |
| nol (3.1-10^9 cells-min)^(-1) |
| 5.6 | 7.2-7.5 | 7.4 |
| 6.0 | 71-74 | 73 |
| 6.5 | 91-108 | 100 |
| 7.0 | 145-151 | 148 |
| 7.2 | 144 | 144 |
| 7.6 | 141-141 | 141 |
| 8.0 | 125-192 | 129 |
| 9.0 | 107-123 | 115 |
| 10.0 | 77-87 | 82 |

The rate of 36Cl efflux was measured by means of the flow-tube technique. The increase of flux with increasing pH from 5.6 to 7.0 at 38°C is similar in both ghosts and erythrocytes. The steep decrease of chloride flux in erythrocytes at pH values above 7.2 (viz. Fig. 5) contrasts with a gradual decrease of 50% over 3 pH units in ghosts.

4. Temperature Dependence of Chloride Exchange at High Chloride Concentrations

The nature of the change in activation energy is further examined in Table II a and b. The activation energies in the two temperature ranges considered are shown in Table II b. They were calculated both from the results obtained with ammonium chloride-loaded cells and from results obtained with resealed erythrocyte ghosts with intracellular chloride concentrations of 165, 320, and 600 mM.
The table compares these results with the results obtained with intact erythrocytes. In all cases the activation energy decreased from 28–32 kcal mol\(^{-1}\) (0–15°C) to 19–21 kcal mol\(^{-1}\) (15–38°C). It is shown in the Discussion that unsaturation of about 35% of the transport system at 38°C only accounts for a decrease of the experimentally determined activation energy by 2–3 kcal mol\(^{-1}\) and it is therefore not surprising that the same values are found in all three sets of experiments. Table IIa also shows the large range of half-times at each temperature, which excludes the idea that the decrease of activation energy is an artifact of the method, becoming manifest when chloride transport is rapid.

5. Temperature Dependence of Bromide Self-Exchange

The experiments were conducted like the experiments of chloride self-exchange reported in Section 1. The Millipore-Swinnex filtration technique was used in experiments at temperatures up to 25°C because the rate of bromide exchange is slower. In Table III the half-times of exchange, the self-exchange fluxes, and the apparent activation energies are shown. It can be seen that the temperature dependence of bromide self-exchange is not well described by a single value of activation energy. The kink in the vicinity of 25°C corresponds to a decrease of activation energy from 32 kcal mol\(^{-1}\) to 22 kcal mol\(^{-1}\).

6. Temperature Dependence of Inhibited Chloride Transport

The following inhibition study elucidates whether the change of activation energy persists when chloride transport is inhibited by DIDS, which is a potent and selective inhibitor of anion transport (Cabantchik and Rothstein, 1974; Lepke et al., 1976).

Two sets of experiments were performed, one where chloride transport was inhibited 99.6%, and another where the inhibition was 66%. Both batches of
### Table I

**HALF-TIMES ($T_1$, s) AND CHLORIDE TRANSPORT ($M$, mol·[3.1·10¹³ cells·min]⁻¹) IN HUMAN ERYTHROCYTES AND THEIR GHOSTS AT 0–38°C (pH 7.2)**

| [Cl]₀ (mM) | Erythrocytes | Ghosts | NH₄Cl-loaded erythrocytes |
|------------|--------------|--------|---------------------------|
|            | 150          | 165    | 200                       | 600 | 150-350 |
| 0 M        | 17.2         | 26.7   | 65.0                      | 103.6 | 27.7 |
| 5 M        | 6.52         | 9.23   | 20.3                      | 33.6   | -   |
| 10 M       | 2.32         | 3.63   | 8.13                      | 12.6   | 3.30 |
| 15 M       | 0.89         | 1.87   | 3.29                      | 5.20   | -   |
| 20 M       | 0.454        | 1.04   | 1.88                      | 2.87   | -   |
| 25 M       | 0.250        | 0.523  | 0.968                     | 1.58   | 0.636 |
| 30 M       | 0.098*       | 0.293  | -                         | 0.551‡ | -   |
| 38 M       | 0.052        | 0.162‡ | 0.324§                    | 0.469§ | 0.221 |

The half-times and the chloride transport between 0°C and 38°C in: (a) erythrocytes washed in medium A having a constant extracellular chloride concentration of 150 mM; (b) ghosts prepared with three cellular KCl concentrations; and (c) erythrocytes washed in medium B with NH₄Cl concentrations that gave maximal fluxes at the temperatures concerned. *, ‡, and § denote that the experiments were performed at 32°C, 35°C, and 37.5°C, respectively.

### Table II

**APPARENT ACTIVATION ENERGIES (kcal mol⁻¹) OF CHLORIDE EXCHANGE IN RED CELLS AT 0–38°C**

| [Cl]₀ (mM) | Erythrocytes | Ghosts | NH₄Cl-loaded erythrocytes |
|------------|--------------|--------|---------------------------|
|            | 150          | 165    | 200                       | 600 | 150-350 |
| 0-15°C     | 30.2±0.6     | 28.1±1.6 | 30.6±0.9                  | 31.2±0.9 | 31.6* |
| (0.999)    | (0.997)      | (0.999) | (0.999)                   |
| 15-38°C    | 19.7±0.5     | 19.8±0.9 | 18.3±0.7                  | 19.4±0.4 | 19.0* |
| (0.999)    | (0.997)      | (0.998) | (0.999)                   |
| 0-38°C     | 23.5±1.3     | 22.8±1.0 | 23.6±1.6                  | 23.7±1.4 | 24.6±1.8 |
| (0.993)    | (0.994)      | (0.998) | (0.992)                   | (0.995) |
ghosts and the efflux medium were titrated to pH 7.2 at 38°C which ensured that chloride transport was measured at pH values in the whole temperature interval where noninhibited chloride transport was maximal. Fig. 7 displays the temperature dependence of chloride transport that was 66% inhibited. It can be seen

\[
\text{TABLE III}
\]

| Temperature (°C) | 0°C | 5°C | 10°C | 15°C | 20°C | 25°C | 30°C | 35°C | 38°C |
|------------------|-----|-----|------|------|------|------|------|------|------|
| \(T_1\) (s)      | 160.3 | 46.8 | 15.0 | 5.0  | 2.0  | 0.97 | 0.400 | 0.227 | 0.174 |
| Range            | 158.2-162.4 | 46.5-47.1 | 15.0-15.0 | 5.0-5.4 | 2.0-2.0 | 0.94-0.99 | 0.396-0.401 | 0.225-0.229 | 0.171-0.177 |
| \(M\) (mol \(5.1 \times 10^{10}\) cells \(\text{min}^{-1}\)) | 0.08 | 0.36 | 0.82 | 2.01 | 5.14 | 10.5 | 20.5 | 36.2 | 47.2 |

The rate coefficients of bromide self-exchange were determined as described in Materials, Methods, and Calculations. The half-times \(T_1 = \ln 2 / k\) in the table are average values of one to two double runs. The ranges of the half-times are also shown. The apparent activation energies (kcal mol\(^{-1}\)) of bromide transport in the two temperature intervals as well as the whole temperature range are calculated according to Eq. (3) in Materials, Methods, and Calculations. The correlation coefficients of the linear regression analyses are stated in brackets.

\[
E_a = 31.5 \pm 1.8 \text{ kcal mol}^{-1} \quad (0.998)
\]

\[
E_a = 21.5 \pm 1.4 \text{ kcal mol}^{-1} \quad (0.998)
\]

\[
E_a = 29.0 \pm 1.9 \text{ kcal mol}^{-1} \quad (0.994)
\]

\[\ln M = -10.26 (\text{SD} \pm 0.25) \times (10^5 \ T^{-1}) + 39.21 (\text{SD} \pm 0.19)\]

**FIGURE 7.** The Arrhenius plot of partially inhibited chloride transport shows that the deflection persisted at 15°C although the transport was 66% reduced with DIDS. There is a good agreement between results obtained with the two methods employed (cf. flux at 25°C). It also appears that the change of activation energy could be determined with the slow filtration method, when the rate of chloride exchange is reduced by inhibition.

that also under these conditions the apparent activation energy decreased from 30 to 18 kcal mol\(^{-1}\) above ca. 15°C as in the case of noninhibited chloride transport (viz. Fig. 2). If chloride transport, however, were almost completely inhibited (99.6%), the relation between transport and temperature could be described by the linear equation: \(\ln M = -10.26 (\text{SD} \pm 0.25) \times (10^5 \ T^{-1}) + 39.21 (\text{SD} \pm 0.19)\)
BRAHM Chloride Transport in Human Red Cells

±0.85), having a constant activation energy of 20.4(SD ±1) kcal mol⁻¹ between 0°C and 38°C.

**DISCUSSION**

*The pH Dependence of Chloride Transport*

In Fig. 8 the pH dependence of chloride self-exchange at 38°C is compared with results obtained at 0°C (Gunn 1972). At each pH value chloride fluxes have been depicted as a fraction of the maximal flux at the appropriate temperature in order to facilitate comparison. It appears that both graphs exhibit a maximum and that the acidic branches have similar positions. In contrast, the alkaline limb was displaced in the acidic direction when temperature was raised. Therefore,

$$M_{\text{frac}} = \left(1 + \frac{[H^+]}{10^{-4.1}}\right)^{-1}.$$  

The alkaline limb is discussed in the text.

Effects of low and high pH values on the fractional fluxes will be discussed separately.

**Chloride Transport Below pH 7** Chloride transport decreases to the same degree at 0°C and 38°C when pH is lowered from 7 to 5.7. It has been concluded from studies at 0°C that the transport system is titrated into a form that does not mediate chloride exchange when one or more hydrogen ions are bound to a proton acceptor in the membrane (Gunn 1972, 1973). Gunn (1973) based his analysis of the acid pH effect on the assumption that the inhibition of chloride transport was caused by the protonation of a single group. Later, Dalmark (1975) proposed that the decrease of the flux was a function of the second power of the hydrogen ion concentration, and suggested that the hydrogen ion inhibition might involve at least two proton binding groups. This second
power dependence of inhibition on H⁺ concentration has not yet been confirmed by other investigators. In resealed ghosts with an intact anion transport system Funder and Wieth (1976) found it sufficient to assume that one H⁺ ion reacts with the transport system to explain the effect of pH on chloride transport at 0°C. The present results at 38°C also agree with the assumption that the binding of one proton is sufficient to block the transport. It may also be noted that chloride transport is only inhibited by 90% at pH 5 (Gunn et al., 1975), although the inhibition should have been 99% if H⁺ reacts with two proton acceptors with apparent pK values of 6 or more. It must be noted, however, that chloride permeability increases spontaneously with time when red cells are incubated at pH values below 6 (Gunn et al., 1975) and this phenomenon makes it difficult to analyze the true nature of the hydrogen ion effect at low pH values. The important thing to note in the present study is that the apparent pK of the hydrogen ion inhibition is very insensitive to variations of temperature between 0°C and 38°C. The ionization enthalpy of a possible proton acceptor group must therefore be very low.

**CHLORIDE TRANSPORT ABOVE PH 7** The maximum of chloride transport is peaked at 38°C compared to the rounded shape found at low temperatures. This is exclusively due to a displacement of the alkaline limb in the acidic direction. It is worth noting that the maximum at both low and high temperatures is found at that pH where the chloride distribution ratio \((\frac{Cl_{cell}}{Cl_{water}}/Cl_{medium})\) is about 0.75, meaning that in both cases intracellular chloride concentration is 110 mM when the extracellular concentration is held constant at 150 mM. Fig. 9 shows both the chloride distribution ratio (left ordinate) and the cellular chloride concentration (right ordinate) as a function of pH. The dashed line represents the chloride distribution at 0°C (Gunn et al., 1973a). At 38°C a parallel line was obtained, but it was shifted ~0.6 pH unit in the acidic direction, in a manner similar to the displacement of the transport maximum.

Gunn (1972, 1973) suggested that the decreasing flux with increasing pH was caused by the deprotonation of a group in the transport system which would function only when the group was protonated. Dalmark (1975) showed that the decrease of flux with increasing pH disappears when chloride transport at 0°C is studied in red cells loaded with high concentrations of potassium chloride. It might be argued that this finding is expected if OH⁻ and Cl⁻ compete for the transporting group, so that Cl⁻ would not be displaced by OH⁻ when the chloride concentration was increased to 600 mM as in the studies of Dalmark (1975). The studies of Funder and Wieth (1976) showed that this explanation cannot be correct. In their study of ghosts with a constant intracellular chloride concentration of 165 mM, an increase of pH from 7 to 10.5 had no effect on chloride exchange flux, and they stressed that the similarity between the ghosts and the KCl-enriched cells of Dalmark was that the effect of pH on intracellular chloride concentration (and thereby also on membrane potential) had been efficiently abolished in both cell preparations.

It is obvious that the effect of alkaline pH on chloride transport in ghosts at 38°C differs from the low temperature results cited above. Table 1 shows that the flux decreases with increasing pH. This observation raises the question of whether the difference between the 0°C and the 38°C results could be caused by a
temperature-dependent change of pK of a functional group in the transport system. Assume, for instance, that the transport system possesses a critical functional group with a pK of 12 at 0°C. This group will not be deprotonated to any measurable degree at 0°C in the accessible pH range, explaining why Funder and Wieth (1976) found a constant exchange flux up to pH 10.5. If the pK of the group changes to 10 when temperature is increased from 0°C to 38°C, one would expect to find a decrease of the flux by 50% at pH 10 as was actually the case (Table I). However, the table also shows that there was an almost linear decrease of the flux, over three pH units, of −15% per pH unit. This gradual decrease has no resemblance to that caused by the deprotonation of a single functional group in the transport system. On the other hand it cannot be excluded that the reduction of chloride exchange is related to a number of groups in the membrane with a pK distribution which would “smear” the titration curve over several pH units. In that case the effect on chloride transport could be indirect, namely by reducing the local chloride concentration through an effect on the surface potential of the membrane. Such an effect would be seen if deprotonation of quarternary ammonium groups and of phosphate groups in the polar heads of the phospholipids are critical for the local chloride concentration in an electrical double layer “feeding” the exchange transport system (Hall and Latorre, 1976). Fig. 5 shows that the flux decreased much more steeply with increasing pH in erythrocytes than in ghosts. This is qualitatively similar to the cited findings at 0°C. Dalmark (1975) showed that the effect of pH on chloride transport in erythrocytes at 0°C could be grossly accounted for by assuming that the reduction of flux is due to a reduced formation of chloride complexes with a transporting group. The number of these complexes was assumed to be determined by equilibrium reactions, as suggested by Gunn (1972), and the ratios between uncomplexed sites at the two surfaces (S− and S+) were assumed to vary according to the relation Cl−/Cl+ = S+/S−. It must be noted that the reported agreement between experimental findings and the model was only qualitative. For instance, the model (Dalmark 1975, Fig. 10) predicts a 20% decrease of flux

![Figure 9. Chloride distribution ratio and internal chloride concentration (mM) as a function of extracellular pH at 0°C and 38°C. At both temperatures the external chloride concentration is 150 mM. The dashed line shows the distribution at 0°C according to the equation: rCl = 3.18(SD ± 0.05) − 0.312(SD ± 0.007)·pH (Gunn et al., 1973 a). At 38°C the distribution is also a linear function of extracellular pH (rCl = 2.89 (SD ± 0.09) − 0.300(SD ± 0.01)·pH) parallel to the curve at 0°C but shifted ~0.6 pH unit in the acid direction.](image)
at pH 9.5, whereas reductions of 30% were found experimentally at the same pH value (Dalmark, 1975, Fig. 5) and a reduction by 70% at the same pH value has been reported by others (Gunn et al., 1973a). Qualitative agreement similar to that reported by Dalmark (1975) applies to the present results obtained at 38°C with the single addition that it is necessary to take into consideration the effect, found in ghosts, of pH on chloride exchange at constant intracellular chloride concentration (Table I). The conclusion, therefore, is that the effect of alkaline pH on chloride transport in red cells can also be interpreted at 38°C in terms of the effects of pH on intracellular chloride concentration and thereby on membrane potential, which may in turn determine the steady-state distribution of transport sites between the two surfaces of the membrane as originally suggested by Dalmark (1975).

The Temperature Dependence of Chloride and Bromide Transport

Self-exchange of chloride in red cells has not previously been measured at 38°C. Various authors have assumed that the rate of exchange at body temperature can be extrapolated from results obtained at 0-10°C if one assumes that the activation energy of chloride transport remains constant over the whole temperature interval. However, the present study (Fig. 2) shows that the activation energy decreased from about 30 to about 20 kcal mol \(^{-1}\) when the temperature was increased. The value obtained by extrapolation of low-temperature results exceeds the actual chloride flux at 38°C by a factor of four (600 vs. 150 mol [3.1 \(\times\) 10\(^{10}\) cells \(\cdot\) min\(^{-1}\)]). Chloride self-exchange above 10°C has previously been determined in a single investigation at 25°C by Tosteson (1959) who found a half-time of 220 ms at pH 7.4. The corresponding flux was 1.4 \(\times\) 10\(^{-8}\) mol cm\(^{-2}\) s\(^{-1}\) to be compared with a value of 1.5 \(\times\) 10\(^{-8}\) calculated from the present results.

POSSIBLE "ROLE OF UNSTIRRED LAYER" The decrease of activation energy is much smaller than that reported for chloride-bicarbonate exchange by Chow et al. (1976). Nevertheless, it is necessary to consider whether the decrease reported here might be an experimental artifact. One could ask whether diffusion of chloride through an unstirred layer might contribute significantly to chloride permeability when membrane transport is rapid. The unstirred layer has been estimated to correspond to an aqueous layer of 5-6 μm in a stop-flow apparatus (Sha’afi et al., 1967). This value may be taken as an upper limit under the present conditions, where flow is turbulent. With a diffusion coefficient for chloride of 2 \(\times\) 10\(^{-5}\) cm\(^2\) s\(^{-1}\) the permeability coefficient (\(P = D/\Delta x\)) of the unstirred layer is 4 \(\times\) 10\(^{-2}\) cm s\(^{-1}\), almost 100 times larger than the permeability measured at 38°C, pH 7.2 (\(P_c\): 5 \(\times\) 10\(^{-4}\) cm s\(^{-1}\)). Another proof that the deflection of the activation energy is not directly related to the rate coefficient of chloride exchange is found in the experiments with raised cellular chloride concentrations. It can be seen from the results in Table IIb that activation energy also decreased in high-chloride ghosts and in ammonium chloride-loaded cells where the half-time of exchange is up to 10 times longer than in cells at physiological ionic strength.

THE POSSIBLE ROLE OF PARTIAL SATURATION It was shown in Fig. 6 that the chloride transport system of human red cells with an intracellular chloride concentration of 110 mM is not saturated at 38°C. The transport system is operating at only 60-70% of its maximum capacity, and one must consider how
much this could decrease the apparent activation energy. Calculations show that
the activation energy would only increase by 2-3 kcal mol$^{-1}$ between 15°C and
38°C if the chloride transport system was saturated in the whole range between
15°C and 38°C. In this context it is worth noting that the activation energy was in
fact not significantly different in cells with an intracellular chloride concentra-
tion of 110 mM and in chloride-loaded cells or ghosts, where saturation of
transport was achieved in the whole temperature interval.

**Critical Temperature or Critical Rate**

The evidence thus shows that
the change of activation energy with temperature is in fact real. It cannot be
excluded that the change is more gradual than indicated by the straight lines of
the regression analyses (viz. Fig. 2). However, it simplifies the evaluation if one
assumes that the curves can be resolved into two straight lines with a distinct
point of intersection. There appear to be at least two possibilities: (a) that the
energy barrier of chloride transport decreases at a critical temperature (e.g., due
to a phase transition of some membrane component); or (b) that chloride
exchange is rate limited by one step in the transport process when the rate is slow
(below 15°C), whereas another step with a somewhat lower activation energy
becomes rate limiting at high temperatures.

**Critical Temperature**

It cannot be excluded that the state of the membrane lipids indirectly may affect
the transport rates of ions and hydrophilic molecules by modifying the function
of membrane lipoproteins as suggested by Warren et al. (1975). Studies of anion
transport in red cells from various animals show a correlation between mem-
brane lipid composition and rate of transport (Deuticke and Gruber 1970; Wieth
et al., 1974). Changes of the activation energy of sugar transport in *Escherichia
coli* strains correlate with the temperatures at which membrane lipids undergo a
phase transition (Linden and Fox 1973). It is not clear whether a phase transition
of erythrocyte membrane lipids actually occurs between 0°C and 38°C. Aloni et
al. (1974) determined the microviscosity and fusion energy of lipids in intact
erthrocytes, in ghost membranes, and in liposomes of the lipid extracts without
finding a phase transition between 0°C and 40°C. Several results reviewed by
Oldfield and Chapman (1972) support this observation. However, other investi-
gators have found evidence of a phase transition occurring around 10–20°C
(Johnson, 1975; Zimmer and Schirmer, 1975; Bieri and Wallach, 1976). If it is
assumed that the change of activation energy is due to a phase transition, it is a
necessary consequence that the critical temperature is influenced by the anions
of the medium. The deflection in the Arrhenius diagram occurred at 15°C in the
case of chloride transport, at 25°C for bromide transport, and it does not appear
to be present for anions that are transported more slowly than bromide (Dal-
mark and Wieth, 1972). The effects of anions on the temperature of phase
transitions in lipids recently described by Chapman et al. (1977) appear to be too
small to account for the present effects.

**Critical Rate**

A second possibility is that chloride exchange is rate limited by two different rate
coefficients. The Arrhenius diagram in Fig. 10 shows that the change of activa-
tion energy of chloride and of bromide transport did not occur at the same temperature. It can be seen that the kink appeared at the two temperatures where chloride and bromide fluxes were identical: i.e. at the same transport rate. For chloride transport the deflection was found at 15°C where chloride transport was 11.5 mol (3.1 · 10^13 cells · min)^{-1}, equalling 3.7 · 10^9 ions (cell · s)^{-1}. At 25°C, where the deflection was found in case of bromide, the transport amounted to 3.5 · 10^9 ions (cell · s)^{-1}.

If it is accepted as a working hypothesis that the activation energy changes when a critical turnover number is reached, the simplest explanation of this phenomenon would be that the overall transport rate is determined by two “reactions” with different activation energies, in such a way that one step with a high $E_A$ is rate limiting at low temperatures, whereas another step with a lower

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E_A \text{ becomes limiting at higher temperatures. Although nothing is known about the molecular nature of chloride exchange it is likely that it involves more than one rate process. A classical carrier model includes at least three rate coefficients: (a) the formation of the anion-carrier complex; (b) translocation of the complex; and (c) liberation of the anion from a site on the carrier. The deflection of $E_A$ could then be the result of a highly temperature-dependent step that is rate limiting at low temperatures ($E_A \sim 30$ kcal mol^{-1}), whereas a less temperature-dependent process becomes rate limiting at the temperature where the critical turnover rate of the transport system is exceeded.}

At this point it must be interpolated that the amount of evidence showing that a 10^6 dalton membrane protein is involved in anion transport makes it unlikely that anion transport in red cells is mediated by a simple mobile carrier (Rothstein et al., 1976). However, the general considerations above may as well be applied to a model where (a) the binding of an anion to a site in a membrane protein

![Figure 10. Arrhenius plot of the temperature dependence of chloride and of bromide self-exchange at 0–38°C. The logarithmic righthand ordinate shows the turnover number of ions per cell per second. The data of chloride self-exchange are repeated from Fig. 2. The results of bromide self-exchange were obtained by means of the Millipore-Swinnex filtering technique at 0–25°C and by means of the flow-tube method above 25°C. The pH of the experiments was 7.2. The extracellular halide concentration was 150 mM in all experiments.](image-url)
BRAHM Chloride Transport in Human Red Cells

leads to (b) a conformational change in the protein which (c) allows the further passage of the anion through the membrane.

It is implied by the present interpretation that the transport system functions at a slower rate when it is transporting bromide instead of chloride. It appears from Fig. 10 that the exchange flux of bromide at any temperature is 3-10 times smaller than that of chloride. According to the present interpretation the smaller flux of bromide at any given temperature cannot be due to a decreased number of transport sites all functioning with the rates characteristic for chloride transport. The differing rates of halide transport have been observed by Tosteson (1959) and by Dalmark and Wieth (1972). Determinations of the half-saturation constants for chloride, bromide, and iodide (Gunn et al., 1973b) clearly indicate that these rates are not due to differing affinities of the anions for the transport system. Therefore, it is reasonable to assume that the rate of translocation is modified by the chemical nature of the halide.

ACTIVATION ENERGIES FOR TRANSPORT OF ANIONS OTHER THAN CHLORIDE AND BROMIDE It is therefore concluded that the activation energy of anion transport remains constant and high until a critical transport rate is exceeded, and that it decreases from 30 to 20 kcal·mol⁻¹ when the turnover of anions exceeds 4·10⁹ ions (cell·s)⁻¹. This hypothesis would also explain why the activation energies of more slowly transported anions have been found to remain constant even at higher temperatures. Dalmark and Wieth (1972) found that self-exchange of iodide has a constant activation energy of 36 kcal·mol⁻¹ between 0°C and 38°C, and studies of sulfate transport have also demonstrated constant activation energies of 33-38 kcal·mol⁻¹ between 0°C and 38°C (Lepke and Passow, 1971; Wieth, 1970a). These findings agree with the concept of a critical turnover number that will not be reached by I⁻ or by SO₄²⁻, which are transported 10⁸-10⁹ times slower than chloride.

THE ACTIVATION ENERGIES OF OTHER TRANSPORT SYSTEMS IN RED CELLS The anion transport system is not the only transport mechanism of the red cell membrane that shows a decrease of activation energy between 0°C and 38°C. Sen and Widdas (1962) found an activation energy of glucose transfer of 20 kcal·mol⁻¹ around 20°C, decreasing to 7-10 kcal·mol⁻¹ around 37°C. This is interesting because glucose transport, according to Taverna and Langdon (1973), is ascribed to that group of integral membrane proteins which are believed to be involved in anion transport. Recent studies do not exclude this possibility (Kasahara and Hinkle, 1976; Zala and Kahlenberg, 1976). Hoare (1972) studied the activation energy of the facilitated diffusion of L-leucine and found a decrease from 47 to 17 kcal·mol⁻¹ above 20°C. These changes in activation energy have been found in NaCl media in transport systems which presumably function considerably more slowly than the chloride-transporting system. If the activation energies of sugar and amino acid transport become constant in, e.g., iodide media, one might hypothesize that the thermodynamic properties of the transport systems depend on the nature of the anions present. In this context it may be noted that facilitated diffusion of erythritol through the hexose transport system is inhibited when chloride is replaced by the foreign anions thiocyanate or salicylate (Wieth, 1971) and that very complex changes of
the activation energy of passive sodium and potassium transfer have been found when chloride in the cells and medium has been replaced by the same anions (Wieth, 1970b).

**Activation Energy of Inhibited Chloride Transport**

If the above interpretation is correct it follows that the change of activation energy should persist if chloride transport is inhibited by an inhibitor which reduces the maximum flux by removing a fraction of the transport sites without affecting the transport rate of the remaining sites.

Attempts have been made to characterize the inhibition caused by the amino reagent DIDS which is an irreversible inhibitor of anion transport (Cabantchik and Rothstein, 1974; Lepke et al., 1976). It is shown in Fig. 7 that a similar temperature dependence of chloride transport was found when the exchange was reduced by two-thirds with DIDS. The results therefore strongly suggest that DIDS inactivated two-thirds of the anion-transporting sites, whereas the remaining fraction of sites operated with a turnover rate as high as in the case of noninhibited chloride transport.

If the chloride transport was reduced by more than 99%, however, a constant activation energy of 20 kcal mol\(^{-1}\) was found in the whole temperature interval between 0°C and 38°C. Previous studies have shown that the almost complete inhibition of chloride transport with salicylate (Dalmark and Wieth, 1972) and with trinitrocresolate (Dalmark et al., 1972) also caused a constant activation energy. This is surprising if one believes that the amino reagent and the aromatic anions are "non-competitive inhibitors" in the sense that they act by reducing the rate of transport of the sites. However, it must be stressed that these inhibition studies were all performed under conditions where chloride exchange was inhibited by more than 99%. It cannot be excluded that there exist minor classes of transport sites which always function at a rate lower than the critical turnover rate and which are more resistant to inhibitors than the vast majority of transport sites. In that case the nature of inhibition may be obscured when almost complete inhibition is achieved.

**The Turnover Rate of Chloride Ions**

It is shown in Table II that chloride transport at 0°C was about 0.7 mol·(3.1·10\(^{13}\) cells·min\(^{-1}\)) which equals 2.3·10\(^{8}\) ions·(cell·s\(^{-1}\)). At 38°C the chloride transport of 150 mol·(3.1·10\(^{13}\) cells·min\(^{-1}\)) corresponds to a value of 4.9·10\(^{10}\) ions·(cell·s\(^{-1}\)). The transport rate thus increased about 200 times when temperature was raised from 0°C to 38°C.

It has previously been mentioned that there is some evidence that an integral membrane protein with a molecular weight of about 10\(^{6}\) daltons is involved in anion transport. Steck (1974) estimated that there are about 10\(^{6}\) copies of these protein molecules per cell. The number of transport sites may be smaller if anions are transported through a dimer or a tetramer of the membrane protein.

It has been suggested (Cabantchik and Rothstein, 1974; Rothstein et al., 1976) that each cell membrane carries about 3·5·10\(^{8}\) inhibitor binding sites. The same order of magnitude was found by Ho and Guidotti (1975) using the sulfanilate anion or the isothiocyanate derivative of the sulfanilate anion as inhibitor.
However, it has been suggested by Zaki et al. (1975) that the actual number may be higher because their studies of the competition between binding of a stilbene derivative and dinitrofluorobenzene revealed a number of 0.8–1.2 \times 10^6 sites cell\(^{-1}\). A recent study of Lepke et al. (1976) shows that the band 3 proteins bind as many as 1.2 \times 10^6 \text{H}2\text{DIDS} molecules per cell.

It may be of interest to compare the turnover rate for chloride transport in red cell membranes with the turnover rates of ion transport through modified bimolecular lipid membranes. If one assumes that each red cell membrane carries \(10^6\) transport sites, the turnover number per site is \(5 \cdot 10^4\) ions s\(^{-1}\) at 38°C. For bimolecular lipid membranes data are available both for potassium transport mediated by valinomycin, which is believed to be a cycling carrier, and for potassium transport mediated by gramicidin, which is believed to form pores or channels through the membrane. The carrier transport of potassium by valinomycin had a turnover number of \(10^4\) ions s\(^{-1}\) at room temperature (Läuger, 1972). When an activation energy of 14–19 kcal mol\(^{-1}\) is used (Benz et al., 1973) the turnover rate at 38°C is about \(5 \cdot 10^4\) s\(^{-1}\). In contrast, the pore-mediated potassium transport in gramicidin-treated membranes amounts to \(2 \cdot 10^7\) ions s\(^{-1}\) at 25°C (Hladky, 1974). According to Ginsburg and Noble (1974) the net activation energy of pore formation and cation transfer is 9.3 kcal mol\(^{-1}\), resulting in a turnover rate of \(4 \cdot 10^7\) s\(^{-1}\) at 38°C.

It is obvious that a comparison of the turnover rate of chloride transport with the rates found with ionophores in bimolecular lipid membranes does not lend information about the molecular nature of chloride transport. Still it is of interest to note that if one assumes the number of transport sites to be of the order of 1 million, the turnover rate of the biological transport system is two to three orders of magnitude smaller than that found in the narrow gramicidin pore, and appears to be within one order of magnitude from the turnover rate of the valinomycin carrier. Chloride transport is therefore not so rapid that it is necessary to postulate a rapid transfer of chloride through membrane pores. On the contrary the transport process appears to be slow enough to accommodate several successive steps, as e.g., the binding to a site followed by a minor conformational change in a protein molecule which, as suggested by Rothstein et al. (1976), may precede the actual penetration of the membrane barrier.

**The Hetero-Exchange of Chloride and Bicarbonate**

It must be emphasized that the activation energies of chloride and bromide transport are high in the whole temperature range of 0–38°C. The lower activation energy of 20 kcal mol\(^{-1}\) above the critical turnover rate means that \(Q_{10}\) is 3–4 and not 5 as predicted from results at low temperatures. \(Q_{10}\) of 1.2–1.7 (\(E_A\) 4–9 kcal mol\(^{-1}\)) between 24°C and 38°C, as reported by Luckner (1948) and by Chow et al. (1976) for the hetero-exchange of chloride for bicarbonate, is significantly lower than the present values. In both these studies anion movements were registered electrometrically by an electrode system: silver-silver chloride electrode (Luckner, 1948), and pH-glass electrode (Chow et al., 1976). The low activation energies are very close to those found for free diffusion of electrolytes in water. It is obvious that the effect of diffusion on the response time might
have been a real problem in the experiments of Luckner, which were carried out with a layer of unstirred red cells. A similar explanation does not appear to apply to the experiments of Chow et al. (1976), because the response time of their pH stop-flow technique has been reported to be below 5 ms (Crandall et al., 1971). As stated above, it has not yet been excluded that individual anion species can exert specific effects on the activation energy of anion transport at physiological temperatures. If there is such an effect, the results of Chow et al. may be due to a specific effect of bicarbonate on the thermodynamics of the transport process at high temperatures.

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