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ADRENALIN-INDUCED Na^+/H^+ EXCHANGE IN TROUT ERYTHROCYTES AND ITS EFFECTS UPON OXYGEN-CARRYING CAPACITY

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SUMMARY

Addition of adrenalin (10^{-4} mol l^{-1}) to trout erythrocytes in an unbuffered saline resulted in a rapid acidification of the extracellular medium. This process was inhibited by amiloride ($K_{1/2} \sim 10^{-4}$ mol l^{-1}) and by the removal of extracellular Na^+. The rate of acidification was a saturable function of extracellular Na^+ concentration.

When extracellular pH was maintained constant by continual titration with KOH, adrenalin induced a transient burst of H^+ efflux. During this period the loss of cellular H^+ equivalents was approximately equal to the net gain of Na^+, providing evidence for a Na^+/H^+ exchange with a stoichiometry of 1. The steady state following stimulation with adrenalin could be disturbed by changes in extracellular pH. After the addition of adrenalin, intracellular pH (pH_i) was increased by 0·2–0·3 units but did not exceed extracellular pH, as required if the Na^+ and H^+ concentration ratios came into equilibrium. The increase in pH_i in stimulated compared with control cells was maintained approximately constant over a wide range of pH_o, suggesting that pH equilibration by the Jacob-Stewart cycle was operating normally and that the activation of Na^+/H^+ exchange provides an offset to the normal relationship between pH_i and pH_o. The steady state results from a balance of an increase Na^+/H^+ and Cl^-/HCO_3^- exchange with an increased rate of Na^+ pumping and next KCl efflux.

In a buffered saline, adrenalin caused a 22–46 % increase in the oxygen-carrying capacity of trout erythrocytes. It is suggested that this was due to a Root effect of trout haemoglobin caused by cellular alkalinization when the Na^+/H^+ exchange mechanism was activiated. This observation suggests that many published values for oxygen-carrying capacity of fish blood require re-evaluation.

INTRODUCTION

Erythrocytes of rainbow trout undergo a distinct swelling when incubated in an isosmotic medium containing adrenalin (Nikinmaa, 1982*b*; Bourne & Cossins, 1982; Baroin, Garcia-Romeu, Lamarre & Motais, 1984*a*). This swelling is

Key words: Trout erythrocytes, Na^+/H^+ exchange, amiloride, HCO_3^-/Cl^- exchange, adrenalin.

accompanied by a dramatic increase of both active and passive K^+ fluxes (Bourne & Cossins, 1982). However, it seems likely that this is a secondary effect and that the swelling is brought about by an increased Na^+ permeability, which, because of the inwardly-directed Na^+ concentration gradient, results in a net Na^+ uptake together with osmotically obliged water.

These ideas have been confirmed by Baroin *et al.* (1984*a*), who showed that the net Na^+ uptake following adrenalin-stimulation was accompanied by net Cl^- uptake. These authors have identified two different pathways of salt entry. The first was inhibited by amiloride but was insensitive to DIDS and furosemide, whilst the second was Cl^--dependent and was sensitive to amiloride, DIDS and furosemide. The first system corresponds to the putative Na^+/H^+ exchange mechanism observed in amphibian red cells (Cala, 1980, 1983), whilst the second may be a tightly-coupled (Na^+-Cl^-) co-transport.

An experimental distinction between these two transport systems is not easy when based upon Na^+ fluxes alone, but they may be separated by studying the co-transported species. Until recently, the evidence for the Na^+-dependent H^+ transport was circumstantial rather than direct (Nikinmaa, 1983). In this communication, we describe the induction by adrenalin of a net efflux of H^+ equivalents which is dependent upon the presence of external Na^+ and is completely inhibited by amiloride. We also present evidence in support of an exchange mechanism which depends upon the combined concentration gradients of Na^+ and H^+. Whilst this work was in the final stages of preparation, Baroin *et al.* (1984*b*) published their studies of adrenalin-induced Na^+/H^+ exchange, also in trout erythrocytes.

METHODS

Animals

Rainbow trout (*Salmo gairdneri*, Richardson, 0·5–0·75 kg) were obtained from a commercial source and were maintained in 1300-litre fibreglass aquaria. Water temperature varied between 10 and 20 °C during the experimental period and photoperiod was maintained constant at 16L:8D. Fish were fed twice daily with commercial pelleted trout food (B.P. Nutrition, Ltd).

Chemicals

Inorganic compounds, D-glucose, choline chloride, D-sucrose, trichloracetic acid, dibutylphthalate and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid) were obtained from BDH Chemicals Ltd (Poole, Dorset) and were of analytical reagent grade. Bovine serum albumin (fraction V), dimethylsulphoxide and adrenalin (L-epinephrine bitartrate) were obtained from Sigma Chemicals Ltd (Poole, Dorset). Imidazole was obtained from Koch-Light Laboratories (Sharnworth, Beds). Furosemide was a gift of Hoechst Pharmaceuticals (Hounslow, Middlesex) and amiloride was a gift of Merck, Sharp & Dohme Ltd (Hoddesdon, Herts).

Blood removal and treatment

Fish were stunned by a sharp blow to the head and blood was drawn from the caudal vein by a hypodermic syringe (21 gauge needle). Clotting was prevented by gently shaking the blood in disposable heparinized tubes.

Cells were washed at least four times in a buffered trout saline by centrifugation (1000 ***g***, 5 min). The osmolality of the saline was precisely adjusted to 300 mosmol kg^{-1} by mixing appropriate quantities of solutions containing either 180 or 100 mmol l^{-1} NaCl, together with (in mmol l^{-1}) KCl, 6; $CaCl_2$, 1; $MgCl_2$, 1; D-glucose, 5; and imidazole-HCl, 15; pH 7·6 (room temperature). Osmolality was measured with a freezing-point depression osmometer (Advanced Instruments, MA, U.S.A.). Erythrocytes were finally suspended in buffered saline at 25–30 % haematocrit and left overnight at 4 °C to ensure that the cells were at a steady state with respect to volume and ion content, and were not in a catecholamine-stimulated condition (Bourne & Cossins, 1982).

H^+ fluxes in unbuffered media

Cells were washed three times in an unbuffered trout saline (prepared as for normal trout saline but substituting 15 mmol l^{-1} mannitol for imidazole, 300 mosmol kg^{-1}). They were then resuspended either in an unbuffered 0·3 mol l^{-1} sucrose solution (containing 0·3 mmol l^{-1} KCl) or in the unbuffered trout saline. A sample (0·2 ml) of this suspension was added to 1·6 ml unbuffered sucrose or unbuffered trout saline, respectively. The extracellular pH (pH_o) of this suspension was monitored with a semi-micro combination pH electrode connected to a Beckman 3550 pH meter. The suspension was stirred continuously with a small magnetic follower. Where necessary, $[Cl^-]_o$ was measured using a Radiometer CMT10 chloride titrator.

Stock solutions of SITS and adrenalin were freshly prepared in unbuffered trout saline and kept under nitrogen in the dark. Before use they were titrated to the same pH as that established by the cells in an unbuffered solution. Amiloride were freshly prepared in dimethylsulphoxide. In the latter case, the solution was diluted with unbuffered trout saline and was also titrated. Extracellular Na^+ concentration ($[Na^+]_o$) was varied by mixing solutions containing either Na^+ or choline as principal cation (other constituents as for buffered trout saline, 300 mosmol kg^{-1}).

H^+ fluxes at constant, pH_o

The rate of net H^+ loss from a suspension of erythrocytes (8–10 % haematocrit) was measured by manual titration with 0·1 mol l^{-1} KOH from a 10 μl Hamilton syringe. Ouabain was present at a final concentration of 1 mmol l^{-1}. External pH was maintained between 7·40 and 7·45. The rate of net H^+ loss was calculated by dividing the mmoles of KOH injected by the time taken for pH_o to return to its original value. This was normalized by haematocrit to yield

mmol l^{-1} pcv h^{-1} (pcv = packed cell volume). In some experiments imidazole (1–1·5 mmol l^{-1}) was added to reduce the rate of changes of pH_o to a convenient level.

The net loss of cellular H^+ as a result of adrenalin stimulation was calculated by measuring the area of the peak on the graph relating H^+ efflux to time, by the cut-and-weigh method. The contribution from residual H^+ efflux was estimated by drawing a baseline to the peak by eye and was subtracted. The resulting value was normalized to haematocrit to give mmol l^{-1} pcv.

Intracellular Na^+ content was measured at intervals before and after adrenalin stimulation by rapidly centrifuging 2-ml aliquots of the erythrocyte suspension through a layer of dibutylphthalate in an Eppendorff microcentrifuge. The supernatant and oil layer were carefully removed and the pellet lysed in 0·5 ml 0·05 % Triton X-100. The lysate was deproteinized by addition of 0·5 ml 5 % trichloracetic acid. After centrifugation the clear supernatant was diluted and the Na^+ concentration was measured by emission flame spectroscopy.

Oxygen-carrying capacity of erythrocytes

2·5 ml of a suspension of erythrocytes (10–12 % haematocrit) in the buffered, isotonic trout saline, was placed in the main chamber of a manometric flask either with or without adrenalin (final concentration, 10^{-4} mol l^{-1}). 0·5 ml of a solution containing 2 % (w/v) potassium ferricyanide and 0·05 % (v/v) Triton X-100 was placed in the side-arm. The suspension was equilibrated against air by vigorous shaking for at least 30 min. The volume of oxygen evolved when the side-arm contents were poured into the main flask was determined at 25 °C on a Gilson Respirometer. Values were normalized to haematocrit and recalculated to yield ml O_2 evolved per 100 ml blood, assuming 40 % haematocrit.

Intracellular pH (pH_i)

A suspension of red cells (~10 % haematocrit) was centrifuged (10 000 ***g*** for 2 min, Eppendorf centrifuge) over a layer of dibutylphthalate. The pH of the extracellular medium (pH_o) was measured with a small combination pH electrode (4 mm diameter, Russell Ltd, Auchtermuchty, Scotland) and then discarded. The pellet was immediately frozen and thawed at −20 °C and, after the dibutylphthalate had been aspired, the pH of the haemolysate was determined with the same electrode.

RESULTS

Cl^-/HCO_3^- exchange

The demonstration of H^+ fluxes in vertebrate erythrocytes is complicated by the presence of a Cl^-/HCO_3^- exchange system of very great capacity. This is because the unhindered movements of HCO_3^- tend to neutralize changes in extracellular pH (pH_o) that result from H^+ movement (Hladky & Rink, 1977). In view of some uncertainty regarding the precise status of this anion exchange

process in fish erythrocytes (Haswell & Randall, 1976), it was necessary to demonstrate that a hetero-exchanger was indeed functional and that it was blocked by the established inhibitors of anion exchange in mammalian red cells.

This has been achieved by following the movements of HCO_3^- (as revealed by changes in pH_o) that occur as the concentration gradient for Cl^- is experimentally altered (Wieth, Brahm & Funder, 1980). Fig. 1A shows that when trout erythrocytes were suspended in an unbuffered 0·3 mol l^{-1} sucrose solution the pH_o rapidly fell to 5·4 as Cl^- passed out of the cell down its concentration gradient in exchange for HCO_3^-. Successive additions of aliquots of an isotonic KCl solution caused a stepwise increase in pH_o to new steady state values where (Hladky & Rink, 1977)

$$[Cl^-]_i/[Cl^-]_o = [HCO_3]_i/[HCO_3]_o. \quad (1)$$

Fig. 1B shows a linear relationship between $\log[Cl^-]_o$ and steady state pH_o with a slope of 1·09 ± 0·086 (mean ± S.D., $N = 5$). This demonstrates that a 10-fold change in $[Cl^-]_o$ induces a change in pH_o of one unit, all of which is consistent with an exchanger having a Cl^-/HCO_3^- stoichiometry of 1:1. Identical results have been obtained also with cells following treatment with adrenalin. SITS (10^{-4} mol l^{-1}), a potent inhibitor of anion exchange in human red cells (Knauf & Rothstein, 1971), caused a blockage of HCO_3^- redistribution following addition of KCl. This, together with the data of Haswell, Zeidler & Kim (1978), Romano & Passow (1984) and Baroin *et al.* (1984*a*), establishes that fish erythrocytes

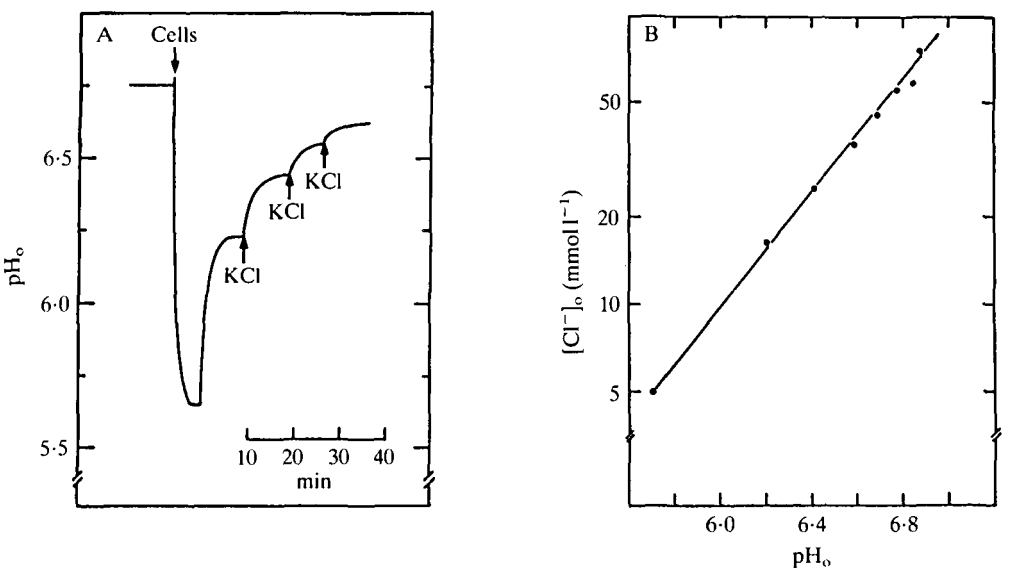

Fig. 1. Hydrogen ion equilibration *via* the Jacobs-Stewart cycle by trout erythrocytes suspended in a 0·3 mol l^{-1} sucrose solution. (A) The addition of cells to the medium caused a rapid fall in pH_o. Subsequent additions of increasing volumes of isotonic KCl caused pH_o to increase and plateau at new steady state levels. (B) Graph of pH_o at each steady state against extracellular $[Cl^-]$ (see text for details).

exhibit similar anion hetero-exchange properties to those of human erythrocytes which lead to similar equilibrium distributions of Cl^- and HCO_3^-.

Na^+/H^+ exchange in unbuffered media

Fig. 2A illustrates the effects of adrenalin upon pH_o of a suspension of erythrocytes in unbuffered saline. Addition of cells to the medium caused pH_o to stabilize at approximately 6·6–6·8. Addition of adrenalin (10^{-4} mol l^{-1}, final concentration) caused a rapid but transient acidification of the external medium with pH_o stabilizing approximately 0·1 unit lower than before addition of adrenalin.

Fig. 2B shows that addition of SITS to erythrocytes led to a slow, but maintained decrease in pH_o; the SITS-dependent acidification. The subsequent addition of adrenalin led to a rapid and dramatic increase in the rate of extracellular acification until pH_o reached a plateau at approximately pH 6. That this plateau represents a steady state involving changes in the distribution ratios of H^+ was suggested by the resumption of extracellular acidification when the saline was neutralized (not shown).

Fig. 3 illustrates the dose-response curve for adrenalin upon the initial rate of acidification (dpH/dt). Half-maximal rate was achieved at 3×10^{-7} mol l^{-1} adrenalin, which is reasonably close to the $K_{1/2}$ for stimulation of ouabain-insensitive K^+ influx (8×10^{-7} mol l^{-1}) obtained by Bourne & Cossins (1982).

Fig. 2C shows that, in the absence of extracellular Na^+, adrenalin had no effect. In fact, there was a small but measurable *increase* in pH_o. The subsequent addition of isotonic NaCl re-established the rapid acidification of the extracellular medium. This experiment clearly established an absolute requirement for extracellular Na^+.

If net transport through the proposed Na^+/H^+ exchange mechanism was a function of the combined concentration gradients of Na^+ and H^+ (Cala, 1983), the H^+ efflux and the steady state pH_o should both be functions of $[Na^+]_o$. We have found that the rate of change of pH_o was, indeed, a saturable function of Na^+ (data not shown), in agreement with the observations of Baroin *et al.* (1984*b*). The rate became zero at $[Na^+]_o \sim 2{\cdot}5$ mmol l^{-1} and below this concentration there was a slow extracellular alkalinization. By contrast, the SITS-dependent rate showed a linear dependence upon $[Na^+]_o$, and was not abolished in the absence of $[Na^+]_o$.

A linkage of the concentration gradients for H^+ and Na^+ also predicts that pH_o at the steady state (i.e. the plateau in Fig. 2B) should depend upon $[Na^+]_o$. Fig. 4 shows that this is the case. The relationship between pH_o and $\log[Na^+]_o$ was almost linear, but in contrast to the steady states observed with the anion exchange, the slope was only about 0·3. This was probably due to the significant variation of $[Na^+]_i$ with variations of $[Na^+]_o$. The solid line in Fig. 4 was calculated assuming that $[Na^+]_i$ was a linear function of $[Na^+]_o$ and that pH_i was constant. This produces a curve which matches the slope of the observed relationship.

Many putative Na^+/H^+ exchange mechanisms are inhibited by the diuretic

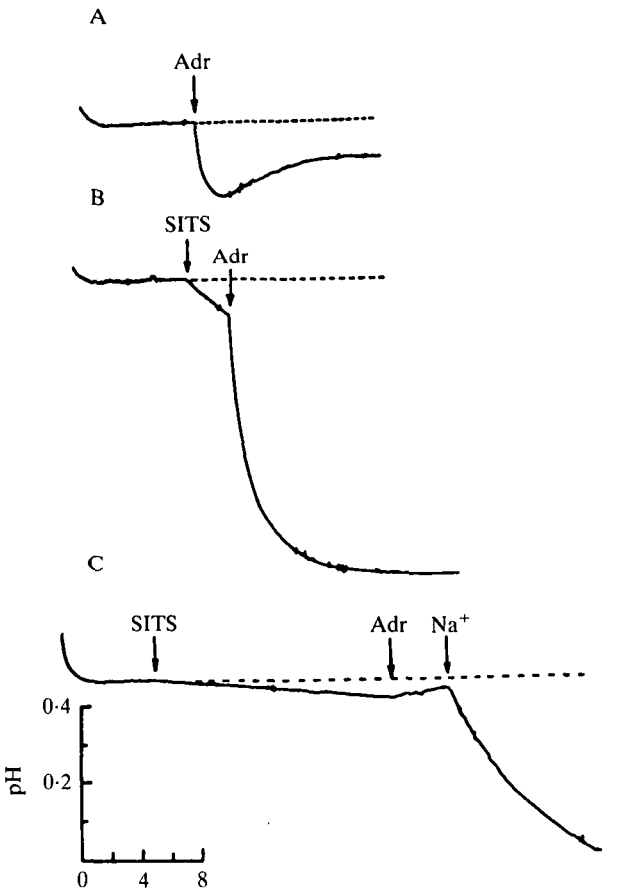

Fig. 2. Traces illustrating the acidification of the extracellular medium by adrenalin-treated trout erythrocytes in an unbuffered saline. (A) Addition of adrenalin (Adr, 10^{-4} mol l^{-1} final concentration), caused a transient acidification. (B) SITS (10^{-4} mol l^{-1}, final concentration), caused a gradual decrease in pH_o. Subsequent addition of adrenalin caused a dramatic increase in the rate of acidification until pH_o plateaued at approximately pH 6. (C) In the absence of external Na^+ (using choline as the replacement cation) SITS again caused an acidification of the external medium. Addition of adrenalin produced a small alkalinization. Subsequent addition of isotonic NaCl to give $[Na^+]_o$ of 70 mmol l^{-1} led to an immediate rapid extracellular acidification.

drug amiloride (reviewed by Benos, 1982). In trout erythrocytes, amiloride caused a complete blockage of the adrenalin-induced acidification. Fig. 5 shows a typical dose-response curve with a half-maximal inhibition at 10^{-4} mol l^{-1}. The SITS-dependent acidification was completely unaffected, even at the highest amiloride concentrations (10^{-3} mol l^{-1}).

Na^+/H^+ exchange at constant pH_o

Because plasma is significantly buffered, the steady states observed in unbuffered media have little relevance to *in vivo* conditions. Consequently, the progress of Na^+/H^+ exchange at constant pH_o has been monitored by measuring the rate of addition of KOH required to maintain pH_o constant (i.e. between 7·40 and 7·45).

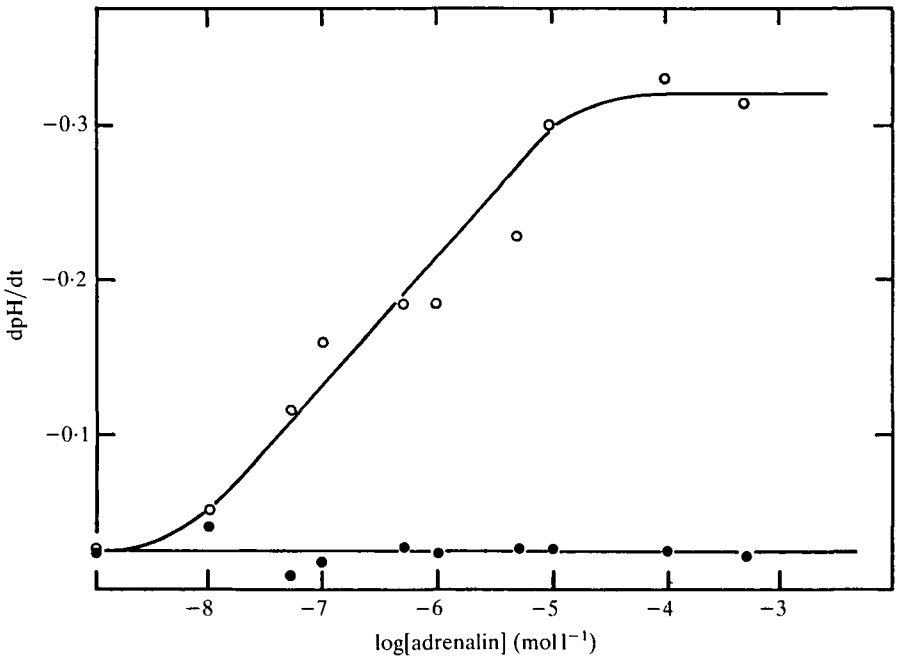

Fig. 3. The dose-response curve for adrenalin upon H^+ efflux in trout erythrocytes. The values represent the initial rate of change of pH_o: (●) in the presence of SITS; (○) in the presence of both SITS and adrenalin (both 10^{-4} mol l^{-1} final concentration). Similar results were obtained in two other experiments.

Fig. 6A shows that in control cells there is a significant and continuing net efflux of H^+ equivalents. Upon addition of adrenalin this is rapidly but transiently increased. Over a series of experiments the net H^+ flux before addition of adrenalin was 72 ± 10 mmol l^{-1} pcv h^{-1} (mean ± S.E., $N = 12$), whilst after stimulation by adrenalin the peak value was 434 ± 47 mmol l^{-1} pcv h^{-1}, an increase of six-fold.

The short-lived efflux of H^+ may be due to the rapid establishment of a new steady state or to a loss of sensitivity to adrenalin. The former alternative is favoured by the experiment shown in Fig. 6B. Following the transient H^+ efflux caused by adrenalin, the external pH was rapidly shifted to and maintained at 8·1 by addition of KOH. This increased the outwardly-directed concentration gradient for H^+ and led to a second transient pulse of H^+ efflux. Evidently, there was no refractoriness of the Na^+/H^+ exchange mechanism following the initial pulse of H^+ efflux after addition of adrenalin.

The net loss of cellular H^+ caused by stimulation with adrenalin was calculated from the area under the curves shown in Fig. 6. The net Na^+ uptake caused by stimulation with adrenalin was simultaneously estimated by measuring cellular Na^+ content immediately before addition of adrenalin and after the pulse of H^+ efflux. Ouabain (1 mmol l^{-1}, final concentration) was included to prevent active removal of cellular Na^+. Net Na^+ uptake was $13{\cdot}27 \pm 2{\cdot}91$ mmol l^{-1} pcv (mean

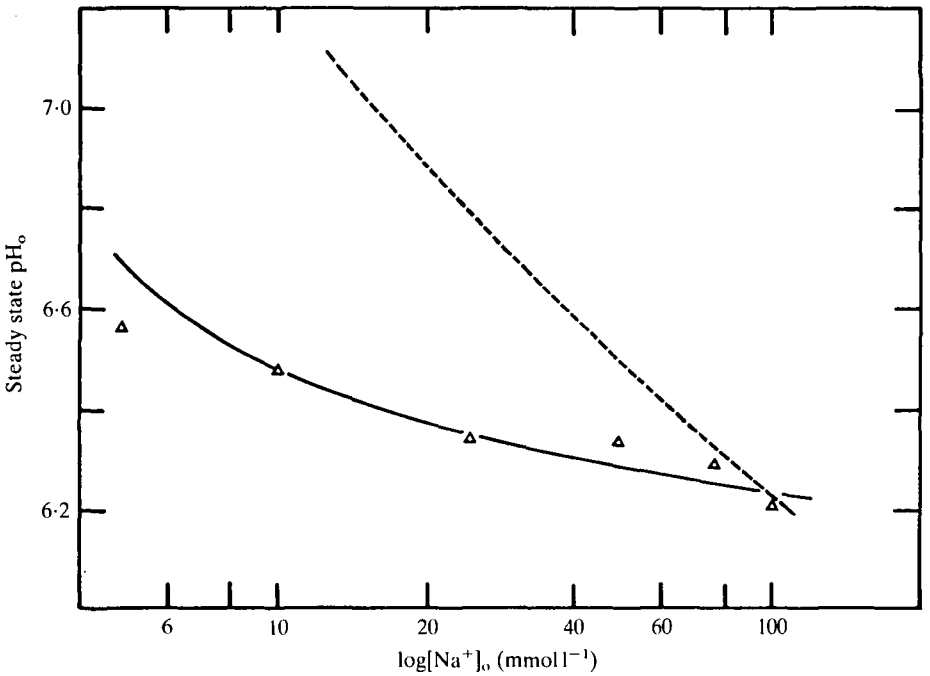

Fig. 4. Graph showing the effect of extracellular Na^+ concentration upon the steady state pH_o in adrenalin-treated trout erythrocytes. The data points were obtained from the plateaus shown in Fig. 2. The solid line was calculated from the relationship $[H^+]_o = ([Na^+]_o/[Na^+]_i) \times [H^+]_i$, where $pH_i = 6{\cdot}6$ and $[Na^+]_i = 0{\cdot}5[Na^+]_o + 0{\cdot}005$. The dashed line was calculated assuming a constant $[Na^+]_i$ of 0·05 mol l^{-1} and $pH_i = 6{\cdot}5$. This graph is typical of two other experiments.

$\pm$S.D., $N=7$) whilst net H^+ extrusion was $10{\cdot}19 \pm 2{\cdot}83$ mmol l^{-1} pcv. The stoichiometry of net H^+/Na^+ exchange was $1{\cdot}35 \pm 0{\cdot}45$ which again agrees closely with the results of Baroin *et al.* (1984*b*). This value was greater than the expected value of unity, probably because of a slight overestimation of Na^+ uptake due to the slow, continuing, passive net Na^+ uptake in the absence of Na^+ pump activity (Bourne & Cossins, 1984).

Intracellular pH

Over the normal range of pH_o, intracellular pH was between 0·3 and 0·4 units lower than pH_o, in agreement with the results of Tetens & Lykkeboe (1981). The values for erythrocytes treated with adrenalin were typically between 0·2 and 0·3 above that of control erythrocytes, when measured at a constant pH_o. Fig. 7 shows the relationship between pH_i and pH_o for control and adrenalin-treated erythrocytes. It is clear that, over this wide range of pH_o, the relationship is non-linear and that, at low pH_o, pH_i became greater than pH_o. Over a limited range of pH_o (7·2–8·0), the relationship was approximately linear and the calculated slopes for control and adrenalin-treated cells were similar; for control erythrocytes the regression equation was $pH_i = 0{\cdot}899pH_o + 0{\cdot}276$ ($N=19$, $r=0{\cdot}996$), whilst for

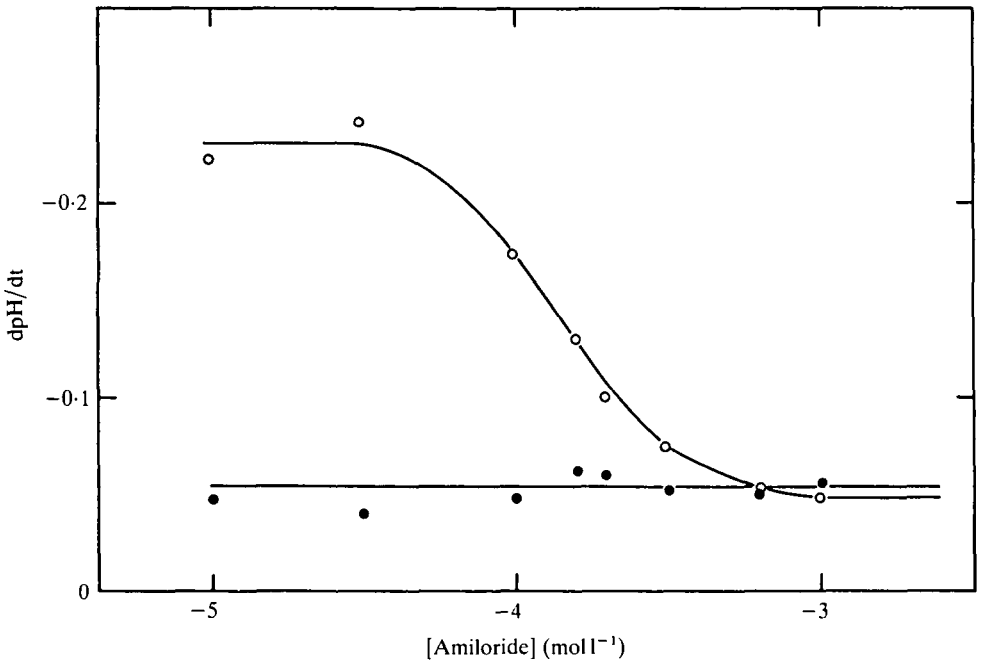

Fig. 5. The dose-response curve for amiloride inhibition of H^+ efflux of trout erythrocytes: (●), in the presence of SITS; (○) in the presence of boths SITS and adrenalin. Similar results were obtained in two other experiments.

adrenalin-treated erythrocytes it was $pH_i = 0{\cdot}813pH_o + 1{\cdot}074$ ($N = 20$, $r = 0{\cdot}998$). These equations are very similar to those observed in carp by Albers, Goetz & Hughes (1983), in European catfish by Albers, Goetz & Welbers (1981) and in rainbow trout by Tetens & Lykkeboe (1981).

Fig. 7 also shows that SITS had very little effect upon pH_i of adrenalin-treated erythrocytes over the normal range of pH_o. Below this range, however, it caused a progressively greater increase in pH_i.

Oxygen-carrying capacity of adrenalin-stimulated cells

During the course of these experiments it was obvious to us that adrenalin enhanced the red colouration of the erythrocyte suspension. This suggested that after stimulation by adrenalin the cells bind additional oxygen. Consequently, we have measured the oxygen-carrying capacity of trout erythrocytes in the absence and presence of adrenalin (Table 1). In all experiments, adrenalin caused a substantial increase in the bound oxygen compared to that in control erythrocytes; the percentage increase varying between 22 and 46 %. The absolute increase in carrying capacity upon stimulation by adrenalin was highly significant ($P > 0{\cdot}002$, Student's paired t-test). The percentage increase in carrying capacity was maintained constant over the range of extracellular pH_i of 6–8 (data not shown).

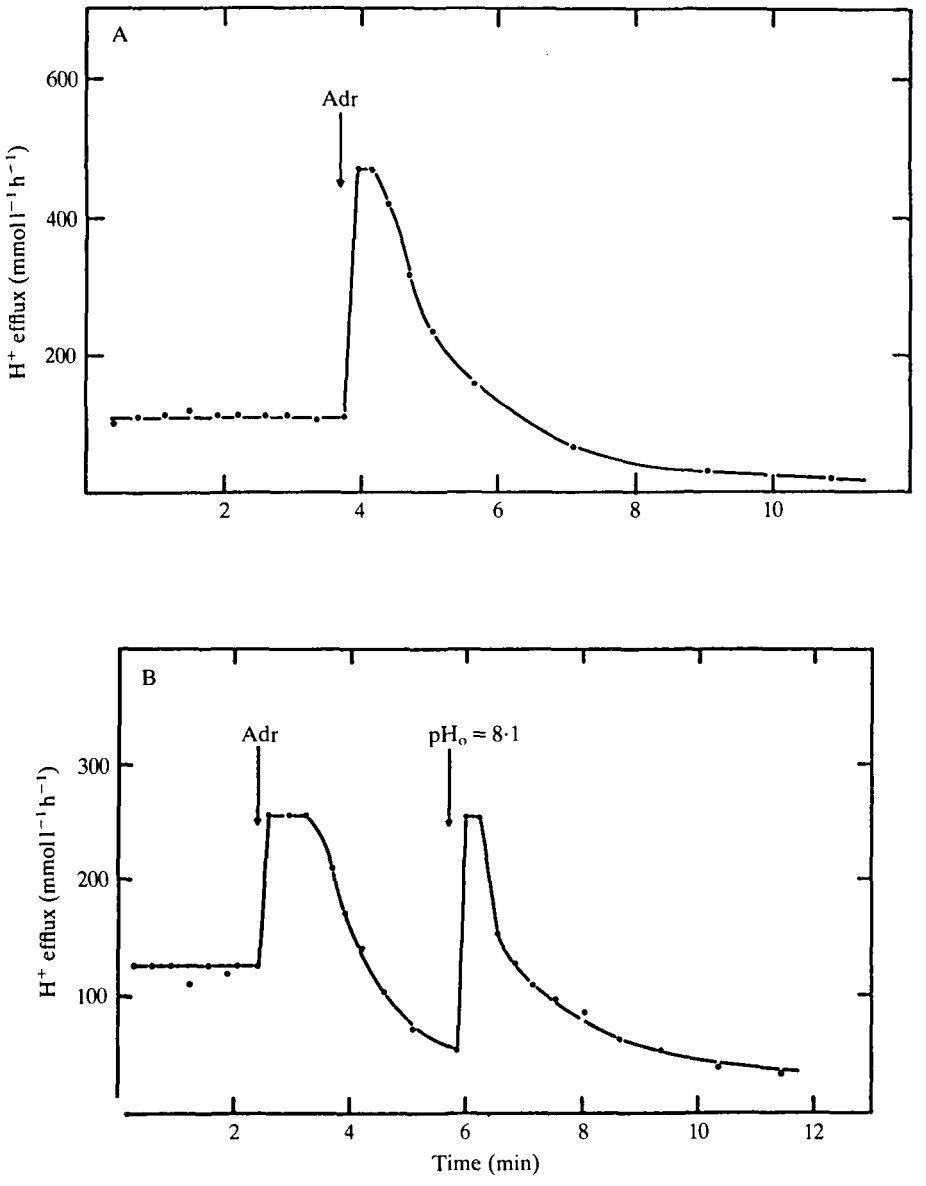

Fig. 6. The time course of net H^+ efflux at constant pH_o from trout erythrocytes. Cells were pre-incubated with 1 $mmol\,l^{-1}$ ouabain for 15 min and SITS ($10^{-4}\,mol\,l^{-1}$, final concentration) was added immediately before the experiment. Adrenalin was added (Adr, $10^{-4}\,mol\,l^{-1}$, final concentration) as indicated. H^+ efflux was estimated by measuring the rate of addition of 0·1 $mol\,l^{-1}$ KOH necessary to maintain pH_o constant (i.e. between 7·4 and 7·45). In (B) the second arrow represents the point at which pH_o was rapidly increased to 8·1 and maintained by addition of 0·1 $mol\,l^{-1}$ KOH.

DISCUSSION

The apparent net proton efflux in trout erythrocytes can be resolved into two distinct components. The first component is revealed in unbuffered media when

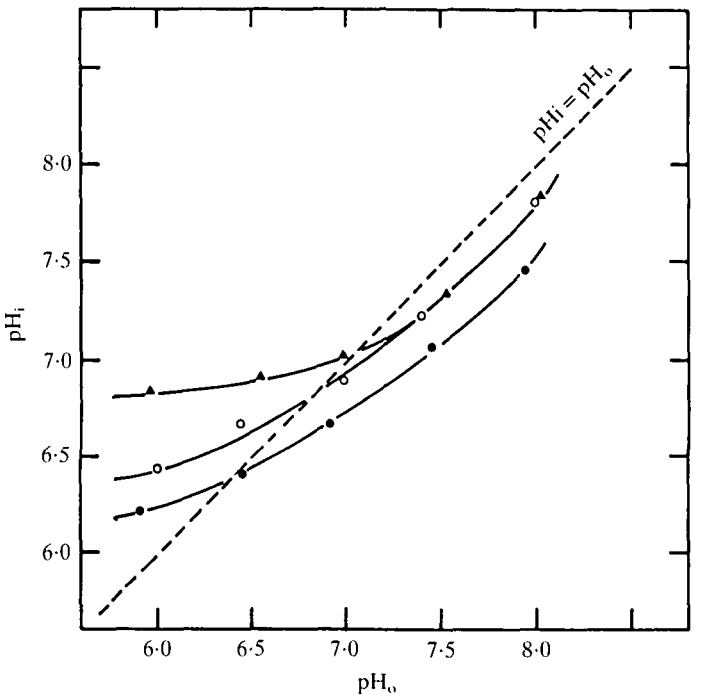

Fig. 7. The relationship between pH_i and pH_o in trout erythrocytes: (●) refers to a control suspension, (○) to a suspension with adrenalin (10^{-4} mol l^{-1}, final concentration) and (△) to a suspension containing adrenalin and SITS (10^{-4} mol l^{-1}, final concentration). Similar results were obtained in three other experiments.

the anion exchanger is inhibited by SITS, whilst the second is not normally operative but is induced by adrenalin and may be observed in the presence of normal anion exchange. The underlying mechanisms for each component are apparently different in that only the second mechanism is totally inhibited at low $[Na^+]_o$ or by the addition of amiloride.

It may be that component 1 represents the removal of metabolically-produced acid (see Zhuang *et al.* 1984), such as CO_2, rather than a SITS-induced H^+-transport. This could occur either by the diffusion of CO_2 across the cell

Table 1. *The effect of adrenalin (10^{-4} mol l^{-1}, final concentration) upon the oxygen-carrying capacity of trout erythrocytes*

Animal	Control	+Adrenalin	% Increase
1	11·0*	15·7	43·4
2	6·0	8·7	45·6
3	14·5	18·3	26·8
4	13·1	16·0	22·0
5	15·0	20·5	36·3

*Values presented as ml O_2/100 ml blood, assuming 40 % haematocrit.

membrane and its subsequent hydration to yield an extracellular proton, or, in cells with a functional anion exchanger, by the intracellular hydration of CO_2 and the coupled exit of HCO_3^- through the anion exchanger with H^+ by some other electroneutral route.

The slight but noticeable dependence of component 1 upon $[Na^+]_o$ is puzzling since Na^+ is not directly involved in either of these proposed mechanisms. Two possible explanations come to mind. The first is that the Na^+-dependence represents continuing Na^+/H^+ exchange in unstimulated cells in addition to the metabolic production of H^+. However, the linear dependence of component 1 upon $[Na^+]_o$ and the lack of inhibition by amiloride both argue against a specific mechanism of this sort. The second and more likely explanation is that, by changing $[Na^+]_o$, the rate of passive Na^+ uptake is altered and this affects the rate of Na^+ pumping necessary to maintain a constant $[Na^+]_i$. Thus, changes in $[Na^+]_o$ may alter the demand of ATP for ion regulatory purposes thereby altering the production of respiratory CO_2.

The efflux of H^+ caused by adrenalin has all the characteristics expected of a Na^+/H^+ exchange mechanism. Firstly, it was abolished in the absence of extracellular Na^+ and was a saturable function of $[Na^+]_o$ (Baroin *et al.* 1984*b*). Secondly, the pH_o at which a steady state in an unbuffered medium containing SITS was observed showed a strong dependence on $[Na^+]_o$. Thirdly, in certain situations the net movement of H^+ was reversed by manipulating the concentration gradient of Na^+. Fourthly, the efflux of H^+ caused by adrenalin was inhibited by amiloride at concentrations which agree well with those observed in other studies (Benos, 1983; Siebens & Kregenow, 1978; Pouyssegur *et al.* 1983; Zhuang *et al.* 1984). Finally, intracellular sodium content increased dramatically as a result of stimulation with adrenalin. The stoichiometry of net H^+ and net Na^+ changes was close to 1.

The putative Na^+/H^+ exchanger has some interesting characteristics. The exchange is not operative in non-stimulated, volume-static erythrocytes. In human lymphocytes and other cells an ongoing Na^+/H^+ exchanger acts to regulate pH_i (Grinstein & Furuya, 1984), though in vertebrate erythrocytes this function seems largely redundant because of the high-capacity anion exchanger and the Jacob-Stewart cycle. Activation of the Na^+/H^+ exchange in trout erythrocytes is very rapid and shows no noticeable lag, such as occurs in a mammalian cell line (Cassel *et al.* 1983). A steady state is reached within 3–5 min and this persists for some considerable time after the initial transient burst of net Na^+/H^+ exchange, since a subsequent disturbance of the H^+ concentration ratio led to renewed net exchange. This, together with the observed effects of $[Na^+]_o$ upon steady state pH_o in unbuffered media containing SITS (Fig. 4), suggests that in these experiments an equilibrium was established in which the concentration ratios for Na^+ and H^+ were equal (Cala, 1983), i.e.

$$[Na]_i/[Na]_o = [H^+]_i/[H^+]_o. \quad (2)$$

It is obvious from this relationship that because $[Na^+]_o$ is greater than $[Na^+]_i$ in

cells treated with adrenalin (Baroin *et al.* 1984*a*) that at equilibrium the $[H^+]_o$ should be greater than $[H^+]_i$; that is, pH_i becomes greater than pH_o. This is in contrast to the normal, unstimulated condition where Na^+/H^+ exchange is inoperative and only the Cl^-/HCO_3^- exchanger is operative. Because of the linkage between H^+, HCO_3^- and Cl^- distributions and the net negative charge on intracellular polyanions (Hladky & Rink, 1977), pH_i is generally 0·3–0·4 units less than pH_o (Albers *et al.* 1983; Nikinmaa, 1983; present results). Nikinmaa & Huestris (1984), using the DMO technique, have observed an elevation of pH_i above pH_o in erythrocytes of striped bass after treatment with adrenalin in the presence of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid). In addition, Nikinmaa & Weber (1984) noted higher intraerythrocytic pH than plasma pH in blood from hypoxic lamprey. Although in the present experiments pH_i was significantly elevated following addition of adrenalin, it did not exceed pH_o over the physiological range of pH_o (see also Nikinmaa, 1983). It is thus clear that the steady states observed in the experiments at constant pH_o values between 7·2 and 8 were not accounted for by equation 2 and that other processes are important. One obvious possibility is that HCO_3^- movements that occur as a result of the altered H^+ distribution tend to dampen out the changes in pH_i and pH_o despite addition of SITS. That this process is important in cells with a functional anion exchanger is clear from the small extracellular acidification that occurs upon treatment with adrenalin in unbuffered media (Fig. 1A).

Because the Na^+/H^+ exchange does not reach an equilibrium it follows that there is a significant and continuing net exchange during the steady state following stimulation with adrenalin which induces a net Cl^-/HCO_3^- exchange. The resulting net efflux of H_2CO_3 can easily be balanced by the Jacobs-Stewart cycle, whilst the net NaCl influx must be balanced by other routes of net efflux. We have previously shown that the rate of K^+ (Rb^+) transport by the Na^+ pump and the bidirectional Cl^--dependent passive mechanism are both enhanced by approximately 250 % following stimulation by adrenalin (Bourne & Cossins, 1982) and remain so for a considerable time. Assuming that the latter process represents a co-transport of KCl, which, as previously shown by Bourne & Cossins (1984) occurs in a net outward direction, then activation of this co-transport and the Na^+/K^+ pump provides the appropriate stoichiometry and direction to balance the net NaCl uptake. The pump is probably activated by the increase in intracellular Na^+, whilst the KCl-co-transport may be triggered either by indirect means or, more likely, by a direct effect of the swelling that occurs as a result of treatment with adrenalin (Bourne & Cossins, 1983; Baroin *et al.* 1984*a*) (Fig. 8).

A rather surprising feature of the Na^+/H^+ exchanger was its very high transport capacity. The maximal efflux of H^+ equivalents was 20–100 times greater than other volume-induced or continuing co-transport systems in trout erythrocytes (Bourne & Cossins, 1984) and approaches the exchange capacity of the anion exchanger. This being the case, it is important to determine the role of anion exchange in the estimation of H^+ efflux in the presence of SITS. In our hands, SITS at 10^{-4} mol l^{-1} reduced the rate of pH_o equilibration by 99 % when

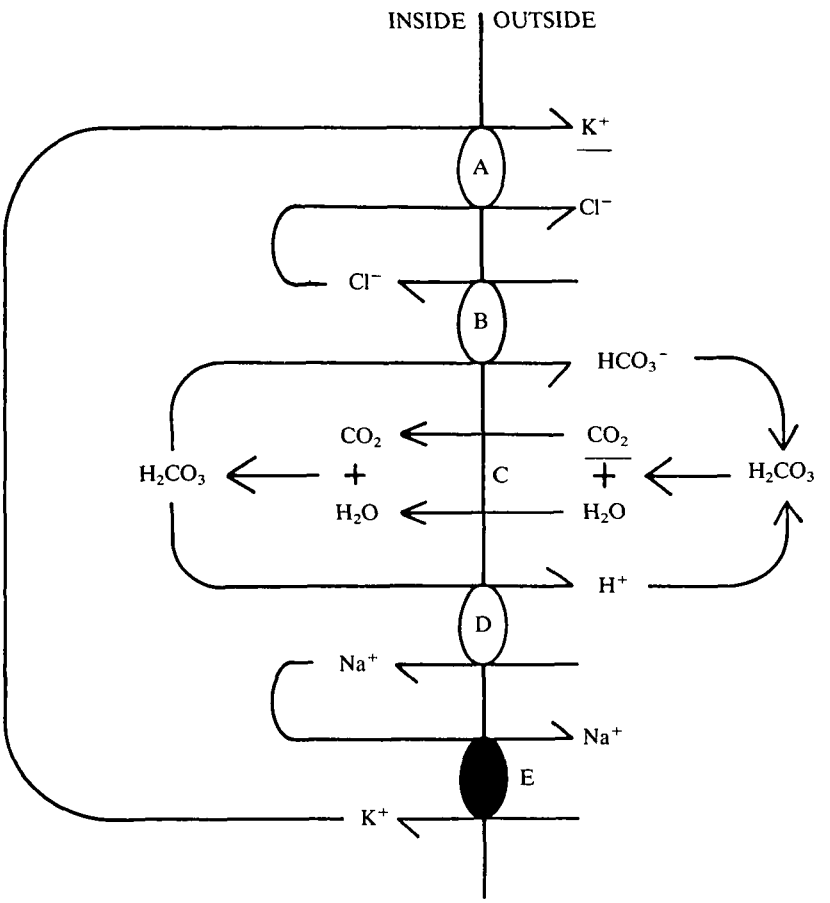

Fig. 8. Scheme to illustrate the proposed net movements of H^+, HCO_3^-, Cl^- and monovalent cations during the steady state following adrenalin-stimulation. (A) Represents the putative KCl co-transport (i.e. Cl^--dependent, passive K^+ transport), (B) the anion exchanger, (C) the Jacob-Stewart cycle, (D) the Na^+/H^+ exchanger and (E) the Na^+/K^+ pump.

acid or alkali was added to an unbuffered medium, but certainly did not block the process completely. Romano & Passow (1984) have also noted a significant DIDS-insensitive sulphate exchange in trout erythrocytes. The residual anion exchange may well be sufficient to influence the steady states observed in the present experiments and account for the previously mentioned failure to measure equal concentration ratios for Na^+ and H^+ following stimulation by adrenalin in the presence of SITS. By neutralizing transported H^+, the residual HCO_3^- movements will lead to an underestimation of H^+ efflux that is mediated by Na^+/H^+ exchange, particularly in the steady state following the initial burst of Na^+/H^+ exchange. The observation of $pH_i > pH_o$ in DIDS-treated erythrocytes of striped bass (Nikinmaa & Huestris, 1984) may be due to more complete blockage

of anion exchange. The apparent agreement of the Na^+ and H^+ gradients with equation 2 in the experiments where pH_o was not held constant (Fig. 4) may be accounted for by a greater potency of SITS at lower pH_o. This effect of pH upon SITS-inhibition is supported by the substantial increase in pH_i of adrenalin-treated erythrocytes that was caused by SITS at lower pH_o values (Fig. 7).

Adrenalin caused an increase in pH_i of 0·2–0·3 in agreement with the increases found in fish erythrocytes (Nikinmaa, 1983; Nikinmaa & Huestris, 1984) and during activation of Na^+/H^+ exchange in other cellular systems (lymphocytes: L'Allemain, Paris & Pouyssegur, 1984; neutrophils: Grinstein & Furuya, 1984). Nikinmaa (1983) observed a reduction in the dependence of pH_i on pH_o on stimulation by adrenalin. This suggests a pH-dependent change in the nature of the processes which determine H^+ distribution and perhaps in the potency of the Jacobs-Stewart cycle. In contrast, the present experiments indicate firstly that the increase in pH_i after adrenalin treatment was maintained constant over a wide range of pH_o and secondly that pH_i is as dependent upon pH_o in stimulated as in unstimulated erythrocytes. We have confirmed that Cl^-/HCO_3^- exchange, and therefore the Jacobs-Stewart cycle, was indeed operative in erythrocytes stimulated by adrenalin. Together, these results suggest that activation of the Na^+/H^+ exchanger leads to an offset of pH_i with respect to pH_o and to the establishment of a new steady state due to combined activity of both Na^+/H^+ and Cl^-/HCO_3^- exchangers.

The functional significance of adrenalin-induced swelling and alkalinization seems to be due to changes in the oxygen-binding properties of haemoglobin. Nikinmaa (1982*b*, 1983) has correlated the cellular alkalinization with a decrease in P_{50} that occurs as a result of stimulation with adrenalin by suggesting that the hormone induces a Bohr effect. Our studies show that the carrying capacity for oxygen is also greatly augmented. This is most simply explained by invoking the marked Root effect observed for the predominant haemoglobin isoform in trout (Brunori, 1975), since the pH range over which the Root effect is most apparent (Giardina, Antonini & Brunori, 1973) corresponds to the changes in pH_i that occur during stimulation with adrenalin (Nikinmaa, 1982*b*, 1983). A pronounced Root effect is apparent in the *in vitro* oxygen equilibration curves for trout erythrocytes obtained by Nikinmaa (1983) and is consistent with an increase in *in vivo* blood oxygen content after injection of trout with adrenalin (Nikinmaa, 1982*a*) despite there being no change in arterial P_{O_2}. Quiescent erythrocytes *in vivo* thus display sub-maximal oxygen-carrying capacities and greater P_{50} values compared to those of adrenalin-treated erythrocytes. The increase in pH_i may serve to offset the effects of reduced pH_o caused by lactic acid production during times of stress.

The practical significance of these observations may be great, since they are likely to apply to species other than trout. Thus carp (Bourne & Cossins, 1982) and striped bass erythrocytes (Nikinmaa & Huestris, 1984) display transport systems which are activated by adrenalin. It is clear that much of the published work on the respiratory characteristics of fish and amphibian blood at least,

requires re-evaluation, since the blood in these studies was likely to contain adrenalin. Erythrocytes obtained from fish by the 'grab and stab' method are undoubtedly stimulated by adrenalin (Riddick, Kregenow & Orloff, 1971; Bourne & Cossins, 1982), and unless the erythrocytes are thoroughly washed in a saline and incubated for several hours will remain so. Only in those studies where blood has been obtained by means of chronic catheters from undisturbed fish, can the respiratory properties be any reflection of *in vivo* properties.

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