Chloride and bicarbonate transport in chick embryonic red blood cells

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1. Unidirectional efflux of $^{36}Cl^-$ and $H^{14}CO_3^-$ from erythrocytes of 4- to 16-day-old chick embryos was measured under steady-state conditions at 37 °C and pH 7·7. The efflux rates were high, $> 3\ s^{-1}$, and were, therefore, measured by means of the continuous flow tube method.

2. At day 4 of development the range of permeability coefficients for bicarbonate and chloride (P_{HCO_3} and P_{Cl} was $1–30 \times 10^{-4}$ cm s^{-1}, with average values of respectively 10×10^{-4} and 8×10^{-4} cm s^{-1}. However, the results can be divided into two groups, one with P_{HCO_3} and P_{Cl} above 12×10^{-4} cm s^{-1}, and one with values below 5×10^{-4} cm s^{-1}. The same range of values was also obtained for day 6 erythrocytes, but the overlap is more conspicuous. At day 16, P_{HCO_3} and P_{Cl} were respectively 9×10^{-4} and 6×10^{-4} cm s^{-1} (37 °C, pH 7·7). In adult chicken red blood cells P_{HCO_3} and P_{Cl} were respectively 7×10^{-4} and 4×10^{-4} cm s^{-1}, and in human red blood cells the respective values were $5·6 \times 10^{-4}$ and 4×10^{-4} cm s^{-1}.

3. Chloride self-exchange, measured at 0 °C, was almost completely inhibited by addition of 1 mM 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) at both days 6 and 16 of embryonic development, supporting the finding that the embryonic chick erythrocytes also have a transmembrane anion exchanger similar to that of other red cells.

4. The intracellular pH (pH_i) was measured at constant extracellular pH (pH_o) using the pH-dependent fluorescent dye SNARF AM-1. The difference between pH_o and pH_i decreased from 0·600 units at day 4 to 0·245 units at day 16 of development.

5. The results suggest that a functionally active band 3 protein, AE1, is present in the membrane at very early stages of embryonic development and is capable of transporting monovalent anions. However, at the early stages the anion transporter appears not to be able to regulate intracellular pH as efficiently as in mature cells. During embryonic development the ability of AE1 to regulate pH_i improves.

Just when the different functions of the mature cell are built in and to what extent the functions may change during maturation are open questions. One approach to the study of maturation of cellular processes is to compare different cell lines, and from studies of HL-60 cell lines it appears that cation transport decreases as the cells mature (S. Dissing, personal communication). A previous study characterizing chloride and bicarbonate transport in fetal erythrocytes from humans (Brahm & Wimberley, 1989) led to the conclusion that erythrocytes at the stage of gestation have the same qualitative and almost the same quantitative anion transport properties as those of adults.

In the present study we have determined chloride and bicarbonate transport in chick embryonic erythrocytes with the purpose of describing the anion transport kinetics in the red cells at a very early stage of life. While the presence of band 3 protein in the membrane of primitive embryonic red cells has repeatedly been demonstrated (cf. Chan, 1976, 1985), there have been no direct measurements of band 3-dependent anion fluxes in these cells.

The study was prompted by the previous observation that the proton and chloride distributions of primitive red cells are not in equilibrium (Engelke, Zingel & Baumann, 1988). This result contrasts with the generally accepted model for proton distribution across the membrane of adult mammalian or avian red cells. There protons are in equilibrium across the membrane due to the fact that the membrane acts selectively for anions (the membrane potential being close to the equilibrium potentials for chloride and bicarbonate), and, because of the hydration/

dehydration of bicarbonate, the protons distribute across the membrane in an inverse ratio. However, this model does not apply to immature embryonic chick red blood cells, as the internal pH is too low and the membrane potential too negative (Engelke *et al.* 1988). The membrane potential of these cells could not therefore be caused by the chloride distribution only. In fact the experimental data show that the membrane potential of embryonic red cells from early chick embryos is dominated by a proton conductance (Engelke *et al.* 1988).

Furthermore, when cell pH measurements of early embryonic red cells (day 4) were performed in the absence and presence of CO_2, the disequilibrium between the Cl^- and H^+ distributions persisted (Sieger, Reinhardt & Baumann, 1993), despite the fact that in these experiments band 3 protein was not blocked. The present study was therefore carried out to determine whether band 3 of early embryonic red cells has different anion transport characteristics from those of the adult.

METHODS

Electrolyte media and chemicals

The following media were used (mM). (1) Washing buffer (medium A): 140 NaCl, 5 KCl, 1·5 D-glucose, 50 Tris (pH 7·4, 0 °C). (2) Media for the measurement of radioactive tracer flux: medium B, 100 NaCl, 25 $NaHCO_3$, 25 sodium citrate, 1 acetazolamide, pH 7·7 at 37 °C; and medium C, 100 NaCl, 50 sodium citrate, pH 7·5 at 0 °C. (3) Medium for the determination of intracellular pH (medium D): 140 NaCl, 5 KCl, 1·5 $CaCl_2$, 5 D-glucose, 20 Hepes, pH 7·5 at 0 °C.

The chemicals used besides the above-mentioned components were 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) (a gift of Professor Klaus Schnell, University of Regensburg, FRG); and SNARF AM-1 (Molecular Probes, Inc., Eugene, OR, USA).

Radioactive isotopes and determination of radioactivity

The radioactivity of $^{36}Cl^-$ and $H^{14}CO_3^-$ was measured by liquid β-scintillation using a Packard 1500 Tricarb liquid scintillation analyser in Rotiszint 22 scintillation cocktail (Karl Roth KG, Karlsruhe, FRG).

For determination of the Cl^- self-exchange, 0·5 ml of the filtrate was added to 10 ml of the scintillation cocktail, followed by 0·5 ml 10 % (v/v) trichloroacetic acid (TCA). The volume used for counting in determining the $^{36}Cl^-$ and $H^{14}CO_3^-$ exchange was usually 200 μl.

The cells were loaded with $^{36}Cl^-$ and $H^{14}CO_3^-$ (both obtained as the sodium salt, Amersham Buchler GmbH, Braunschweig, FRG) by incubation at a haematocrit of 1 % in medium A containing 18–37 kBq ml^{-1} of the respective radioactive isotopes. The radioactivity in the cells, the supernatant after precipitation with TCA and the cell-free filtrates of the efflux experiments was determined by liquid scintillation spectrometry (Tricarb 1500). The channel settings (A, 156–710; B, 0–156) made ^{14}C-radioactivity in channel A insignificant, and the contribution from $^{36}Cl^-$ in channel B was corrected for after counting $^{36}Cl^-$ standard samples. The distribution of tracer chloride across the membrane was assumed to equal the ratio of chloride concentration in the cell water phase ($[Cl^-]_i$) to that in the external solution ($[Cl^-]_o$), r_{Cl}, and was used together with the distribution of hydrogen ions, ($r_H = [H^+]_i/[H^+]_o$, see below) to estimate the disequilibrium across the membrane.

Mean cell volume, cell water content and membrane area

The mean cell volume (MCV, fl), the membrane area (A, cm^2 cell^{-1}) and the cell water fraction (F_w) of chick embryonic red blood cells change during maturation and were determined at each stage using standard haematological and microscopic techniques. The calculations of the apparent permeability coefficient and flux depend on A and the cell water volume (V_o) ($V_w = F_w$ x MCV, corrected for trapped volume between the cells (Brahm & Wieth, 1977)) and proper values were used accordingly. The values are summarized in Table 1.

Preparation of cells

Fertilized eggs from White Leghorn chickens were incubated for the desired period, i.e. 4–16 days, at 37 °C and 60 % relative humidity in air. Blood was aspirated after dissection of the embryo and resuspended in ice-cold buffer (medium A). The cells were pelleted by centrifugation and washed 3 times in medium A.

Chicken and human red cells were collected by venipuncture in heparin (chickens were restrained during this procedure). After centrifugation the supernatant and upper layers of cells were sucked off, the pelleted cells were resuspended in medium B and titrated to pH 7·7 and then washed 3 times in titrated medium B. After the last wash the cells were incubated with isotopes and isolated by centrifugation at 48000 g (Sorvall RC-5B) in nylon tubes (Funder & Wieth, 1967) for determinations of cell water content and tracer distribution and in 8 ml tubes for efflux measurements in the continuous flow tube apparatus (cf. below).

Chloride–bicarbonate efflux at 37 °C

The washed and packed erythrocytes were resuspended in medium B to a haematocrit of 10 %. The medium contained 1 mM acetazolamide, which inhibits carbonic anhydrase activity and hence minimizes the loss of radioactivity owing to the formation of $^{14}CO_2$. Because the carbonic anhydrase activity is mainly localized intracellularly, the cells were incubated for 1 h at 37 °C to ensure equilibration of the enzyme inhibitor. Subsequently $^{36}Cl^-$ and $H^{14}CO_3^-$ were added to the suspension that was incubated for another 30 min. The cells were next centrifuged, and the isolated and packed erythrocytes were used for the measurement of $^{36}Cl^-$ and $H^{14}CO_3^-$ efflux rates at 37 °C with the rapid flow tube technique, as described elsewhere (Brahm, 1977, 1989). In short, the radioactively labelled cells were continuously injected into a mixing chamber and mixed with a continuously injected non-radioactive medium (350 ml).

The suspension flowed with a constant velocity along a pipe with inserted filtration ports at predetermined distances, which made it possible to obtain cell-free filtrates at predetermined times after mixing.

Because of the limited amount of cells of younger embryos (younger than day 12), the experiments were scaled so that as little as 70–100 μl of cells was used per run. Furthermore, a regaining procedure was used, so that cells were used once or twice again for efflux experiments (cf. Table 2)

Chloride self-exchange at 0 °C

The washed and pelleted cells were weighed and incubated for 30 min at room temperature in medium C, to which $^{36}Cl^-$ was

added. After the incubation period aliquots were taken for the determination of the specific activity and the intracellular activity and for the measurements of chloride self-exchange with the Millipore–Swinnex filtering technique accordingly to Dalmark & Wieth (1972). Due to the limited number of cells we used 'Schwarzrand–Filterhalter' (Schleicher & Schüll, Dassel, FRG) with a small dead space to reduce the loss of cells. The time point of sampling was recorded with a tape and a stopclock. In another series of experiments the cells were incubated as described above, and subsequently washed in medium C containing 1 mM DIDS. The efflux experiments were carried out in medium C that also contained 1 mM DIDS.

Determination of pH_i

Measurements of pH_i were done according to Eidelman & Cabantchik (1989) and Sieger *et al.* (1993). Erythrocytes were resuspended in medium D with a haematocrit of 0·25 %. The fluorescent pH indicator SNARF AM-1 was added to the solution to give a final concentration of $1{\cdot}5 \times 10^{-7}$ M. The cells were incubated for 45 min at 37 °C and pH 7·5, centrifuged and washed twice in medium D (without SNARF) which contained 5 % (w/w) Ficoll to remove the extracellularly trapped dye. The packed erythrocytes were resuspended in medium D (without Ficoll) and the emission ratio was measured with a Shimadzu spectrofluorophotometer RF-5000.

The excitation wavelength was 540 nm; the emission wavelengths were 640 and 603 nm. Calibration of the signal was performed by lysis of the cells with 20 μl of 20 mM digitonin solution (in dimethyl sulphoxide, DMSO) and titration with 0·1 N HCl or NaOH, respectively. In a preceding paper (Sieger *et al.* 1993) we compared the pH measurements carried out with SNARF with two different methods (freeze–thaw and digitonin null point). The correspondence between the various sets of data was excellent. SNARF accumulates in the cytoplasm, since SNARF accumulation in organelles is checked with Probenecid. In addition, light scattering does not affect the measurement (Sieger *et al.* 1993).

Determination of the rate of anion efflux

The experiments showed that the increase of radioactivity in the filtrates, conveniently transformed into the intracellular decrease of radioactivity in accordance with a two-compartment model with constant volumes, followed first-order kinetics:

$$\ln[(a_t - a_\infty)/(a_o - a_\infty)] = -kt, \quad (1)$$

where a_t, a_o and a_∞ are the extracellular radioactivities (c.p.m. ml^{-1}) at time t, time zero and at equilibrium, respectively. Hence, $(a_t - a_\infty)/(a_o - a_\infty)$ is the tracer that remains in the cells at time t. The slope of the semilogarithmic plot, k (s^{-1}), of the left-hand expression of eqn (1) *vs.* t, represents the numerical value of the rate coefficient of the efflux process. Since the experiments were carried out at a very low haematocrit, 0·03–0·4 %, $-k$ is equal to the rate coefficient for the unidirectional tracer efflux.

Permeability

The permeability coefficient, P ($cm\ s^{-1}$), of the red cell membrane was calculated by:

$$P = kV_w/A, \quad (2)$$

where V_w/A is the ratio (in cm) of the cell water volume to the cell membrane area that changes during maturation (cf. above and Table 1).

RESULTS

Area and volume of chick embryonic erythrocytes

The apparent permeability of the chick embryonic red cell membrane cannot be determined directly, but is calculated from determinations of the efflux rates of the radioactive labelled ions and the ratio of the cell water volume to the membrane area (cf. the Methods section). Hence, because the cell volume and area changes during the maturation it is of critical importance to determine cell water volume and membrane area at each day of development. Table 1 shows the results of a series of determinations in chick embryonic erythrocytes. The table also includes numbers for chicken red cells as well as adult and fetal human red blood cells.

Table 1. Areas and volumes of erythrocytes from embryonic chick, chicken and human fetus and adult

	A (10^{-8} cm^2)	MCV (fl)	F_w (n)	V_w (fl)	V_w/A (10^{-5} cm)
Embryonic chick (days)					
4	363	650	0·78 ± 0·021 (3)	494	13·9
6	249	370	0·72 ± 0·018 (4)	256	10·6
8	163	196	0·66 ± 0·039 (4)	125	7·8
10	170	208	0·65 ± 0·022 (4)	131	7·9
12	155	182	0·65 ± 0·069 (3)	115	7·5
14	138	153	0·69 ± 0·019 (3)	103	7·6
16	140	155	0·69 ± 0·028 (2)	104	7·6
Chicken	175	134	0·62	82	4·7
Human					
Fetal	153	—	—	—	4·7
Adult	142	79	0·61	48	3·4

F_w is the cell water fraction determined by drying a cell sample to constant weight. The values of A and MCV for chicken red cells are from Brahm & Wieth (1977), and for fetal cells from Brahm & Wimberley (1989). The experiments were carried out at pH 7·7.

Chloride and bicarbonate self-exchange at 37 °C

Chick embryonic erythrocytes

Chloride and bicarbonate self-exchange was determined in erythrocytes from 4- to 16-day-old chick embryos. Figures 1 and 2 show the efflux of radioactive isotopes from preloaded cells into an initially isotope-free solution. The experimental conditions imply that self-exchange was studied by measuring the unidirectional efflux of tracers.

Figure 1 shows that in red cells from days 4 and 6 both fast efflux rates (*A* and *C*), similar to those of older cells, and slow efflux rates (*B* and *D*) were determined. Figure 2

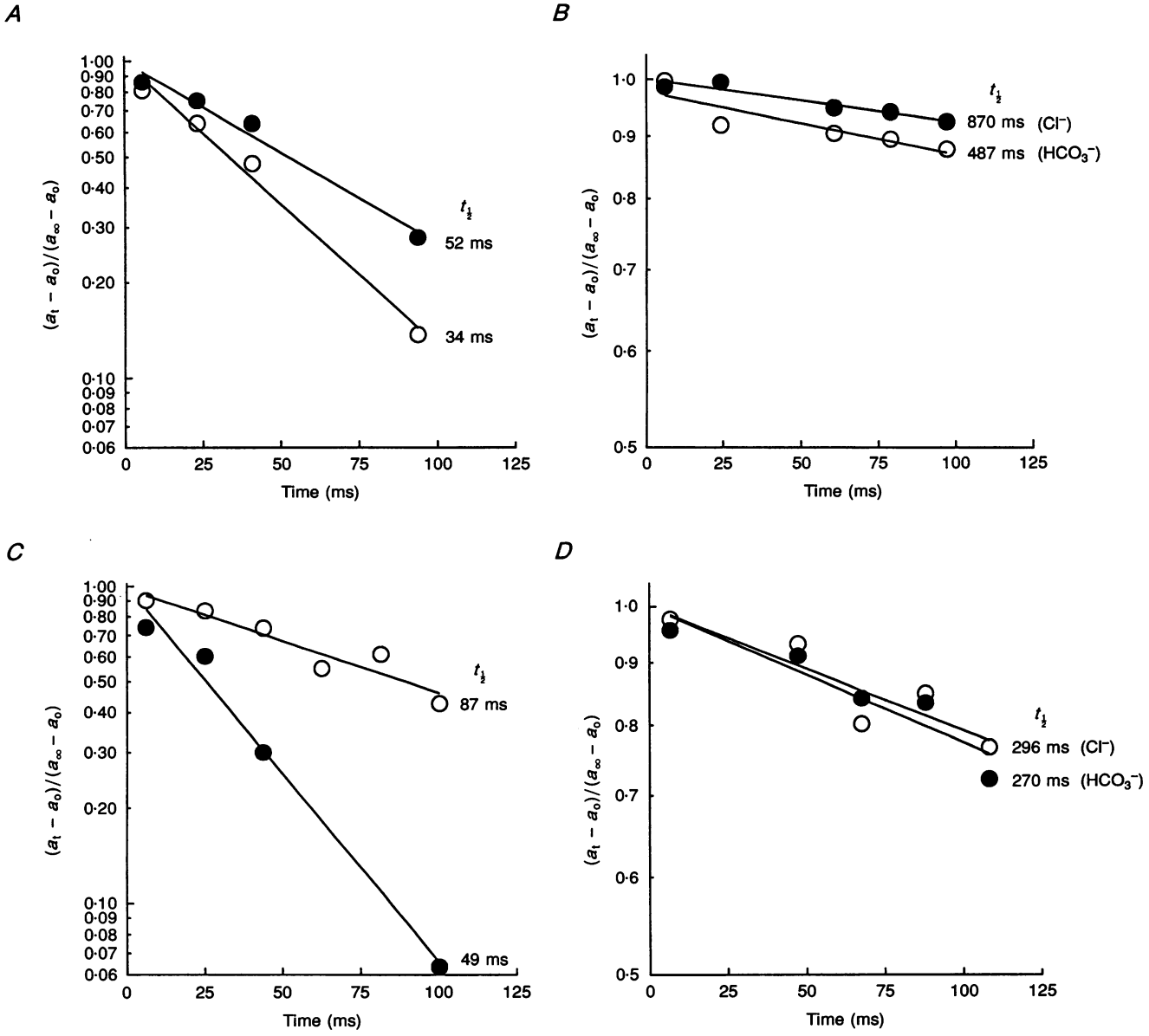

Figure 1. Chloride and bicarbonate self-exchange in chick embryonic red blood cells
Semilogarithmic plots of chloride and bicarbonate efflux as a function of time from erythrocytes of 4- (*A* and *B*) and 6-day-old chick embryos (*C* and *D*). ○, Cl^- efflux; ●, HCO_3^- efflux. The ordinate expresses the fraction of radioactivity that remains in the cells at time *t*, where a_t, a_0 and a_∞ are the extracellular radioactivities (c.p.m. ml^{-1}) at time *t*, time zero and at equilibrium (for a detailed discussion, see Wieth & Brahm (1980)). The negative value of the slopes of the plot equals the rate coefficient (*k*, s^{-1}) of the anion self-exchange transport. The half-time of the exchange, $t_{\frac{1}{2}}$ (s), is related to *k* by $t_{\frac{1}{2}} = (\ln 2)/k$. Different experiments carried out at days 4 and 6, of which represantative data are presented in *A–D*, show that the results could be classified into two groups: (a) fast-transporting red cells at days 4 and 6 (*A* and *C*) with half-times for Cl^- and HCO_3^- self-exchange similar to those of adult chicken and human red blood cells, and (b) slow-transporting red cells (*B* and *D*) with half-times of 0·5 s (at day 4) and 0·25 s (at day 6). Note also that at day 4, $t_{\frac{1}{2}}$ for Cl^- is lower than for HCO_3^-, both in fast- and slow-transporting cells. The experiments were performed at pH 7·7 and 37 °C.

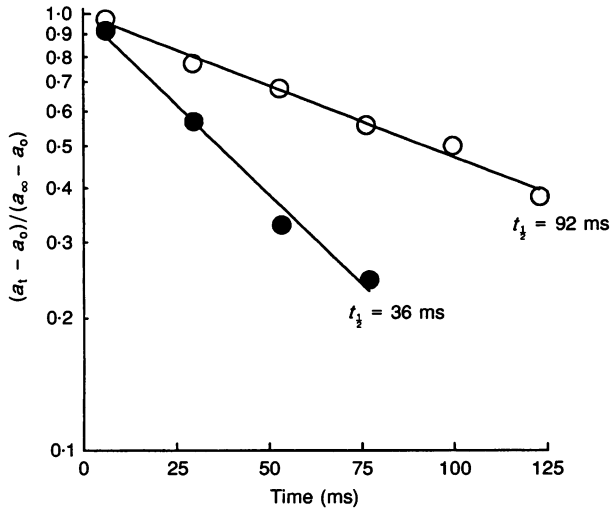

Figure 2.
Semilogarithmic plots of chloride and bicarbonate self-exchange at 37 °C and pH 7·7 in red blood cells collected from a 16-day-old chick embryo. ○, Cl^- efflux; ●, HCO_3^- efflux.

depicts efflux curves from 16-day-old embryonic red blood cells. The figure is representative for the efflux experiments with cells from day 8 and older embryos.

Figure 3 depicts the apparent bicarbonate and chloride permeabilities as a function of embryonic age. The scatter in the data is conspicuous, in particular at days 4 and 6, and suggests marked functional heterogeneities of band 3 in early development.

Re-use of cells for efflux experiments.

After some of the efflux experiments the cells were recovered and used for more efflux experiments. Table 2 shows the reproducibility in three experiments with erythrocytes from day 4, day 6 and day 12 embryos.

Chicken and human red blood cells.

Figure 4 illustrates that the efflux rates in chicken red blood cells changed very little when pH was changed from a physiological value of 7·2 (*A*) to 7·7 (*B*). Because the cell water content is not much altered by a change of 0·5 pH units (5·6 %), the efflux rate coefficients can be regarded as proportional to the relative apparent permeabilities. Figure 5 depicts the efflux curves in the absence of the carbonic anhydrase inhibitor acetazolamide (*A*), and after an incubation procedure (1 mM for 45 min) that results in complete inhibition of the intracellular catalysed hydration/dehydration of CO_2 (*B*). The data in Fig. 5 are not adjusted for the extracellular trapping of radioactivity, which – by extrapolation of the chloride efflux curves – appears to be about 20 % (the trapped extracellular radioactivity at time $t = 0$ does not participate in the efflux process, and does not contribute to the slope of the curve; for more details see Brahm, 1989). If the cells were not treated with acetazolamide, a certain fraction of the intracellular ^{14}C-radioactivity, *ca* 25 % (the difference between the intracellular chloride and bicarbonate radioactivity at the first sampling time, was lost 'instantaneously' and probably as $^{14}CO_2$.

The non-linear pattern of the depiction in the semilogarithmic plot is similar to that described and explained recently by Gasbjerg & Brahm (1991). Here the experiments were carried out to illustrate the importance of inhibiting the enzyme in order to get linear efflux curves representing $H^{14}CO_3^-$ efflux, and to show that inhibition of the hydration/dehydration of CO_2 does not affect the bicarbonate or chloride flux. The differences in half-times of 55 and 45 ms (Fig. 5) in the presence of acetazolamide are due to normal experimental variation of results and do not indicate a specific effect of acetazolamide on Cl^- efflux (Gasbjerg & Brahm, 1991).

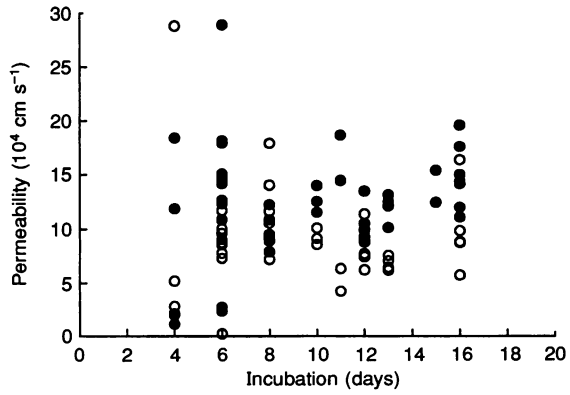

Figure 3.
The apparent chloride and bicarbonate permeability of red cells from chick embryos as a function of age at pH 7·7 and 37 °C. ○, Cl^- efflux; ●, HCO_3^- efflux.

Inhibition of chloride and bicarbonate self-exchange at 0 °C

We examined whether the high anion transport rates that were determined in this study can be inhibited by 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS), which is an efficient inhibitor of AE1-mediated transport in other red cells. The experiments were conveniently carried out at 0 °C, where fewer cells were required than at body temperature. Table 3 shows the inhibition of chloride transport by 1 mM DIDS in embryonic erythrocytes from days 6 and 16.

Determination of pH_i

Internal pH was determined by means of the fluorescent dye SNARF AM-1, and measured in 4- to 16-day-old red blood cells. Table 4 demonstrates the decrease of the difference between pH_o and pH_i as a function of number of days of incubation. It can be seen that ΔpH at $pH_o = 7{\cdot}5$ decreases from about 0·6 at day 4 and day 6 to about 0·25 at day 16.

Table 2. The apparent chloride and bicarbonate permeability in red blood cells from chick embryo at different days

	P_{Cl} (10^{-4} cm s^{-1})	P_{HCO_3} (10^{-4} cm s^{-1})
Day 6		
Control	10·02	12·66
Re-use 1	8·54	10·81
Re-use 2	—	10·87
Day 8		
Control	7·31	4·95
Re-use 1	6·64	6·83
Re-use 2	4·98	5·59
Day 12		
Control 1	7·41	7·85
Control 2	7·52	11·41
Re-use 1	6·53	8·89
Re-use 2	5·26	8·39
Re-use 3	6·25	7·81

On each day one batch of cells was used for one or two control experiments, as described in the Methods section. The cells were next recovered, reloaded with radioactive chloride and bicarbonate (cf. Methods section) and used for another efflux experiment. The re-use procedure and flux experiment were repeated two or three times.

DISCUSSION

Anion transport

It has previously been shown that the transport of chloride and bicarbonate, the physiologically most important anions, is rapid and similar in human red blood cells from adult and late fetuses (Brahm, 1977; Wieth & Brahm, 1982; Brahm & Wimberley, 1989). A similar high rate of anion transport has also been demonstrated in adult chicken red blood cells (Brahm & Wieth, 1977) as well as in red blood cells from all other warm-blooded animals so far studied. Apparently the rapid exchange of anions, which is a partial step in the CO_2 transport by the blood from tissues to the lungs, is essential for life. The extent to which the anion transport function of band 3 protein is a functional necessity in early development is an unsettled question. Interestingly the anion transport system is absent in red cells from lampreys (Ohnishi & Asai, 1985; Ellory, Wolowk & Young, 1987). The study by Brahm & Wimberley (1989) shows that human fetal red cells at the time of parturition have an anion transport system with kinetics very similar to those of adult red cells.

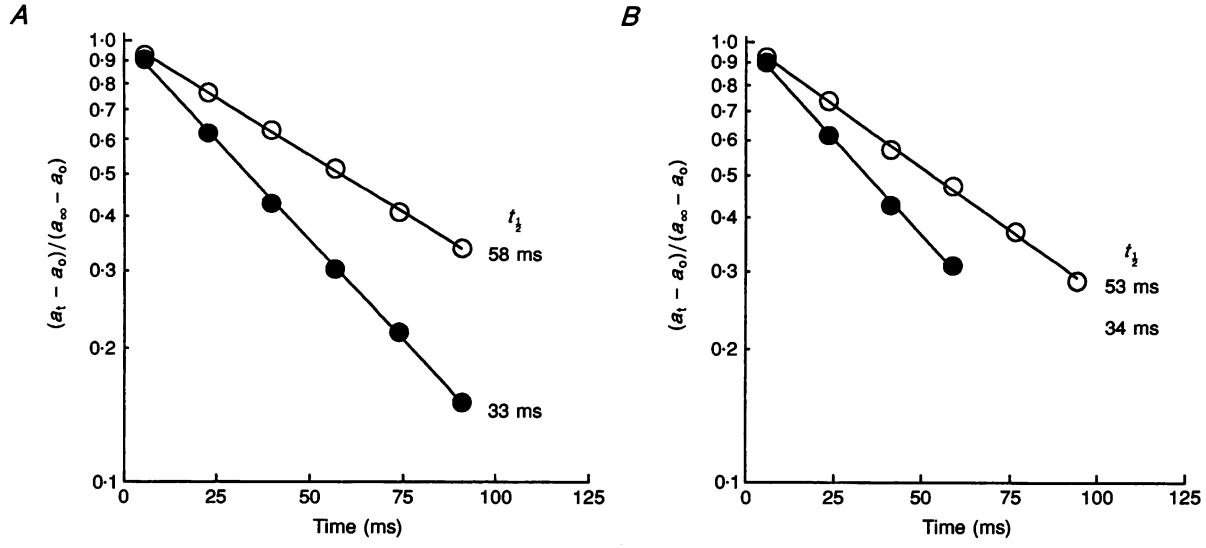

Figure 4.
Semilogarithmic plots of chloride and bicarbonate self-exchange at 37 °C and pH 7·2 (*A*) and pH 7·7 (*B*) in red blood cells collected from a chicken. ○, Cl^- efflux; ●, HCO_3^- efflux.

Table 3. Inhibition of chloride self-exchange in red blood cells collected from 6- and 16 day-old embryos

	Day 6	Day 16
^{36}Cl efflux (without DIDS)	$2{\cdot}902 \times 10^{-12}$	$9{\cdot}800 \times 10^{-12}$
n	7	4
S.D.	0·905	1·521
^{36}Cl efflux (with DIDS)	†	†

The cells were washed and the subsequent efflux experiments were performed in medium C with 1 mM DIDS at 0 °C and pH 7·5. The $^{36}Cl^-$ efflux is expressed as mol (g cells)$^{-1}$ min^{-1}. † The c.p.m. did not exceed the background signal.

Table 4. pH difference ($\Delta pH = pH_o - pH_i$) of chick embryonic erythrocytes as a function of stage of development (days of incubation)

Day	ΔpH	n
4	0·605 ± 0·043	4
6	0·590 ± 0·060	5
8	0·343 ± 0·062	4
10	0·366 ± 0·044	4
12	0·300 ± 0·056	4
14	0·315 ± 0·033	4
16	0·254 ± 0·035	4

Values are means ± S.D. The experiments were carried out at $pH_o = 7{\cdot}5$. Previous experiments (Sieger *et al.* 1993) have shown that ΔpH depends on pH_o according to the following equation: $pH_i = 0{\cdot}73085 + 0{\cdot}833pH_o$.

In the present study we were able to determine anion transport in red cells from chick embryos between 4 and 16 days of development. During this period the composition of the embryonic blood undergoes major changes with respect to red cell type and haemoglobin composition (Bruns & Ingram, 1973). In brief the first red cell population – primitive red cells – are released into the circulation as immature erythroid precursors and they complete their differentiation inside the circulation. Thus primitive red cells from day 4 are still in a proliferative cycle whereas primitive red cells from day 6 are terminally differentiated postmitotic cells. Beginning with day 6 a second population of red cells – the definitive red cells – enters circulation and rapidly replaces the primitive cell type, so that by day 16 the circulating blood contains only marginal numbers of primitive red cells (Bruns & Ingram, 1973). The results clearly show that even at the earliest stage (day 4) the cell membrane transports chloride and bicarbonate with rates that unquestionably demonstrate the presence of transport mechanism(s) in the membrane.

The apparent permeability coefficients are in the range of $1–30 \times 10^{-4}$ cm s^{-1} as compared to permeability coefficients of the order of $10^{-10}–10^{-12}$ cm s^{-1} determined for lipid bilayer membranes (Toyoshima & Thompson, 1975) and 4×10^{-4} cm s^{-1} in human red blood cells (Brahm, 1977). While very little variation is observed in mature adult red cells from humans and chicken, the data for chick embryo red cells show marked differences, in particular at days 4 and 6. The scatter of data (cf. Fig. 3) from days 4 and 6 may reflect primarily a different regulation of band 3-mediated anion transport during the proliferative stage of primitive erythrocytes. Compared to this the overall changes of

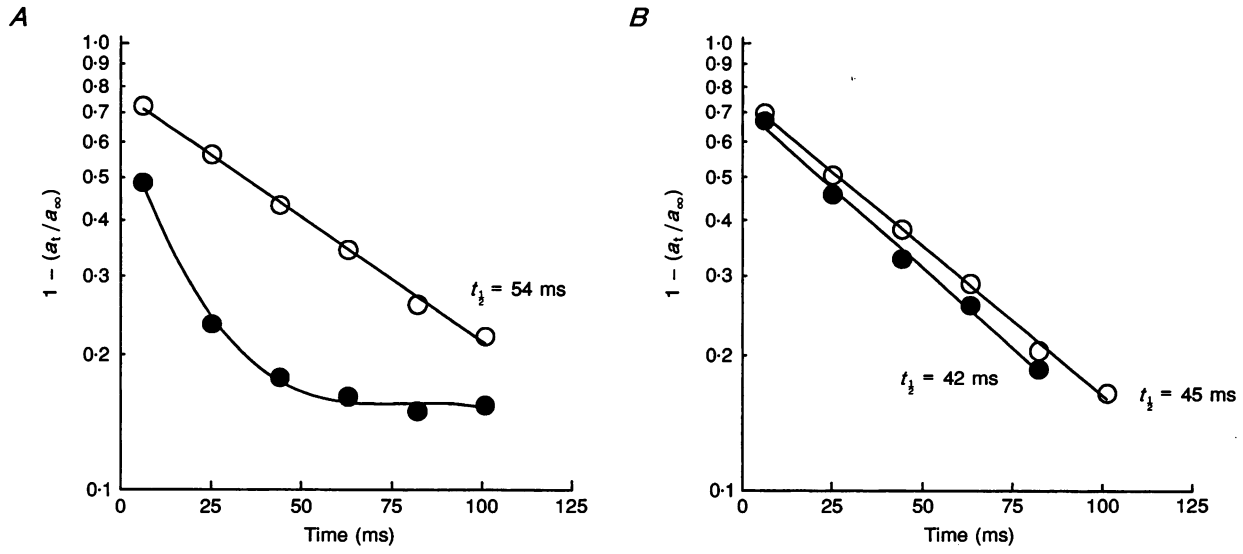

Figure 5. Semilogarithmic plots of chloride and bicarbonate self-exchange at 37 °C and pH 7·2 in human red blood cells in the absence (*A*) and presence (*B*) of the carbonic anhydrase inhibitor acetazolamide

In these plots the extracellular fraction of tracer trapped during the package of the cells has not been subtracted, and the intersection with the ordinate at $t = 0$ is, therefore, less than 1·0. ○, Cl^- efflux; ●, HCO_3^- efflux.

transport kinetics are small between days 6 and 16 when primitive red cells are replaced by definitive red cells.

Embryonic red cell pH

While the chloride distribution ratio between embryonic red cells and plasma is nearly constant during development ($r_{Cl} = 0{\cdot}6$ at pH_o 7·4; Baumann, Fischer & Engelke 1987; Engelke, 1988) there are marked changes of the proton distribution ratio. Previous measurements of red cell pH at days 4 and 6 gave values of ΔpH of 0·6 units (Engelke *et al.* 1988; Sieger *et al.* 1993) in good agreement with the measured membrane potential (Engelke *et al.* 1988). The data shown in Table 4 reveal that ΔpH decreased in a non-linear fashion from day 4 to day 16. At the early stages ΔpH decreased steeply from day 4 ($\Delta pH = 0{\cdot}60$) towards day 12. Subsequently the change become less pronounced, as pH_i apparently tends to stabilize at 0·25 pH units below pH_o. This pattern of pH change contrasts with the comparatively small changes observed for band 3 transport function after day 6. On the other hand the membrane potential of chick embryonic cells shows the same developmental change as ΔpH, decreasing continuously from −36 mV at day 6 to −15 mV at day 16 (Baumann *et al.* 1987; Engelke *et al.* 1988).

Based on the results of the present study one can exclude the possibility that the inability of band 3 to transport bicarbonate is the major cause for the H^+–Cl^- disequilibrium. However, it should be borne in mind that the adjustment of r_H to r_{Cl} under physiological conditions depends on a tightly coupled 1:1 Cl^-–HCO_3^- heteroexchange. The disequilibrium between Cl^- and protons reflects a different organization of the membrane of embryonic red cells from that of adult red cells.

An electrogenic proton conductance exist in parallel with band 3 protein (Engelke *et al.* 1988). The contribution of Cl^-, as well as K^+ and Na^+, to the membrane potential is negligible (Engelke *et al.* 1988). In the presence of CO_2 the resulting distribution of H^+ is due to the relative contributions of heteroexchange via band 3, which tends to equilibrate H^+ with Cl^- and the electrogenic H^+ transport across the conductance.

Indirect evidence suggests that while HCO_3^- and Cl^- transport function in the self-exchange mode of band 3 is present throughout, the heteroexchange function may be compromised in early embryonic red cells. The organic anion transporter tributyltin, which carries out electrosilent exchange of OH^- for Cl^-, has been shown to depolarize the membrane of red cells from 4- and 6-day-old chick embryos, and to increase substantially red cell pH (Engelke *et al.* 1988). As tributyltin tends to equilibrate the distributions of OH^- and Cl^-, the depolarization reflects the difference in E_H and E_{Cl} ($E_H = -36$ mV and $E_{Cl} = -14$ mV at day 6) (Engelke *et al.* 1988). As these experiments were carried out in the absence of DIDS, it follows that Cl^-–HCO_3^- heteroexchange capacity must be restricted at this time.

The present data clearly indicate band 3-mediated transport of HCO_3^- and Cl^- and as such offer no explanation for the apparent failure to carry out sufficient heteroexchange for equilibration of H^+ and Cl^-. However, even in adult red cells only a small part of the transport capacity of band 3 is used for the purpose of heteroexchange and a coupling ratio of 1:1 requires exact matching of the kinetic constants for HCO_3^- and Cl^- transfer. The heteroexchange function of band 3 is normalized during development, so that at day 16 the permeabilities of chloride and bicarbonate reach similar values to those in adult red cells. This is compatible with the fact that by then r_{Cl}, r_H and E_m are in equilibrium (Baumann *et al.* 1987; Engelke *et al.* 1988) presumably due to both normal heteroexchange function of band 3 and a decreased importance of the proton conductance.

Further experiments should clarify the mechanism of developmental regulation of band 3, in particular the role of protein phosphorylation, as band 3 of adult chicken red cells is the substrate for a membrane-associated protein kinase (Hillsgrove, Shores, Parker & Meuers, 1987).

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