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CHLORIDE TRANSPORT IN HUMAN RED CELLS

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SUMMARY

1. The chloride equilibrium flux (chloride self-exchange) was determined by measuring the rate of ^{36}Cl efflux from radioactively labelled human red cells. The cellular chloride concentration was varied between 5 and 700 mM by the nystatin technique (Cass & Dalmark, 1973). The chloride transport capacity was not affected by the nystatin technique.

2. The chloride equilibrium flux showed saturation kinetics in the pH range between 6·2 and 9·2 (0° C). The chloride transport decreased at chloride concentrations higher than those which gave the maximum transport.

3. The apparent half-saturation constant, ($K_{\frac{1}{2}}$), depended on the pH and whether the chloride transport was perceived as a function of the chloride concentration in the medium or in the cell water. The $(K_{\frac{1}{2}})_m$ increased and the $(K_{\frac{1}{2}})_c$ decreased with increasing pH. The dependence of the chloride transport on the chloride concentration was described by Michaelis–Menten kinetics at pH 7·2, but at values of pH outside pH 7–8 S-shaped or steeper graphs were observed.

4. The chloride equilibrium flux varied with the pH at constant chloride concentration in the medium (pH 5·7–9·5). The transport had a bell-shaped pH dependence at chloride concentrations below 200 mM. At chloride concentrations between 300 and 600 mM the chloride transport increased with increasing pH to reach a plateau around pH 8. The position of the acidic branches of the pH graphs was independent of the chloride concentration (25–600 mM), but the position of the alkaline branches moved towards higher values of pH with increasing chloride concentration (5–150 mM). Thus, the position of the pH optimum increased with increasing chloride concentration. The chloride transport at low pH values was a function of the inverse second power of the hydrogen ion concentration. The pK of the groups which caused the inhibition was approximately 6 and independent of the temperature (0–18° C).

5. The chloride equilibrium flux as a function of chloride concentration,

pH, and temperature could be described by a transport model with a mobile, positively charged, chloride binding carrier with a single chloride dissociation constant of 33 mM, a transport capacity of 900 m-mole/3×10^{13} cells.min (pH 7·2, 0° C), and an Arrhenius activation energy of 30 kcal/mole. The pH dependence of the transport of inorganic monovalent and divalent anions is discussed in relation to the suggested model.

INTRODUCTION

It appears from investigations carried out in the last years that the chloride transport in red cells takes place as facilitated diffusion. This suggestion is based on (1) saturation kinetics (Gunn, Dalmark, Tosteson & Wieth, 1973), (2) a chloride tracer 'permeability' four orders of magnitude higher than the chloride *ion* permeability (Harris & Pressman, 1967; Hunter, 1971), (3) a high electrical resistance of the red cell membrane (Lassen, 1972), (4) competition between the chloride transport and the transport of other monovalent anions (Gunn *et al.* 1973), and (5) a high Arrhenius activation energy of the chloride transport (Dalmark & Wieth, 1972).

The purpose of the present work was to demonstrate the influence of the chloride concentration, the pH, and the temperature on the chloride equilibrium flux.

The experiments were carried out with human red cells suspended in salt solutions with chloride as the only anion. The cellular content of chloride was altered by treating the cells with salt solutions containing nystatin (Cass & Dalmark, 1973; Dalmark, 1975). Nystatin increased the permeability of the cell membrane towards monovalent, inorganic cations and anions. After equilibration of salt between the cells and the medium, nystatin was washed out of the cell membrane, and the low permeability of the cell membrane towards monovalent cations and anions was restored. The cellular concentration of KCl varied between 5 and 700 mM.

The results are discussed in relation to a model of the chloride transport (Gunn *et al.* 1973). This model was based on the interpretation of the red cell membrane as a liquid anion exchange membrane (Wieth, 1972), the chloride transporting components of which were hydrogen ion titratable (Gunn, 1972).

METHODS

All experiments were performed by determining the rate of ^{36}Cl efflux from radioactively labelled human red cells. Conditions were arranged so that there was no net flux of chloride across the cell membrane, i.e. the net loss of radioactive chloride was balanced by an equal uptake of non-radioactive chloride.

Preparation of the cells. Freshly drawn, heparinized human blood was titrated

with CO_2 to pH 7·2 (0° C). The CO_2/bicarbonate was removed from the cells by four washes with a solution containing 150 mM-KCl, 1 mM-NaCl, 27 mM sucrose. After removal of CO_2/bicarbonate the cell suspensions were only buffered by the cellular content of haemoglobin, and the pH of the cell suspensions was stable (± 0·01 pH units) for more than 24 hr when exposed to atmospheric air. The chloride distribution ratio was one at pH 7·2 (0° C) (Cass & Dalmark, 1973; Dalmark, 1975).

The concentrations of KCl and NaCl in the cells were altered by the nystatin technique as previously described (Cass & Dalmark, 1973; Dalmark, 1975). The electrolyte solutions employed for the preparation of the cells, for the incubation of the cells with ^{36}Cl, and for the efflux experiments had the following composition (mM): 5–700 KCl, 1 NaCl, 27 sucrose.

Titration of the cells. After the removal of nystatin from the cells the cell suspensions were titrated with HCl/KOH dissolved in the salt solution with which the cells were equilibrated. After the titration the cells were washed thrice with the salt solution to restore the concentrations in the medium of KCl, NaCl, and sucrose.

Labelling and isolation of the cells. The cells were incubated at the appropriate temperature with ^{36}Cl (haematocrit 0·3–0·4 v/v). The pH of the cell suspension was measured after a period of more than 10 half-times of the chloride exchange. The cell suspension was pipetted into nylon tubes kept at the temperature of the experiment. The cell suspension was centrifuged at the temperature of the experiment in a MSE Superspeed 40 centrifuge (MSE Ltd., Crawley, England) until a 85,000 *g* force was achieved (10 min). After centrifugation the column of packed cells (100–200 mg wet weight) was isolated by cutting the tube 1 mm below the cell-medium interface. The trapped extracellular volume was 2% (wt./wt.). After isolation the cells were kept at the temperature of the experiment until the experiments were carried out.

Determination of the rate of tracer efflux. 40 ml. of the efflux medium were incubated at the appropriate temperature and pH, and stirred vigorously by a Teflon-coated magnetic bar. The pH of the salt solution was adjusted to the appropriate pH by addition of HCl/KOH to the salt solution buffered with 50 μM phosphate. The cell suspension was buffered by the cellular content of haemoglobin after the cells were injected into the salt solution. At the start of the efflux experiment 100–200 mg of packed, labelled cells were injected into the medium by flushing the small sleeve of the nylon tube containing the cells with 5–10 ml. of the medium. The efflux of ^{36}Cl from the cells to the medium was followed during the experiments by serially isolating cell-free medium by rapid filtration from the cell suspension as previously described (Dalmark & Wieth, 1972).

Isotopes. ^{36}Cl was obtained as NaCl with a specific activity of 0·1–0·6 mc/m-mole (Amersham, England). The amount of radioactivity employed for labelling the cells was 0·5 μc/ml. cell suspension (haematocrit 0·3–0·4 v/v).

Determination of radioactivity. After labelling of the cells for the efflux experiments the concentration of ^{36}Cl in the cells and in the medium was measured. Duplicates of the clear supernatant of perchloric acid precipitated packed cells and medium were added to the scintillation fluid (toluene 70% (v/v), ethanol 30% (v/v), 2,5-diphenyl-oxazole 3·5 g/l., 1,4-bis, 2-(4-ethyl-5-phenyl-oxazolyl) benzene 0·07 g/l.) and counted in a liquid scintillation spectrometer (Tricarb 3310, Packard Instrument Co., Downers Grove, Ill.). The rate of ^{36}Cl efflux was determined by counting 300 μl. samples of cell-free medium.

Determination of cell water and the chloride concentration in cells and medium. The percent of wet cell weight which is intracellular water, was determined by

drying a column of packed cells to constant weight. The apparent water fraction was then corrected for 2% extracellular trapped water. Duplicate cell columns of known wet weight were added to 1 ml. 7% perchloric acid. Similar, duplicate samples (200 μl.) of the suspending medium were precipitated in perchloric acid. The chloride concentration in the medium was determined in the clear supernatant by coulometric titration (CMT Chloride Titrator, Radiometer, Denmark). The cellular chloride concentration was determined as the product of the distribution ratio of radioactive chloride and the chloride concentration in the medium. The distribution ratio of chloride was determined as the ratio of radioactive chloride between cell water and the medium.

Calculations. All experiments were performed under steady-state conditions, i.e. the net flux of ^{36}Cl was balanced by an equal and opposite movement of non-radioactive chloride. The exchange kinetics of chloride was in all experiments well described by a closed two compartment model with constant volumes (Fig. 1*a* and *b*). The equation describing the time dependence of the specific activity in the medium was

$$a_t = a_\infty(1-e^{-bt}), \tag{1}$$

where a_t was the specific activity at time t, a_∞ the specific activity in both phases at isotopic equilibrium, and the exponent b was equal to the sum of the rate coefficients for isotope efflux (k^0) and influx (k^i). The rate coefficient k^0 approaches the value of b (eqn. (1)), when the haematocrit is low. The haematocrit was below 1% in the present experiments, and k^0, therefore, constitutes more than 98% of the value of b. The rate coefficient of chloride was calculated from the relation between $\ln(1-a_t/a_\infty)$ and the time, t, by a least-square linear regression analysis. The slope of the graph was put equal to $-k^0$.

The chloride equilibrium flux (efflux = influx) was calculated from the relation:

$$M^{Cl} = k^0 \times V \times (Cl^-)_c \times F(\text{m-mole Cl}/3\times10^{13}\text{ cells.min}), \tag{2}$$

where k^0 is the rate coefficient of tracer efflux (min^{-1}), V is the cellular water content (kg water/kg solids), $(Cl^-)_c$ is the cellular chloride concentration (m-mole/kg cell water), and F is a factor correcting 1 kg cell solids to 3×10^{13} cells (Dalmark, 1975); 3×10^{13} cells with a surface area of 4890 m^2 (Ponder, 1948) contain 1 kg cell solids of normal cells at an ionic strength of 0·15 (Funder & Wieth, 1966*a*). The factor F was introduced in order to correlate the chloride equilibrium flux to an identical amount of cells, since the amount of cell solids per single cell varied with the cellular content of KCl.

The Arrhenius activation energy of the chloride transport was calculated by linear regression analysis of the relation between the natural logarithm of the rate coefficient of efflux (k^0) and the reciprocal of the absolute temperature. The activation energy (E) was calculated from the relation: $\ln k^0 = (-E/R)(1/T)+C$, where k^0 (min^{-1}) is the rate coefficient of ^{36}Cl efflux, E is the Arrhenius activation energy (in cal/mole), R is the universal gas constant (1·987 cal/mole.°K), and T is the absolute temperature (°K).

RESULTS

Control experiments

It has previously been demonstrated that the chloride equilibrium flux was not affected by the nystatin treatment (Cass & Dalmark, 1973). The chloride equilibrium flux was a reversible function of chloride concentration. After restoring the concentration to a given initial value the chloride

transport made up 100% (S.D., 6, $n = 4$) of the transport, measured before the alteration of concentration. The standard deviation of the transport in cells from the same donor was 6% of the average transport ($n = 8$) at constant chloride concentration.

It will be shown (Fig. 5) that the chloride equilibrium flux was a function of pH. Since cell volume is also influenced by pH (Dalmark,

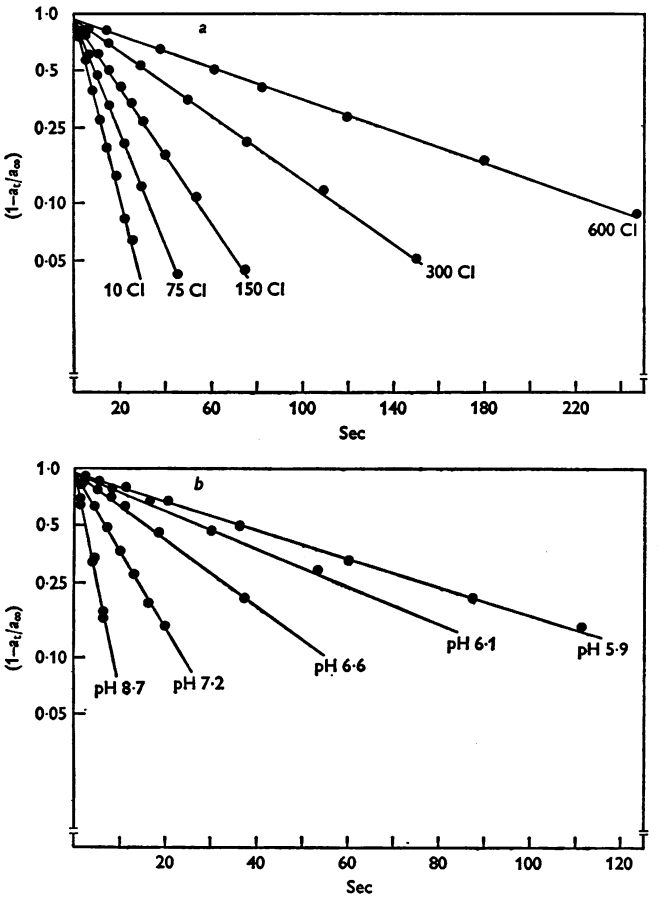

Fig. 1*a* and *b*. The rate of ^{36}Cl efflux from radioactively labelled human red cells under conditions with no chloride net flux. The experiments of Fig. 1*a* were performed at pH 7·2 (0° C) at various KCl concentrations (10–600 mM) in the cells and in the medium. The experiments of Fig. 1*b* were performed at a constant KCl concentration in the medium (50 mM) at various values of the pH (0° C) in the medium. The rate coefficients of the chloride self-exchange were calculated from the slopes of the graphs of ln $(1-a_t/a_\infty)$ *vs.* time as described in the Methods section, a_t and a_∞ being the specific activity in the medium at the time of sampling and at isotopic equilibrium.

1975), it was necessary to study the effect of cell volume *per se* on chloride flux. This was done by using various concentrations of sucrose during nystatin treatment to prepare cells, all with a chloride distribution ratio of 1·0 (pH 7·2), all with intra- and extracellular KCl concentrations of 400 mM, but with water contents varying from 1·2 to 2·7 kg water per kg cell solids. After washing the cells free of nystatin it was demonstrated that the chloride equilibrium flux was not affected by the large variations in cell volume.

The rate of chloride exchange

The chloride equilibrium flux was determined by measuring the efflux of ^{36}Cl from human red cells into the medium. Some results are shown

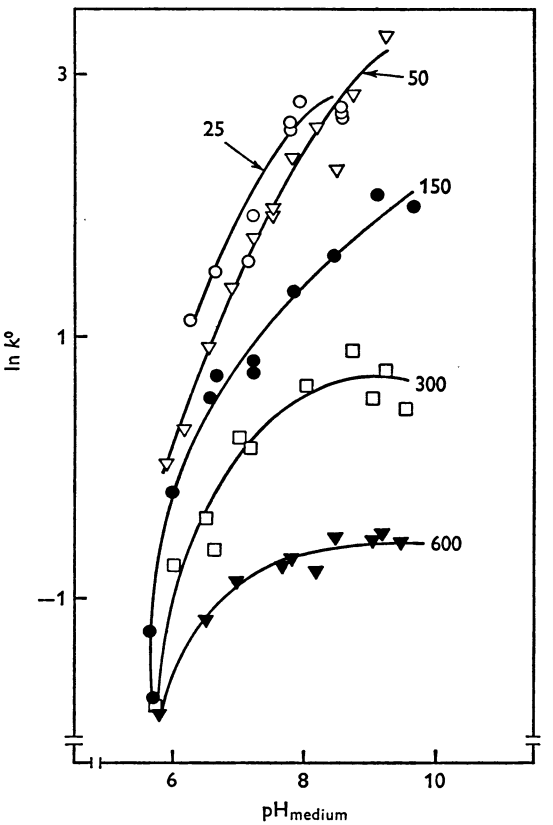

Fig. 2. The influence of pH on the natural logarithm of the rate coefficient k^0 (min^{-1}) of ^{36}Cl efflux from radioactively labelled human red cells to the suspending medium at five different KCl concentrations (mM) shown with each line under conditions where the chloride net transport was zero (0° C).

in Fig. 1 *a* and *b*. Fig. 1 *a* shows the rate of ^{36}Cl efflux (pH 7·2, 0° C) from cells into media having the same KCl concentration (10–600 mM). The Figure shows the effect of the chloride concentration on the rate of ^{36}Cl efflux. The half-time for equilibration of ^{36}Cl was 6 sec at a chloride concentration of 10 mM increasing to 16 sec at 150 mM and to 65 sec at 600 mM chloride.

Fig. 1 *b* shows the effect of pH on the rate of ^{36}Cl efflux at a constant chloride concentration in the medium (50 mM-KCl). The Figure shows that the half-time of the ^{36}Cl efflux decreased with increasing pH. The half-time was 36 sec at pH 5·9 decreasing to 7 sec at pH 7·2, and to 3 sec at pH 8·7.

The rate coefficients of the ^{36}Cl efflux were determined from the slopes of the graphs in Fig. 1 *a* and *b* (cf. Methods section). Fig. 2 shows the natural logarithm of the rate coefficients of ^{36}Cl efflux at five different KCl concentrations (25–600 mM) in cells and medium at different values of the pH in the medium (0° C). The cells, except at normal ionic strength, were prepared by the nystatin technique. The composition of the media was constant at each single chloride concentration except with respect to pH which was varied between 5·6 and 9·5.

Fig. 2 shows that the rate coefficient at a given KCl concentration increased with increasing pH, but the slope of the graph decreased as the pH increased. This confirmed previous investigations on the effect of the pH on the rate coefficient at normal ionic strength (Gunn *et al.* 1973). The pattern of the graphs was a fan starting at pH 5·6. The variation of the rate coefficient with increasing pH was a function of the chloride concentration: the increase was smaller at high chloride concentrations than at low concentrations, and at high chloride concentrations the rate coefficient was independent of the pH above pH 7.

Effect of chloride concentration on chloride transport

The distribution of chloride between cells and medium varies with pH (Dalmark, 1975). Therefore, the relationship between chloride flux and chloride concentration at a given pH depends on whether extracellular or intracellular chloride is taken as the independent variable. Fig. 3 shows the chloride equilibrium flux (cf. Methods section) as a function of the extracellular chloride at six different pH values. In each case the graphs showed saturation kinetics, a finding which has been previously reported at pH 7·2 (Wieth *et al.* 1973; Gunn *et al.* 1973; Cass & Dalmark, 1973). Furthermore, the extracellular chloride concentration required to saturate the mechanism was lowest (50 mM) at pH 6·2 and increased with increasing pH, being over 200 mM at pH 9·2.

Fig. 4 demonstrates that when chloride flux was plotted as a function of intracellular chloride concentration, the pH response pattern of Fig. 3

was reversed: The cellular chloride concentration required to saturate the chloride transport mechanism was lowest (50 mM) at the highest pH and highest at the lowest pH.

For subsequent analysis it is convenient to define two apparent half-saturation constants, $(K_{\frac{1}{2}})_c$ and $(K_{\frac{1}{2}})_m$. These will refer to the chloride concentration (respectively in the cell or medium water) at which chloride flux was half maximum at a given pH. These constants cannot be rigorously

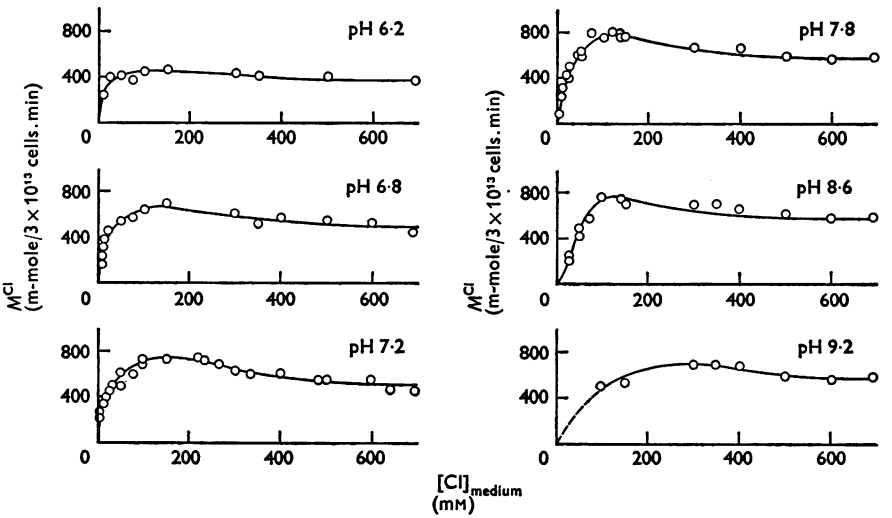

Fig. 3. The dependence of the chloride equilibrium flux on the chloride concentration in the medium at different values of pH in the medium (0° C).

equated to Michaelis constants, since, as shown in Fig. 4, the graphs relating chloride flux and chloride concentration were for the most part sigmoid in shape. The $(K_{\frac{1}{2}})_m$ (mM) was 10 (pH 6·2), 15 (pH 6·8), 18 (pH 7·2), 23 (pH 7·8), 48 (pH 8·6), and around 70 (pH 9·2) (Fig. 3).

However, at external pH 7·2, where the values for extra- and intracellular chloride were quite similar, the data permitted Michaelis–Menten analysis, presented in Table 1. The half theoretical maximum was obtained at 26 mM-KCl in the medium, which at this pH was in equilibrium with a cellular chloride of 44 mM. These different values of the $(K_{\frac{1}{2}})$ reflected the fact that even at pH 7·2 (0° C) the cellular chloride concentration was different from the extracellular concentration when the ionic strength was low. The cellular chloride concentration was 1·7 times higher than the extracellular chloride concentration at pH 7·2 when the chloride concentration in the medium was 26 mM (Dalmark, 1975).

Fig. 3 shows that at each pH level chloride flux passed through a

maximum and then decreased as the chloride concentration was further raised. It was not possible to decide whether the chloride transport was going towards zero or a limit different from zero. The inhibition of the chloride transport per 100 mM increase of the KCl concentration in cells

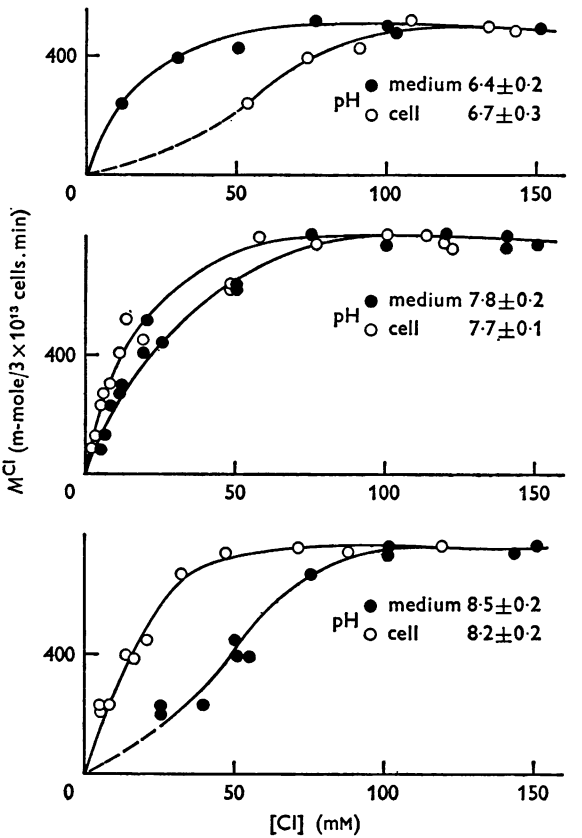

Fig. 4. The chloride equilibrium flux as a function of the chloride concentration in the cell water (○) and in the medium (●) at different values of pH (0° C). The composition of the media was 5–150 mM-KCl, 1 mM-NaCl, and 27 mM sucrose.

and medium decreased with increasing KCl concentration and decreasing pH. The inhibition of the chloride transport was at pH 7·2 (0° C) 70 m-mole $Cl/3 \times 10^{13}$ cells.min per 100 mM increase in the chloride concentration at 300 mM-KCl in the medium and 30 m-mole $Cl/3 \times 10^{13}$ cells.min per 100 mM increase in the concentration at 600 mM-KCl in the medium. At pH 6·2 (0° C) the inhibition was only 25 m-mole $Cl/3 \times 10^{13}$ cells.min

TABLE 1. The kinetic parameters of the chloride equilibrium flux, M^{Cl}, at pH 7·2 (0° C) when the chloride transport was perceived as a function of either the chloride concentration in the medium or the chloride concentration in the cells (Eadie-Woolf plot, Dixon & Webb, 1971). The parameters, $(K_{\frac{1}{2}})$ and M^{Cl} (max), were calculated by a linear regression analysis of the relation $M^{Cl}/(Cl^-) = (-1/(K_{\frac{1}{2}}))(M^{Cl}) + M^{Cl}(\max)/(K_{\frac{1}{2}})$, where M^{Cl} was the chloride equilibrium flux (m-mole Cl/3 × 10^{13} cells. min) at a chloride concentration of (Cl^-), M^{Cl} (max) was the theoretical maximum transport and $(K_{\frac{1}{2}})$ was the theoretical half-saturation constant (mM). The cells, except at normal ionic strength, were prepared by the nystatin technique. Media (mM): 2–170 KCl, 1 NaCl, 27 sucrose

M^{Cl} as a function of the chloride concentration	In the medium	In the cells
Regression coefficient $(-1/(K_{\frac{1}{2}}))$	−0·0386	−0·0227
S.D. of regression coefficient	0·0035	0·0016
Interception with ordinate $(M^{Cl}(\max)/(K_{\frac{1}{2}}))$	34·04	20·61
S.D. of interception	1·85	0·68
Correlation coefficient r	−0·94	−0·96
$(K_{\frac{1}{2}})$ (mM)	26	44
Theoretical M^{Cl} (max) (m-mole Cl/3 × 10^{13} cells.min)	881	907
Maximum M^{Cl} measured (m-mole Cl/3 × 10^{13} cells.min)	770	770

per 100 mM increase in the chloride concentration at 300 mM-KCl in the medium and 11 m-mole Cl/3 × 10^{13} cells.min per 100 mM increase in the concentration at 600 mM-KCl.

Effect of pH on chloride transport

Fig. 5*a* shows that the chloride flux had a bell-shaped pH dependence at chloride concentrations below 200 mM. This was previously demonstrated at normal ionic strength by Gunn *et al.* (1973).

However, the chloride transport at chloride concentrations of more than 200 mM (Fig. 5*b*) did now show a bell-shaped graph in the investigated range of the pH. At KCl concentrations of more than 300 mM the chloride transport increased with increasing pH to reach a plateau. The pH at which the plateau began, decreased with increasing chloride concentration.

The position of the pH optimum and the alkaline branch of the pH graph of the chloride transport was a function of the chloride concentration. The position of the *alkaline branch* moved towards higher values of pH with increasing KCl concentrations (Fig. 5*a*). Thus, as KCl concentration was increased from 5 to 150 mM, there was a corresponding increase in the alkaline pH at which the chloride flux was half the maximum value seen at a given KCl concentration. At high KCl concentrations (300–600 mM) the chloride transport was independent of the pH in the alkaline range (Fig. 5*b*).

The position of the *acidic branch* of the pH graphs of the chloride

transport was independent of the chloride concentration (Fig. 5*a* and *b*). The chloride transport decreased rapidly with decreasing pH below pH 7 at KCl concentrations between 25 and 600 mM.

The position of the *pH optimum* of the chloride transport varied with the chloride concentration (Fig. 5*a* and *b*) since the position of the alkaline branch was a function of the chloride concentration in contrast

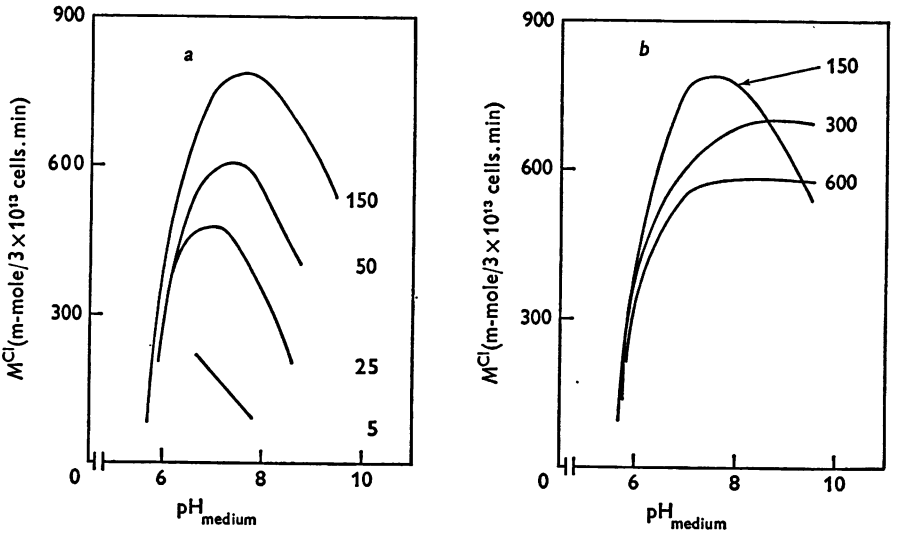

Fig. 5*a* and *b*. The influence of pH on the chloride equilibrium flux at 0° C at KCl concentrations between 5 and 150 mM (Fig. 5*a*) and between 150 and 600 mM (Fig. 5*b*). The composition of the media (5–600 mM-KCl, 1 mM-NaCl, 27 mM sucrose) at each single KCl concentration was constant at the different values of pH.

to the position of the acidic branch. The position of the pH optimum was around pH 7 at 25 mM-KCl, around 7·4 at 50 mM-KCl, and at 150 mM-KCl around pH 7·8. It was not possible for technical reasons to determine the pH optimum at 5 mM-KCl, but Fig. 5*a* shows that the position was below pH 6·7. At KCl concentrations of more than 200 mM the chloride transport had a broad pH optimum (Fig. 5*b*). The pH optimum began at a lower pH when the chloride concentration was increased.

Analysis of the pH-dependent chloride transport

It has been suggested by Gunn (1972) that the bell-shaped pH graph of the chloride transport may reflect the existence of a hydrogen ion titratable chloride binding carrier in red cells. According to this view the pH dependence of the chloride transport may be explained by changes

in the state of the ionization of the chloride binding group and the state of the ionization of saturated carrier-chloride complex. Changes in the state of ionization of the chloride binding group will affect the ($K_{\frac{1}{2}}$), but the effect of pH when all the chloride binding groups are saturated with chloride will reflect the state of ionization of the carrier-chloride complex.

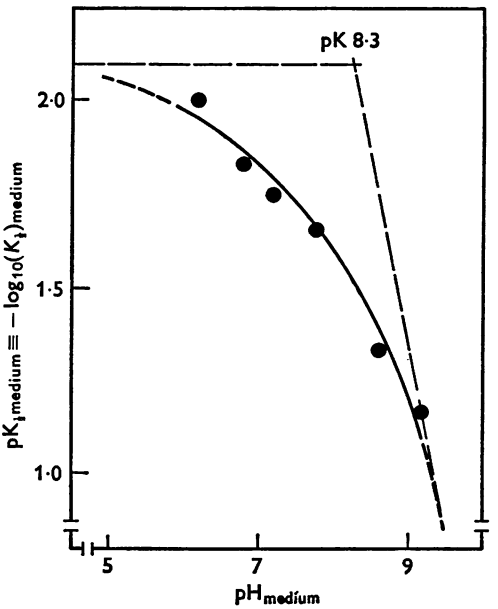

Fig. 6. The influence of pH on the $pK_{\frac{1}{2}m}$ ($\equiv -\log(K_{\frac{1}{2}})_m$) of the chloride equilibrium transport. $(K_{\frac{1}{2}})_m$ was defined as the chloride concentration (M) in the medium at half maximum chloride transport. Analysis of the data by methods commonly used in enzymology might indicate the pK of a hydrogen ion titratable, chloride binding group of 8·3 (0° C) (cf. Discussion section). The pK was determined as the pH of the intersection point between a horizontal line through the extrapolated maximum values of the $pK_{\frac{1}{2}m}$ and a tangent line with a slope of -1 through the measured $pK_{\frac{1}{2}m}$'s at higher values of pH.

The chloride equilibrium flux will be analysed by methods commonly used in enzymology (Dixon & Webb, 1971) in order to determine the hydrogen ion dissociation constants. The prerequisites of the methods are fulfilled when the ($K_{\frac{1}{2}}$) is known at different values of the pH.

The $pK_{\frac{1}{2}m}$ ($= -\log(K_{\frac{1}{2}})_m$) was plotted as a function of the pH, in order to determine the pK of the hydrogen ion titratable, chloride binding group. Fig. 6 shows that the $pK_{\frac{1}{2}m}$ of the chloride transport decreased with increasing pH. The value of the pH at the intersection point between the horizontal line and a tangent line with a slope of -1 was

8·3. This might be interpreted as the pK of the hydrogen ion titratable group of the chloride transport apparatus which in the protonized state is able to bind chloride; but it will be demonstrated below that it was unnecessary to assume that the chloride binding groups was hydrogen ion titratable in the pH range between 6·2 and 9·2.

The pH responsiveness of the chloride transport mechanism at a saturating concentration of chloride (600 mM) is shown in Fig. 5*b*. Firstly, one observes that the saturated chloride transport apparatus was not

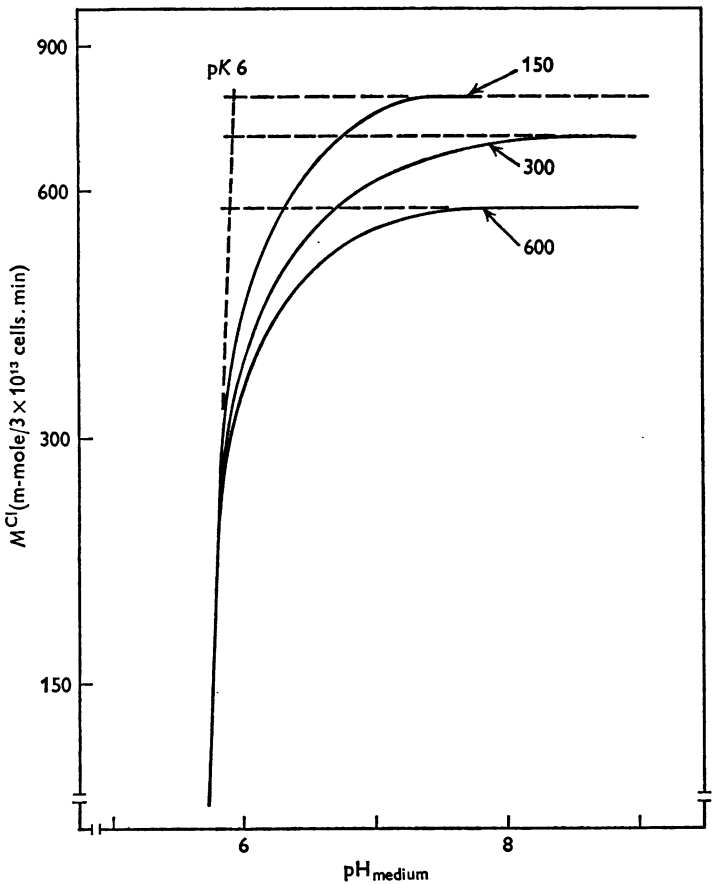

Fig. 7. The influence of pH on the logarithm of the chloride equilibrium flux at three different KCl concentrations (0° C). The pK of the proton binding groups was obtained at the pH of the intersection point between a horizontal line through the values of the maximum transport and a line through the values of the chloride transport at low pH. The pH of the intersection point was approximately 6. The slope ($\log_{10} M^{Cl}$/pH) of the line through the values of the transport at low pH was approximately 2/pH.

hydrogen ion titratable in the pH range between 7 and 9. Secondly, one observes that the flux decreased rapidly below pH 7; this decrease was independent of the chloride concentration (Fig. 5*a* and *b*). The chloride flux was plotted on a logarithmic scale as a function of the pH in the medium at high KCl concentrations (150–600 mM) (Fig. 7) in order to determine the pK of the group(s) which when protonized inhibited the chloride transport. The chloride transport apparatus was more than 90% saturated with chloride at chloride concentrations of more than 150 mM at low pH. The value of the pH at the intersection point between a horizontal line through the maximum values of the transport and a line through the values of the transport at low pH indicated the pK of the inhibition group(s). Fig. 7 informs us of two things: (1) the pK of the inhibiting group(s) was approximately 6, and (2) the slope ($\log M^{Cl}$/pH) of the line through the transport values at low pH was approximately 2/pH. The slope of the line through the transport values at low pH demonstrates that the chloride transport was a function of approximately the inverse second power of the hydrogen ion concentration at values of pH below 7.

In this section the pH effects have been interpreted by likening the chloride transport system to an enzymic reaction. The hazards of this approach are (*a*) that the system only showed Michaelis–Menten kinetics around pH 7 and (*b*) that under many conditions the values for pH, electrical potential, and chloride concentration differed on the two sides of the membrane. In the discussion it will be shown that the variation of $pK_{\frac{1}{2}}$ with pH can as well be described by an alternative formulation, employing a single constant (K_{Cl}) and the pH-dependent changes in chloride distribution ratio between cells and medium.

Effect of the temperature on the chloride transport

The chloride transport (0–10° C) had a high Arrhenius activation energy at pH 7·4 (32 kcal/mole) (Dalmark & Wieth, 1972). The temperature dependence of the chloride transport at 600 mM-KCl concentration in the medium and in the cells was investigated in order to determine the effect of pH on the activation energy.

Fig. 8*a* shows the natural logarithm of the rate coefficient, $k°$, of the ^{36}Cl efflux as a function of the reciprocal absolute temperature (0–18° C). It was not possible to determine the chloride transport at higher temperatures with the filtration technique (Dalmark & Wieth, 1972), since the half time of ^{36}Cl efflux was 3 sec (pH 9·5) at 18° C. Fig. 8*a* shows, (1) that the Arrhenius activation energy was 30 kcal/mole, and (2) that the activation energy was of the same magnitude in the pH range between 5·8 and 9·5.

Fig. 8*b* shows the logarithm of the chloride equilibrium flux as a function of the pH in the medium at 0, 9, 18° C for the same experiments as in Fig. 8*a*. The Figure shows that the influence of pH on the transport was identical in the temperature range between 0 and 18° C. The pK of the inhibiting group(s) was approximately 6 in the temperature range between 0 and 18° C. The ionization enthalpy of this group was zero. This contrasted considerably with the pronounced temperature dependence of the chloride transport itself in the same range of pH.

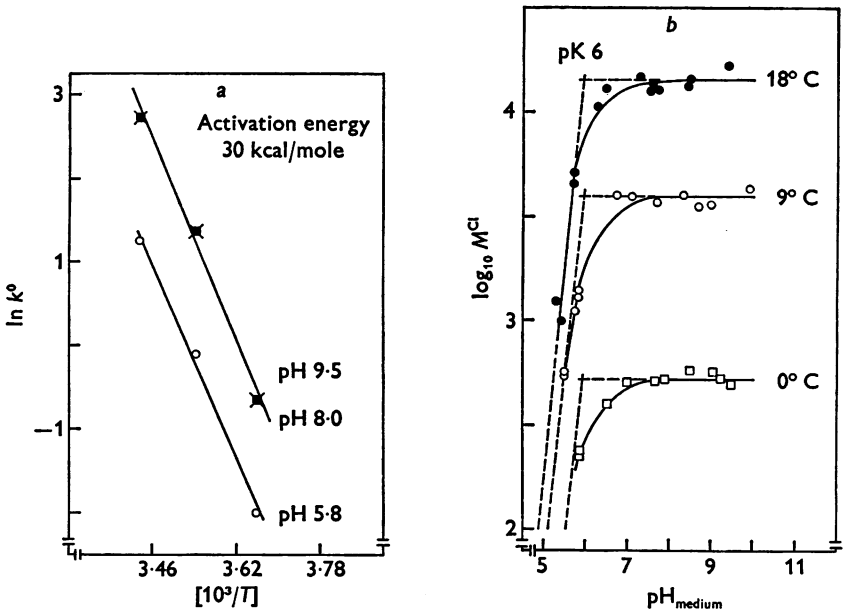

Fig. 8*a* and *b*. The influence of temperature (0–18° C) on the chloride equilibrium flux in human red cells suspended in 600 mM-KCl, 1 mM-NaCl, and 27 mM sucrose. Fig. 8*a* is an Arrhenius diagram of the natural logarithm of the rate coefficient, k^0, of ^{36}Cl efflux *vs.* the reciprocal of the absolute temperature. The relationship between ln $k^0(Y)$ *vs.* $10^3/T$ (X) at pH 8 and pH 9·5 was described by the equation: $Y = -14{\cdot}99X + 54{\cdot}3$, $r = 0{\cdot}99$. The activation energy of the chloride transport was 30 kcal/mole. Fig. 8*b* shows the influence of pH on the logarithm of the chloride equilibrium flux at three different temperatures (0, 9, 18°). The pK of the inhibiting groups of the chloride transport at low pH was independent of the temperature in distinction to the pronounced temperature dependence of the chloride transport itself.

DISCUSSION

The ionization of haemoglobin creates asymmetry across the membrane

The effect of the ionization of haemoglobin on the distribution of anions and on the magnitude of the electrical membrane potential

difference forms the basis of the discussion. It is important to take the ionization of haemoglobin into account since the magnitude of the variables which determine the chloride transport is regulated by the net charge on haemoglobin. The ionization of haemoglobin regulates: (1) the cellular chloride concentration (Dalmark, 1975), (2) the intracellular pH (Funder & Wieth, 1966*b*) and (3) the electrical membrane potential difference which under steady-state conditions is assumed to be equal to the chloride equilibrium potential.

This means that the chloride concentration, the pH, and the electrical potential in the cells are different from those in the medium when haemoglobin carries a net electrical charge. The distribution ratio between the chloride concentration in the cell water and in the medium (r_{Cl}) is used as an expression of the asymmetry caused by the ionization of haemoglobin in the following discussion.

The effect of pH on the maximum chloride transport and the ($K_{\frac{1}{2}}$)

It has been demonstrated that the chloride flux was strongly inhibited at low pH, being proportional to the inverse second power of the hydrogen ion concentration. The concentration independence of the transport inhibition indicated that protons were non-competitive inhibitors.

These results might be interpreted as though protons associate with some groups in the membrane in such a way that protonation of two groups is required for the great reduction of the transport. The two groups involved are probably not the only groups which associate with protons in this range of acidity; it is only assumed that the protonation of these two groups has a much greater effect on the chloride equilibrium flux than protonation of any other groups in this range. The temperature-independent pK (Fig. 8*b*) of these groups which is approximately 6 might indicate that carboxyl or phosphate groups are involved in the inhibition of the transport.

The inhibition of the chloride transport may be correlated to the altered structure of the red cell membrane when the pH decreases from pH 7 towards 5·5 (daSilva, 1972). Membrane particles observed by freeze-fracture electron-microscopy aggregated when the pH was lower than pH 7. This pH-dependent aggregation of membrane particles was independent of the temperature (-16 to $+35°$ C). In distinction to the concentration-independent inhibition of the chloride transport at low pH, the aggregation of the membrane particles was only observed at low ionic strength. Nevertheless, the two phenomena may be consequences of the same primary effect of protons on the cell membrane: the functional activity (the chloride transport) might be a more sensitive measure of conformational changes of components in the cell membrane which after

abolition of a protective effect of a high ionic strength is followed by greater structural changes observed by microscopy as membrane particle aggregation.

It has been demonstrated that $(K_{\frac{1}{2}})_m$ increased and $(K_{\frac{1}{2}})_c$ decreased with increasing pH (Fig. 4). This might indicate that the inside and the outside facing surfaces of the cell membrane had different properties. At first glance this might be correlated to the fact that phlorizin only inhibited the chloride transport when the phlorizin was added to the suspension medium, and no inhibition was observed when the phlorizin was added to the cell water (Schnell, Gerhardt, Lepke & Passow, 1973); but it is not certain that this conclusion was correct. This will be demonstrated below.

An anion transport model

It has been pointed out that there exist similarities between ion transport through a liquid, anion exchange membrane (Shean & Sollner, 1966) and the anion transport in human red cells (Wieth, 1972). It has, furthermore, been suggested that the anion transporting components (carriers) in the cell membrane are hydrogen ion titratable (Gunn, 1972).

The Appendix gives a mathematical description of anion transport based on these suggestions. The chloride flux has been expressed as a function of the chloride concentration in both medium and cell water, since the ratio of these two quantities changes as haemoglobin is titrated with hydrogen ions.

The following discussion distinguishes between two features of the chloride transport system. On the one hand there are the sites on the carrier molecules which bind transported chloride; on the other hand there are various determinants apart from the chloride binding sites which may influence the rate of transport by affecting the carrier molecules or the membrane matrix.

The observed pH-dependent maximum chloride transport, $M\ (\max)_{pH}$, is described by a pH-independent transport capacity, $P(\frac{1}{2}T)$, and a pH function (cf. app., eqns. (7) and (8)). The pH-independent transport capacity is the product of half the maximum number of carrier-chloride complexes and the transmission rate P. The pH function expresses the fraction of the pH-independent transport capacity which is observed at a given pH. The pH function is determined by the hydrogen ion titration of groups on the carrier molecules different from the chloride binding groups or groups on the cell membrane which, without being part of the carrier molecules, are able to inhibit the transport of the carrier-chloride complexes. The pH function has a constant value between pH 7 and 9, but the value decreases below pH 7 when groups with a pK of approximately

6 are titrated. The value of the pH function decreases as the maximum number of carrier-chloride complexes which are able to transport chloride decrease. The pH function may as well express an effect of hydrogen ions on the transmission rate P. It was not possible to test this hypothesis.

The self-inhibition of the chloride equilibrium flux is not taken into account in the description. The effect of a high chloride concentration on the chloride transport can be introduced by application of an empirical factor in the numerator of the eqns. (7) and (8). It was not possible to decide from the experiments whether chloride increased the dissociation constant of the carrier-chloride complex (cf. app., eqn. (2)), or decreased the transport capacity. It has been observed that other halides and bicarbonate inhibited the chloride transport in a competitive and in a non-competitive manner (M. Dalmark, in preparation). This might indicate that the chloride self-inhibition is an effect of chloride on the transport capacity. If so, both positively charged ions (hydrogen ions) and negatively charged ions (F^-, Cl^-, Br^-, I^-, HCO_3^-) are able to complex with groups on the membrane different from the chloride binding groups on the carrier molecules. After complexation these ions inhibit the chloride transport in a non-competitive manner.

The observed pH-dependent half-saturation constant, $(K_{\frac{1}{2}})$, is described by a pH-independent dissociation constant of the carrier-chloride complex, K_{Ci}, and a pH function (cf. app., eqns. (7) and (8)). The equations predict that $(K_{\frac{1}{2}})_c$ is equal to $(K_{\frac{1}{2}})_m$ multiplied by the chloride distribution ratio. This was experimentally confirmed (cf. Results section).

The equations predict that $(K_{\frac{1}{2}})_m$ increases and $(K_{\frac{1}{2}})_c$ decreases with increasing pH; e.g. $(K_{\frac{1}{2}})_m$ increases with increasing pH because (1) the chloride binding groups on the carrier molecules are titratable (cf. app., eqn. (1)), and (2) the carrier molecules accumulate on the inner surface of the cell membrane (cf. app., eqn. (5)). This pH dependence of $(K_{\frac{1}{2}})$ was qualitatively confirmed by experiments (Fig. 4). However, the quantitative agreement was poor between the calculated and measured $(K_{\frac{1}{2}})$ when both the measured K_H (Fig. 6) and the measured r_{Cl} were inserted into eqns. (7) and (8). Fig. 9 shows that when K_H was omitted from the equations, the qualitative and the quantitative agreement between the calculated and the measured $(K_{\frac{1}{2}})_m$ was satisfactory. This agreement led one to conclude: it was not necessary to assume that the chloride binding group on the carrier molecule was hydrogen ion titratable in the pH range between 6·2 and 9·2 (0° C). Thus, the pH function of $(K_{\frac{1}{2}})$ is quantitatively dominated by the asymmetrical distribution of unloaded carrier molecules between the two surfaces of the cell membrane in accordance with the electrical membrane potential difference. This is a consequence of the positive net charge of the unloaded carrier molecules (cf. app., eqn. (5)).

The chloride equilibrium flux as a function of the pH in the medium at different chloride concentrations can be calculated from eqn. (7) without taking K_H into account when the influence of pH on the r_{Cl} is known (Dalmark, 1975). Fig. 10 shows the influence of pH on the calculated chloride transport at four different KCl concentrations in the cells and in the medium. The Figure shows a pH-dependent transport with an acidic and an alkaline branch and a pH optimum. In accordance with the experimental data (Fig. 5*a* and *b*) the Figure shows (1) that the acidic branches converged towards pH 5·6, (2) that the position of the pH optimum moved towards higher values of pH with increasing KCl

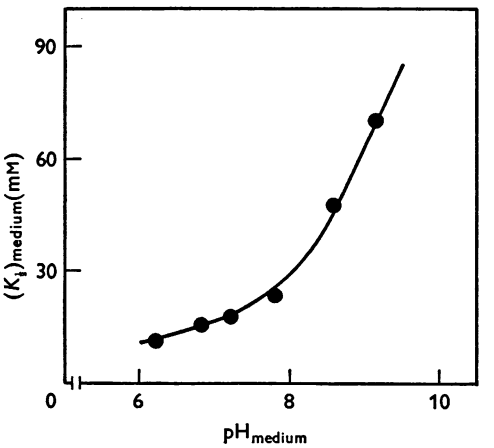

Fig. 9. Comparison between the calculated (−) and the measured (●) $(K_i)_m$ of the chloride equilibrium flux as functions of the pH in the medium (0° C). The half-saturation constant, $(K_i)_m$, was calculated from the equation:

$$K_{Cl}\left[\frac{\frac{1+\frac{1}{r_{Cl}}}{2}}{1+\left[\frac{(H^+)_m}{K_{HH}}\right]^2\left[\frac{1+\frac{1}{r_{Cl}^2}}{2}\right]}\right]$$

assuming that chloride is transported by a mobile, positively charged, chloride binding carrier with a chloride dissociation constant, K_{Cl}. The K_{Cl} was calculated from the observed $(K_i)_m$ and the chloride distribution ratio (r_{Cl}) at pH 7·2 (0° C) to a value of 23 mM. The r_{Cl} is the chloride distribution ratio between the chloride concentration in the cells and in the medium (Dalmark, 1975). The K_{HH} is the dissociation constant of the positively charged, protonized carrier-chloride complex. The magnitude of K_{HH} was determined from Fig. 7 to 10^{-6}. The $(H^+)_m$ was the hydrogen ion concentration in the medium.

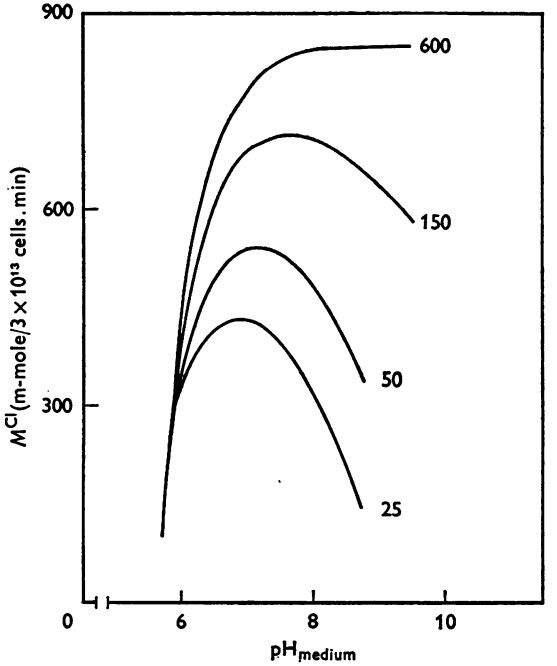

Fig. 10. The influence of the pH on the chloride equilibrium flux in cells with a mobile, positively charged, chloride binding carrier at various chloride concentrations in the cells and in the medium. The chloride concentration (25–600 mM) in the medium was constant at the different values of pH at each single chloride concentration. The chloride equilibrium flux was calculated from the equation:

$$M^{Cl} = \frac{P\left[\frac{T}{2}\right]}{1+\left[\frac{(H^+)_m}{K_{HH}}\right]^2\left[\frac{1+\frac{1}{r_{Cl}^2}}{2}\right]} \cdot \frac{1}{1+\frac{K_{Cl}}{(Cl^-)_m}\left[\frac{\frac{1+\frac{1}{r_{Cl}}}{2}}{1+\left[\frac{(H^+)_m}{K_{HH}}\right]^2\left[\frac{1+\frac{1}{r_{Cl}^2}}{2}\right]}\right]}$$

The $P[T/2]$ and the K_{Cl} were determined from the kinetic data of the chloride equilibrium flux in human red cells at pH 7·2 (0° C) (cf. Table 1). The $P[T/2]$ was 900 mM Cl/3 x 10^{13} cells.min and the K_{Cl} 33 mM. The K_{HH} was 10^{-6} (cf. Fig. 7). The chloride distribution ratio (r_{Cl}) was measured in red cells at a chloride concentration in the medium of $(Cl^-)_m$ and a hydrogen ion concentration in the medium of $(H^+)_m$ (Dalmark, 1975).

concentrations, and (3) that the alkaline branches at high KCl concentrations are independent of the pH.

The bell-shaped pH dependence of the chloride transport at low chloride concentrations is the result of two different mechanisms: the

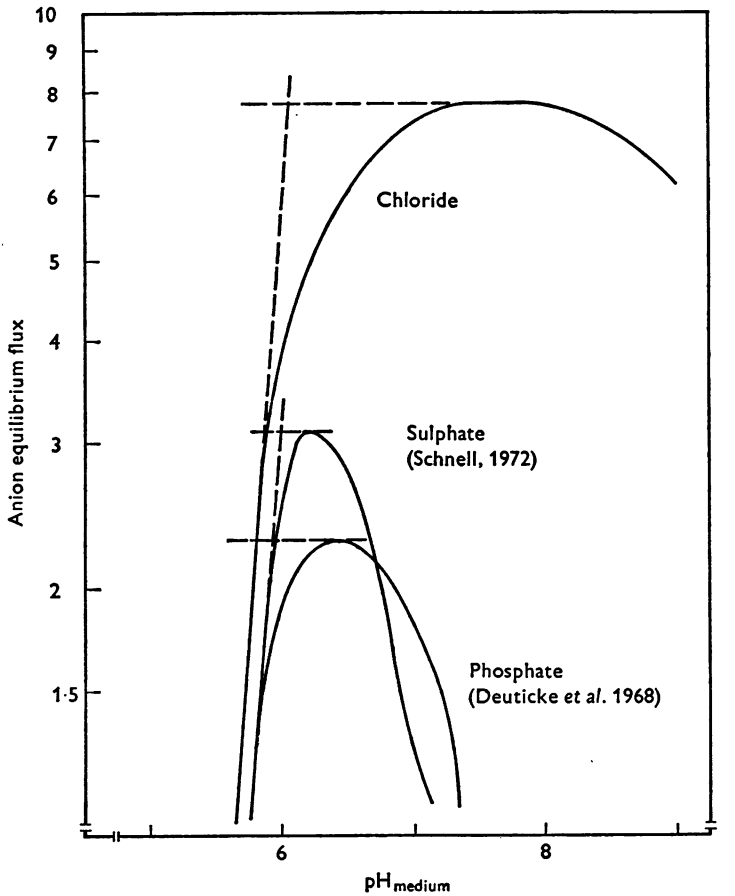

Fig. 11. Comparison between the effect of acidity on the chloride equilibrium flux and the effect of acidity on the phosphate equilibrium flux (Deuticke *et al.* 1968) and the sulphate equilibrium flux (Schnell, 1972). The flux units (logarithmic scale) and the anion concentrations of the experiments: 10^{2} mM $Cl/3 \times 10^{13}$ cells.min at 150 mM chloride, 10^{-8} mM phosphate/kg cells.min at 1 mM phosphate, and 10^{-7} mM sulphate/kg cells.min at 5 mM sulphate. The 3×10^{13} cells are equivalent to 1 kg cell solids (Funder & Wieth, 1966*a*) and to approximately 3 kg cells at a cellular water content of 66% (wt./wt.). The slopes of the dashed lines through the transport values at low pH were approximately 2/pH. The pH of the intersection points between the horizontal dashed lines and the vertical lines was approximately 6.

acidic branch of the pH graphs is caused by a decrease in the number of rapidly transported carrier-chloride complexes, and the alkaline branch of the pH graphs is caused by the asymmetrical distribution of the unloaded carrier molecules. The latter means, e.g. that at a constant chloride concentration in the medium the number of carrier molecules on the outer surface of the membrane decreases with increasing pH, and the chloride transport decreases since the transport is proportional to the number of carrier molecules at the surface of the membrane.

The position of the pH optimum of the chloride equilibrium flux at 38° C in cells suspended in 150 mM-KCl can be predicted from eqn. (7) when the influence of temperature on the chloride distribution ratio is known. It is assumed that the pK of the non-competitively inhibiting groups is constant between 18 and 38° C (Fig. 8*b*). The position of the calculated pH optimum is between pH 7 and 8.

The carrier-chloride complexes are transported across the cell membrane by independent migration of the complexes. The transport of the charged forms of the carrier molecules is much slower through the hydrophobic core of the membrane than the transport of the electroneutral chloride loaded carrier molecules. This agrees with the experimental observation that the red cell membrane has a low electrical conductance (Lassen, 1972).

The titration of red cells from one pH value to another may in the present description take place as either a chloride/hydroxyl ion exchange or a chloride/bicarbonate exchange. The latter possibility must be considered since the cell suspensions were in equilibrium with the carbon dioxide in the atmospheric air during the experiments.

Transport of inorganic divalent anions in red cells

There exist several similarities between the transport of monovalent and divalent anions in red cells (reviewed e.g. by Dalmark & Wieth, 1972 and Wood & Passow, 1974).

One important difference between the transport of mono- and divalent anions is the effect of pH on the equilibrium flux. In distinction to the pH optimum of the chloride equilibrium flux (Fig. 5*a*) the pH optimum of the phosphate and sulphate flux is around pH 6·4 (Deuticke, Dierkesmann & Bach, 1968; Schnell, 1972; Wood & Passow, 1974).

The description of the pH optimum of the chloride equilibrium flux by the present model is based on the idea that the acidic branch of the pH graph was caused by an inhibitory effect of hydrogen ions, but the alkaline branch was caused by the asymmetrical distribution of unloaded, positively charged carriers between the two surfaces of the cell membrane in accordance with the membrane potential difference.

In Fig. 11 the effect of hydrogen ions on the transport of divalent anions at low pH (Schnell, 1972; Deuticke *et al.* 1968) is compared with the effect of hydrogen ions on the chloride transport. One observes three similarities between the graphs of the divalent and the monovalent anions at low pH: (1) the graphs converged towards pH 5·6, (2) the graphs decreased with decreasing pH below pH 6 with slopes of approximately 2/pH, and (3) the pK of the inhibiting groups was approximately 6. Thus, it appears that the transport of monovalent and divalent anions was affected by hydrogen ions in the same manner at low pH.

The principles of the suggested model of the chloride equilibrium flux can be used to calculate the pH dependence of the transport of divalent anions in media containing chloride. The calculated pH optimum of the transport of divalent anions is between pH 6·0 and 6·5 under the assumptions, (1) that two positively charged carriers are necessary to make an electroneutral complex with a divalent anion, and (2) that chloride is a competitive inhibitor of the transport. The position of the calculated pH optimum was only slightly affected by the temperature as is the case with the calculated position of the pH optimum of the chloride transport. A small effect of temperature on the position of the pH optimum of the sulphate flux has been demonstrated (Schnell, 1972; Schwoch, Rudloff, Gut & Passow, 1973). It appears that the alkaline branch of the pH graph of the transport of divalent anions is determined by the asymmetrical distribution of carriers and anions secondary to the ionization of the cellular haemoglobin. This means that monovalent and divalent anions are transported by the same carrier system.

I am glad to express my indebtedness to Mrs Birgitte Olsen for her valued technical assistance and to Dr J. O. Wieth for his comments and suggestions during preparation of the manuscript.

APPENDIX

An anion transport model. The description is based on the assumption that the human red cell membrane contains mobile, anion binding components (carriers) which are able to translocate anions from the cell water to the medium, and vice versa. The different forms of the carrier molecules are restricted in their movements through the membrane by the electrical charge they may carry. They are rapidly transported through the membrane only when they are electroneutral.

The carrier molecules reversibly bind hydrogen ions and anions in the medium and in the cell water at the two surfaces of the cell membrane. At the two surfaces the following mass equations apply for monovalent anions, e.g. chloride

$$A^0 + H^+ \underset{K_H}{\rightleftharpoons} AH^+, \qquad (1)$$

$$AH^+ + Cl^- \underset{K_{Cl}}{\rightleftharpoons} AHCl^0, \tag{2}$$

$$AHCl^0 + H^+ \underset{K_{HH}}{\rightleftharpoons} HAHCl^+, \tag{3}$$

$$HAHCl^+ + H^+ \underset{K_{HH}}{\rightleftharpoons} HHAHCl^{2+},$$

where A^0 is the electroneutral carrier molecule, AH^+ is the protonized form of the carrier molecule which is able to bind chloride, $AHCl^0$ is the rapidly transported electroneutral carrier-chloride complex, and $HHAHCl^{2+}$ is the carrier-chloride complex when other groups than the chloride binding group are titrated with hydrogen ions. The K_H is the hydrogen ion dissociation constant of the chloride binding group on the carrier molecule, K_{Cl} is the chloride dissociation constant of the carrier-chloride complex, and K_{HH} is the hydrogen ion dissociation constant of proton binding groups on the carrier molecules. The latter groups are unable to bind chloride.

The chloride net flux through the cell membrane is zero when chloride equilibrium exists between the medium and the cell water. The efflux and influx of chloride are proportional to the number of carrier-chloride complexes at the two surfaces of the membrane

$$M^{Cl} = P(AHCl^0)_c = P(AHCl^0)_m, \tag{4}$$

where M^{Cl} is the chloride equilibrium flux as determined with radioactive chloride (cf. Methods section), $P(\text{time}^{-1})$ is the transmission rate of the electroneutral carrier-chloride complexes, and $(AHCl^0)_c$ and $(AHCl^0)_m$ are the number of carrier-chloride complexes at the inner and outer surface of the cell membrane.

The eqn. (4) states that the number of the electroneutral carrier-chloride complexes are equal at the two surfaces. The distribution of the electrically charged forms of the carrier molecules between the two surfaces follows from eqns. (1), (2) and (3)

$$r_{Cl} = \frac{(Cl^-)_c}{(Cl^-)_m} = \frac{(H^+)_m}{(H^+)_c} = \frac{(AH^+)_m}{(AH^+)_c} = \sqrt{\frac{(HHAHCl^{2+})_m}{(HHAHCl^{2+})_c}}, \tag{5}$$

where r_{Cl} is the chloride distribution ratio between the chloride concentration in the cell water $(Cl^-)_c$ and in the medium $(Cl^-)_m$, $(H^+)_m/(H^+)_c$ is the ratio between the hydrogen ion concentration in the medium and in the cell water, and $(AH^+)_m/(AH^+)_c$ and $(HHAHCl^{2+})_m/(HHAHCl^{2+})_c$ are the ratios between the different charged forms of the carrier molecules at the outer and the inner surface of the cell membrane.

Assuming a finite number of carrier molecules (T) in the membrane it follows that

$$T = (A^0)_c + (AH^+)_c + (AHCl^0)_c + (HHAHCl^{2+})_c + (A^0)_m + (AH^+)_m + (AHCl^0)_m + (HHAHCl^{2+})_m. \tag{6}$$

The chloride equilibrium flux is calculated from eqn. (4) after eqn. (6) has been solved for the number of carrier-chloride complexes at the two surfaces by application of eqns. (1), (2), (3) and (5). The chloride equilibrium flux (M^{Cl}) can be expressed as a function of the chloride concentration in the medium or as a function of the chloride concentration in the cell water

$$M^{Cl} = \frac{P\left[\frac{T}{2}\right]}{1+\left[\frac{(H^+)_m}{K_{HH}}\right]^2\left[\frac{1+\frac{1}{r_{Cl}^2}}{2}\right]} \Bigg/ 1+\frac{K_{Cl}}{(Cl^-)_m}\left[\frac{\frac{1+\frac{1}{r_{Cl}}}{2}+\frac{K_H}{(H^+)_m}}{1+\left[\frac{(H^+)_m}{K_{HH}}\right]^2\left[\frac{1+\frac{1}{r_{Cl}^2}}{2}\right]}\right] = \frac{M\,(\max)_{pH}}{1+\frac{(K_{\frac{1}{2}})_m}{(Cl^-)_m}}, \quad (7)$$

$$M^{Cl} = \frac{P\left[\frac{T}{2}\right]}{1+\left[\frac{(H^+)_c}{K_{HH}}\right]^2\left[\frac{1+r_{Cl}^2}{2}\right]} \Bigg/ 1+\frac{K_{Cl}}{(Cl^-)_c}\left[\frac{\frac{1+r_{Cl}}{2}+\frac{K_H}{(H^+)_c}}{1+\left[\frac{(H^+)_c}{K_{HH}}\right]^2\left[\frac{1+r_{Cl}^2}{2}\right]}\right] = \frac{M\,(\max)_{pH}}{1+\frac{(K_{\frac{1}{2}})_c}{(Cl^-)_c}}, \quad (8)$$

where $P(\frac{1}{2}T)$ is the pH-independent transport capacity, and $M\,(\max)_{pH}$ is the pH-dependent expression of the observed maximum transport. The observed half-saturation constants are $(K_{\frac{1}{2}})_m$ and $(K_{\frac{1}{2}})_c$ when the chloride transport is perceived as a function of either the chloride concentration in the medium $(Cl^-)_m$ or in the cell water $(Cl^-)_c$ at a given hydrogen ion concentration in the medium $(H^+)_m$ or in the cell water $(H^+)_c$. It is demonstrated (cf. Discussion section) that it was unnecessary to assume that the chloride binding group on the carrier molecule was hydrogen ion titratable (cf. eqn. (1)) in the pH range between pH 6·2 and 9·2 (0° C).

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