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THE SEASONAL MODULATION OF Na^+/H^+ EXCHANGER ACTIVITY IN TROUT ERYTHROCYTES

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Summary

A pH-stat method is described for determining the initial rate of efflux of H^+ equivalents from trout erythrocytes after stimulation with catecholamines. This measure of transport capacity of the Na^+/H^+ exchanger was sensitive to medium pH with a peak at $7{\cdot}24 \pm 0{\cdot}09$ pH units. After washing blood into a trout saline the exchange capacity recovered to a maximal value after 2 h and remained stable for at least 24 h at 5 °C. Internal Na^+ concentration reached a low, stable value also within 2–3 h of washing. The antiporter was comparatively unstable when incubated at room temperature. The concentrations for half-maximal responses to the agonists adrenaline, noradrenaline and isoproterenol were $3{\cdot}0 \pm 1{\cdot}1 \times 10^{-7}$, $2{\cdot}5 \pm 3{\cdot}0 \times 10^{-8}$ and $1{\cdot}5 \pm 0{\cdot}6 \times 10^{-8}$ mol l^{-1}, respectively.

The variation of antiporter capacity with time of the year was followed over a 24 month period. Capacity was highest during early summer at 200–250 mmol h^{-1} l^{-1} packed cell volume (pcv), but declined from November to March to a low of approximately 100 mmol h^{-1} l^{-1} pcv. Exchange capacity increased rapidly during the early spring back to values characteristic of early summer. It appears that both temperature and photoperiod are involved in regulating the spring increase.

Introduction

Our understanding of the respiratory physiology of fish erythrocytes has been greatly enhanced in recent years by the discovery of adrenergically stimulated transport systems. Isoproterenol induces a rapid swelling of trout erythrocytes which is caused by the simultaneous entry of Na^+ and Cl^-. Because the swelling and entry of Na^+ is blocked by amiloride and is associated with a net efflux of H^+, the transport system appears to be a Na^+/H^+ countertransport and, indeed, a 1:1 stoichiometry for Na^+ entry and H^+ efflux has been recorded (Baroin *et al.* 1984*a*; Cossins & Richardson, 1985). Thus, the inwardly directed Na^+ gradient drives the net extrusion of H^+ to produce an intracellular alkalinization of approximately 0·2 pH units at a constant external pH. Under normal circumstances the disturbance to the H^+ distribution induces a net loss of HCO_3^- from cells in exchange for extracellular Cl^- *via* the anion exchange mechanism, so that the continuing H^+

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efflux after the initial burst is effectively titrated. The swelling also appears to activate a Cl^--dependent K^+ transport mechanism, which is sensitive to an enlarged cell volume (Bourne & Cossins, 1982; Borgese *et al.* 1987*a*), as well as an increase in the activity of the Na^+ pump due to the increase in intracellular Na^+ concentration, $[Na^+]_i$. The resulting steady state is complex but results in an increased internal pH (pHi), a dramatically increased $[Na^+]_i$ and a 2·5-fold increase in ATP demand of the Na^+ pump.

As the mechanism of the phenomenon becomes clear it is possible to investigate its physiological significance. One interesting observation is the possible seasonal modulation of adrenergic responsiveness. Thus, Nikinmaa & Jensen (1986) have found that *in vivo* adrenergic responses observed during the summer were undetectable in rainbow trout during the winter. In contrast, Tetens *et al.* (1988) did observe *in vitro* adrenergic responses during the winter months using the blood obtained by cannulation of undisturbed trout. Neither of these studies on erythrocytes directly quantified the transport capacity of the exchange mechanism and it is difficult to compare the sensitivity of the two studies in defining the presence or absence of adrenergic responses.

We describe here a method using a pH-stat to determine the exchange capacity of the Na^+/H^+ exchanger by measuring the initial rate of efflux of H^+ equivalents following adrenergic stimulation. This technique has been used to determine the optimal conditions for the reliable assay of maximal exchange activity and the seasonal variation in transport capacity of suspensions of trout erythrocytes over a period of 24 months.

Materials and methods

Materials

Inorganic salts, D-glucose, trichloroacetic acid, mannitol and imidazole were purchased from BDH Ltd, Poole, Dorset. (−)Adrenaline bitartrate, (−)noradrenaline bitartrate, (−)isoproterenol bitartrate, DL-propanolol–HCl, heparin, bovine serum albumin (BSA, fraction V), Triton X-100 and amiloride hydrochloride were purchased from Sigma Chemical Company Ltd, Poole, Dorset. 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) was purchased from Molecular Probes Inc., Eugene, Oregon.

Fish

Rainbow trout (*Salmo gairdneri*, 0·5–1 kg) were obtained from Chirk Fisheries Ltd, Chirk, Clwyd, North Wales and transported to the laboratory in galvanized metal containers. This stock is of the 'shasta' variety, spawning from October to early December, and has been reproductively isolated at Chirk for over 55 years. Fish were maintained in the laboratory for up to 4 months in 2000 l fibreglass aquaria (2 m × 2 m × 0·5 m) at ambient temperatures between 8 and 18 °C and with a fixed photoperiod of 16 h light: 8 h dark. Aquarium water was treated by vigorous recirculation through a biological filter and a pressurized mechanical filter. Fish

were fed once daily to satiation with trout pellets (BP Nutrition, Northwich, Cheshire, UK). Water temperature of the inlet stream (River Ceiriog) at the trout farm was measured at irregular intervals with a mercury-in-glass thermometer to a precision of 0·5 °C. Water temperature of the River Dee at Manly Hall Hydrographic Station, Clwyd (approximately 7 km downstream on the River Dee) was continuously monitored with a Rotatherm automated chart recorder.

Bleeding

Fish were netted from the tank and stunned with a blow to the head. Arteriovenous blood was rapidly sampled from the caudal vessels with a 21 gauge hypodermic needle into heparinized plastic containers (approximately 45–60 s after netting) and was gently shaken for 2 min. Erythrocytes were washed by centrifugation at 300–400 *g* for 2 min into an isotonic trout saline (room temperature) containing 6 mmol l^{-1} KCl, 5 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} D-glucose, 15 mmol l^{-1} imidazole–HCl at pH 7·6 (measured at room temperature) and sufficient NaCl to give an osmolality of 308 mosmol kg^{-1}. Cells were washed four times with greater than 10-fold dilutions in each case and then transferred to a refrigerator. For overnight storage erythrocytes were washed once into a trout saline containing 1 % (w/v) BSA and kept at a haematocrit of 15–20 % in a refrigerator at 5 ± 1 °C.

Determination of intracellular Na^+ concentration ($[Na^+]_i$)

Samples (0·25 ml) of erythrocyte suspension at 8–10 % haematocrit were centrifuged at 10 000 *g* for 10 s (Eppendorf Microcentrifuge) and the supernatant was removed by aspiration. The pellet was rapidly resuspended in 1 ml of ice-cold isotonic $MgCl_2$ solution (150 mmol l^{-1} $MgCl_2$, 15 mmol l^{-1} imidazole–HCl at pH 7·6, room temperature) mixed thoroughly and centrifuged again. This was repeated three times. The final pellet was lysed by the addition of 0·5 ml of Triton X-100 solution (0·05 %, w/v) and thoroughly mixed. Protein was precipitated by addition of 0·5 ml of 5 % (w/v) trichloroacetic acid followed by centrifugation at 10 000 *g* for 2 min. A sample of the resulting supernatant was diluted into distilled water and the Na^+ concentration determined using a flame emission spectrometer (Model S11, Instrumentation Laboratories, Warrington, Cheshire, UK).

Measurement of Na^+/H^+ exchange activity

The apparent transport of protons from erythrocytes to the extracellular medium was determined using a Radiometer RTS 822 pH-stat. Erythrocytes were washed four times in an unbuffered trout saline (308 mosmol kg^{-1}) in which the imidazole in the normal buffered trout saline was replaced by mannitol. The final pellet was suspended at a haematocrit of 5–10 % and 1·5 ml was transferred to a conical plastic cuvette (Radiometer). The stirring rate of the titration unit was reduced to prevent frothing of the erythrocyte suspension. The titrant was 10 mmol l^{-1} NaOH whose strength was routinely calibrated by titration against 10 mmol l^{-1} potassium hydrogen phthalate. The red cell suspension was manually

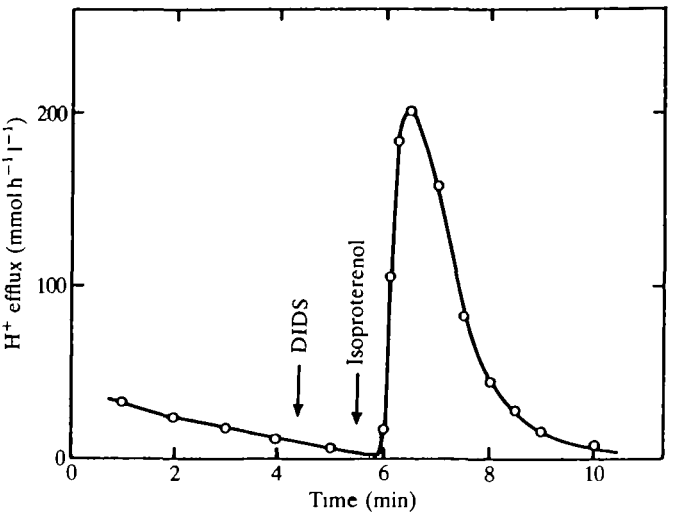

Fig. 1. The time course for the efflux of H^+ equivalents from trout erythrocytes determined using an automatic pH-stat. Cells were washed into unbuffered trout saline and titrated to approximately pH 7·3. The pH-stat was started and the rate of titrant addition allowed to decline to low stable values as the suspension equilibrated. DIDS (5×10^{-4} mol l^{-1}, final concentration) and isoproterenol (5×10^{-5} mol l^{-1}, final concentration) were added as indicated. Efflux was calculated from the slope of the graph of titrant added against time to maintain pHe at 7·30 ± 0·02. In subsequent experiments the peak value achieved after addition of agonist was used as a measure of transport capacity.

titrated with 100 mmol l^{-1} NaOH to approximately pH 7·3 and the automatic titration started. Agonists and inhibitors were added as aqueous solutions as indicated in the figures. A few crystals of ascorbic acid were added to catecholamine solutions to inhibit oxidation. The rate of H^+ production (efflux) was calculated from the slope of the graph of titrant volume added against time. The agonist-stimulated rate was routinely determined as the initial rate following addition of agonist minus the rate immediately before addition. Values were normalized for haematocrit and reported as mmol h^{-1} l^{-1} pcv. It is not possible to distinguish changes in extracellular pH (pHe) caused by transport of HCO_3^- or OH^- from those caused by movements of H^+, so values are reported as the efflux of H^+ equivalents.

Results

Fig. 1 shows a typical graph of the efflux of H^+ equivalents as a function of time determined using the pH-stat. Addition of erythrocytes to the unbuffered solution led to a gradual equilibration to pH 6·0–6·5. Sufficient titrant was added to bring the equilibrium pH to 7·3. Addition of DIDS usually had no effect or occasionally led to a small but transient increase in H^+ efflux. The subsequent addition of

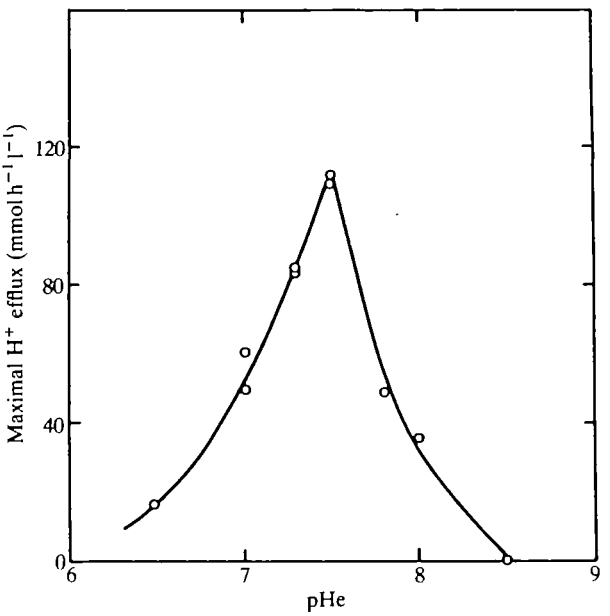

Fig. 2. The effect of external pH (pHe) upon the maximal isoproterenol-stimulated efflux of H^+ equivalents from trout erythrocytes.

isoproterenol caused, after a lag of 15–40 s, a rapid and dramatic increase in H^+ efflux to peak values of 150–300 mmol h^{-1} l^{-1} pcv. This burst lasted for 2–3 min before gradually decreasing over 5–10 min to control levels. Addition of isoproterenol in the absence of DIDS leads to a much smaller, though more sustained, increase in H^+ efflux (not shown).

Characterization of H^+ efflux properties

Fig. 2 shows the effects of pHe upon H^+ efflux. There was a pronounced peak at pH 7·24 ± 0·09 (mean ± S.D., five experiments) with no isoproterenol-stimulated increase at pH 8 or pH 6, in close agreement with the curve described by Borgese *et al.* (1987*b*), who measured the isoproterenol-induced increase in $[Na^+]_i$. All subsequent measurements were performed at pH 7·3.

Fig. 3 illustrates the effects of washing and storage of erythrocytes following bleeding upon the maximal efflux of H^+ equivalents. High values were observed in washed blood immediately after bleeding, though in several experiments this declined to zero after a short period of incubation at 5 °C (not shown) and subsequently recovered within 2–3 h of incubation. The maximal efflux of H^+ equivalents was usually stable for at least 24 h in washed erythrocytes stored at 5 °C in a BSA-containing saline. Storage at room temperature (19–20 °C) led to a rapid loss of exchange capacity. In subsequent experiments washed blood was stored at 5 °C for 2–6 h before maximal H^+ effluxes were determined.

Fig. 4 illustrates the corresponding changes in intracellular Na^+ immediately

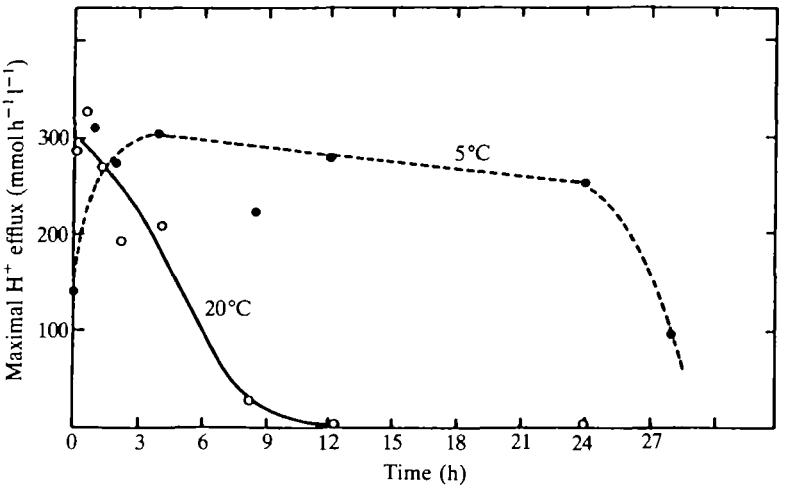

Fig. 3. The time course of recovery of the maximal isoproterenol-stimulated efflux of H^+ equivalents in trout erythrocytes following bleeding and washing of the cells into a buffered trout saline. Suspensions were incubated at 5 ± 1 °C (●) or 20 ± 1 °C (○) and samples taken for assay at the times indicated. Zero time indicates the resuspension of the erythrocytes following their final wash. Similar results were obtained in two other experiments.

following bleeding and washing of the suspension. $[Na^+]_i$ was 23·5 ± 4·1 mmol l^{-1} in freshly drawn blood (mean ± S.D., $N = 3$), a value somewhat smaller than that obtained by Korcock *et al.* (1988) using a similar bleeding technique. It declined over a 2–3 h period to 10·5 ± 1·5 mmol l^{-1} pcv ($N = 18$). Addition of a maximally stimulatory dose of isoproterenol caused a dramatic increase in $[Na^+]_i$ to approximately 70 mmol l^{-1} pcv (Fig. 4), indicating that freshly drawn blood was stimulated by approximately 25 % of its maximal possible stimulation. Subsequent removal of agonist by washing again led to a rapid decrease in $[Na^+]_i$ to control levels.

Fig. 5 shows that *in vitro* exposure of erythrocytes for 1 h to maximally stimulatory doses of agonist caused a loss of approximately 50 % (adrenaline) or 70 % (noradrenaline) in exchange capacity relative to a control suspension not exposed to agonist but otherwise treated in an identical manner. The loss of exchange activity in control cells was not usually observed and its cause in this experiment is not known.

Fig. 6 illustrates the dose–response curves for the agonists, adrenaline, noradrenaline and isoproterenol. The concentrations for half-maximal response were $3{\cdot}0 \pm 1{\cdot}1 \times 10^{-7}$, $2{\cdot}5 \pm 3{\cdot}0 \times 10^{-8}$ and $1{\cdot}5 \pm 0{\cdot}6 \times 10^{-8}$ mol l^{-1}, respectively ($N = 3$). Since these values, which were obtained for washed erythrocytes in trout saline, are similar to those determined by Tetens *et al.* (1988) in blood from undisturbed trout by cannulation, it appears that plasma has no obvious potentiating effect upon catecholamine binding.

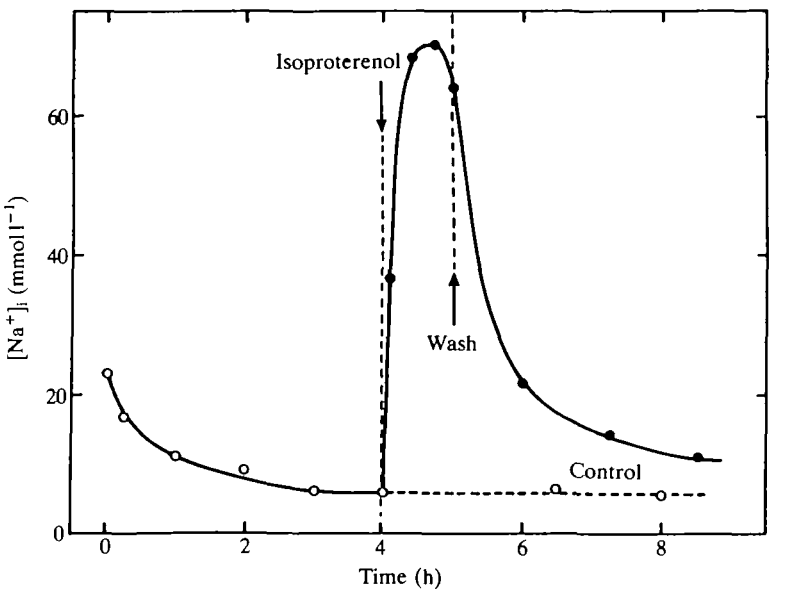

Fig. 4. The time course of changes in $[Na^+]_i$ of erythrocytes immediately following bleeding and washing into trout saline. At 4 h the suspension was divided into two and isoproterenol (5×10^{-5} mol l^{-1}, final concentration) was added to one (●) and an identical volume of saline to the control (○). At 5 h the isoproterenol-treated suspension was washed into trout saline. Incubation temperature for the control suspension was 5±1°C and for isoproterenol-treated suspension was 20°C. Similar results were obtained in two other experiments.

Unusual responses to DIDS

On a number of occasions we have noticed that the addition of DIDS led to an immediate stimulation of H^+ efflux (Fig. 7A) which then gradually declined to low values. Subsequent addition of isoproterenol caused only a small stimulation of H^+ efflux. The DIDS-induced H^+ efflux was completely blocked by amiloride (Fig. 7B), which suggests that it represents an existing efflux of H^+ *via* the Na^+/H^+ exchanger which is normally titrated by HCO_3^- movements *via* the anion exchanger. The small isoproterenol stimulation of H^+ efflux indicates that the existing Na^+/H^+ exchange, if this is indeed the mechanism, was not maximally stimulated. The DIDS-induced H^+ efflux was a stable property of washed cell suspensions and does not appear to be related to the transitory exposure of erythrocytes to catecholamines during the capture and bleeding of the fish or during *in vitro* exposure to agonist (see Fig. 5).

Seasonal dependence of Na^+/H^+ efflux activity

Fig. 8 shows the maximal Na^+/H^+ exchange capacity during the period March 1987 to February 1989. Measurements were routinely made at pH 7·3 and after 2 h incubation at 5°C in buffered saline (pH 7·6). The filled circles represent values for

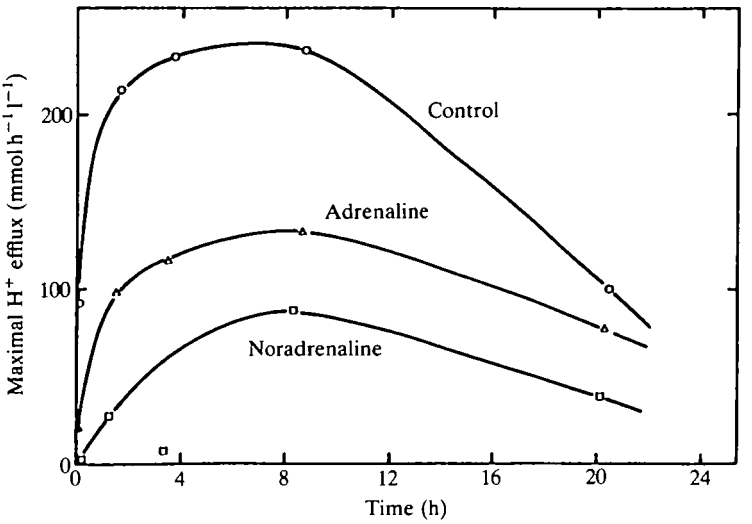

Fig. 5. The effects of exposure to maximally stimulatory concentrations of adrenaline and noradrenaline (10^{-5} mol l^{-1}) upon the subsequent recovery of transport capacity. Suspensions were incubated at 20°C for 1 h in the presence or absence (control) of agonist, washed three times with buffered trout saline and then incubated at 5°C. At the times indicated, samples were removed and the maximal agonist-stimulated efflux of H^+ equivalents determined with the pH-stat technique as described in the legend to Fig. 1. Similar results were obtained in two other experiments. Zero time as in Fig. 3.

fish obtained directly from the farm during the autumn and winter of 1987. The mean values varied considerably from day to day but were generally low, often with a large DIDS-induced H^+ efflux.

In contrast, fish which were held in the aquarium for in excess of 7 days yielded much higher and more consistent values (open circles, Fig. 8) with very little, if any, DIDS-induced H^+ effluxes. During the summer of 1987 consistently high values of 240–280 mmol h^{-1} l^{-1} pcv were observed with little variability between individuals, although in the late summer of 1988 the values had dropped to less than 200 mmol l^{-1} pcv. There was a gradual and progressive decline from November 1987 to March 1988 and during the autumn and winter of 1988, to a low value of approximately 100 mmol h^{-1} l^{-1} pcv. This was followed by rapid increases in exchange capacity during March–April in both 1987 and 1988 as well as during early February 1989. The sudden transition from low to high exchange capacities was recorded over a 2-week period in fish of batch 1 in 1987, in batch 4 in 1988 and in batch 7 in 1989. The increase was approximately 1 month earlier in batch 3, which had been obtained in the previous October and held in the laboratory for 2–3 months (line *A*, Fig. 8), than in batch 4, which was obtained in early March and held for a few weeks (line *B*, Fig. 8).

Fig. 8B shows the corresponding variation in water temperature of the inlet stream at the trout farm from which the fish were obtained. Temperature varied from a winter minimum of 1·5°C to a summer maximum of 16°C, with a typica

day-to-day variation of up to 2°C and a week-to-week variation up to 4°C. Water temperatures in the aquarium showed very small daily and weekly variations: the winter temperatures were typically 9–10°C and the summer temperatures

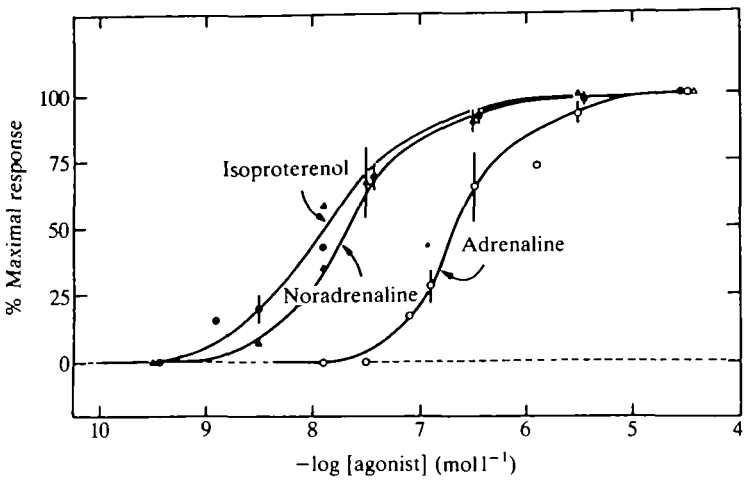

Fig. 6. The dose–response curves for the maximal efflux of H^+ equivalents from trout erythrocytes following addition of adrenaline (○), noradrenaline (△) and isoproterenol (●). DIDS was present at 5×10^{-4} mol l^{-1}. 100% was taken as the rate determined in the presence of 5×10^{-5} mol l^{-1} agonist and measurements recorded at lower agonist concentrations were normalized to this value. The symbols with vertical bars represent the mean ± standard error of the mean for three separate experiments and the symbols without bars were for single experiments. The curves were drawn by hand. The mean values for the concentration for half-maximal stimulation are given in the text.

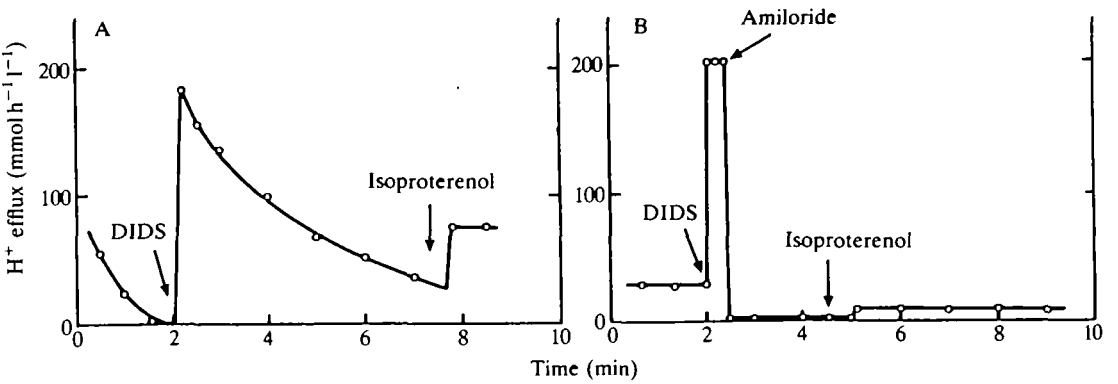

Fig. 7. (A) The time course of efflux of H^+ equivalents in suspensions of trout erythrocytes which exhibited a DIDS effect. The efflux was monitored as described in Fig. 1. DIDS (5×10^{-4} mol l^{-1}, final concentration) and isoproterenol (5×10^{-5} mol l^{-1}, final) were added as indicated. (B) The inhibitory effects of amiloride (5×10^{-4} mol l^{-1}, final concentration) upon the DIDS-induced efflux of H^+ equivalents.

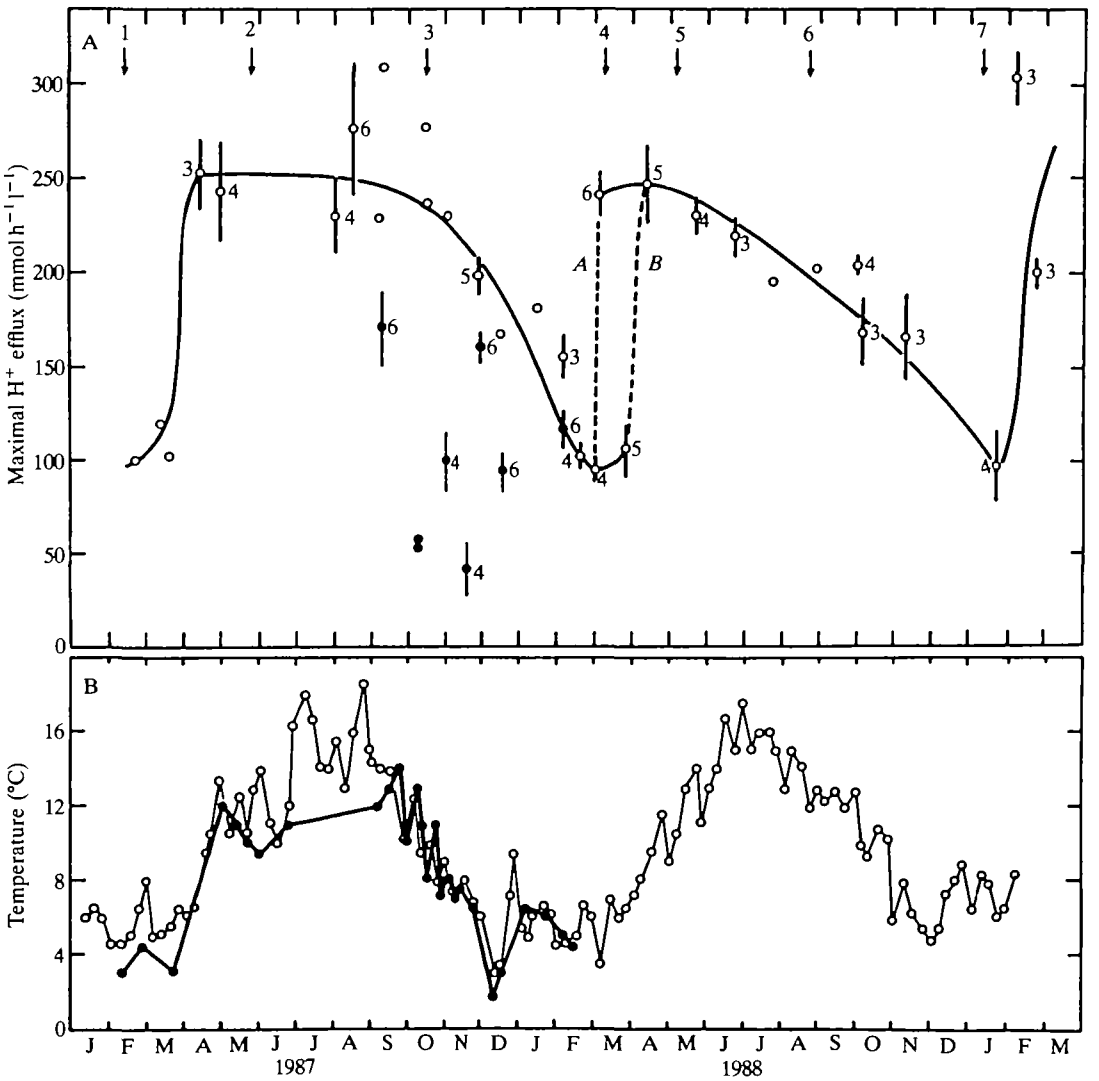

Fig. 8. (A) The seasonal rhythm of maximal transport capacity for isoproterenol-stimulated Na^+/H^+ exchange in trout erythrocytes. The filled circles represent values obtained for fish bled at the trout farm or immediately following transportation to the laboratory. The open circles represent values for fish acclimated in the laboratory for at least 7 days. The vertical bars indicate the standard error of the mean and the adjacent number shows the number of individual fish sampled. Symbols without error bars were for one individual only. The thick line was drawn by hand through the open circles and represents the seasonal variation. The arrows indicate the times at which fresh batches of trout were transferred from the farm to the laboratory. The time between this and the date of the assay therefore represents the period of acclimation. The spring increase in exchange capacity observed in 1988 was followed in two batches indicated by the two thick dashed curves marked *A* and *B*. (B) The seasonal variation of water temperature of the inlet stream at the trout farm from which the fish were obtained (filled circles and thick line) and at Manly Hall (open circles with thin line). The latter data were plotted at intervals of 7 days.

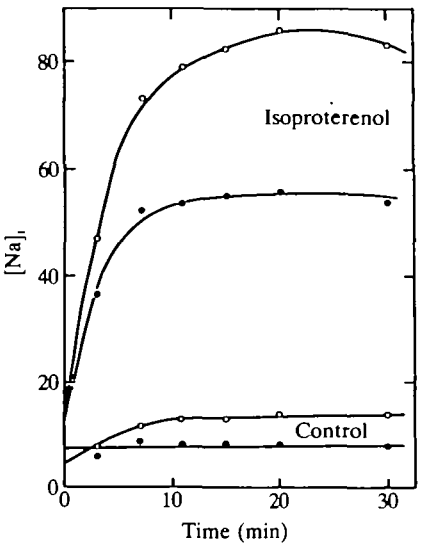

Fig. 9. The time course of increase in $[Na^+]_i$ following addition of isoproterenol (10^{-5} mol l^{-1}, final concentration) to erythrocytes with high (○) and low (●) transport capacities.

16–18 °C. The decline in transport capacity recorded during the autumn and winter of 1987–88 showed a similar time course to the gradual decline in water temperature at the trout farm. Moreover, the spring 1987 increase in transport capacity closely matched an increase in water temperature at the trout farm from 3 °C to 12 °C which occurred within a 5-week period. Fig. 8 also shows a more detailed record of seasonal temperature variations at a Hydrographic Station located approximately 7 km downstream from the tributary which fed the trout farm. Generally, the spring increase in exchange capacity coincided with the onset of the spring increase in water temperature at the Hydrographic Station. The earlier increase noted in spring 1989 may be related to the particularly mild winter experienced in the UK when water temperatures were 1·5–2 °C higher than in the previous winter.

The loss of transport capacity in the winter would be expected to reduce the maximal values of $[Na^+]_i$ following agonist stimulation, since this represents a steady state between the rate of Na^+ entry and active extrusion by the Na^+ pump. Fig. 9 illustrates the time course of changes in $[Na^+]_i$ after addition of isoproterenol in erythrocytes from two individuals with very different agonist-stimulated H^+ effluxes. Stimulation by isoproterenol induced a very rapid increase in $[Na^+]_i$ to a plateau of 85 mmol l^{-1} pcv in the erythrocyte suspension with a high transport capacity (170 mmol h^{-1} l^{-1} pcv) but to only 55 mmol l^{-1} pcv in a suspension of lower transport capacity (40 mmol h^{-1} l^{-1} pcv). The half-times for the increase were similar at 2·2 and 2·3 min, respectively.

Discussion

The pH-stat technique offers a major advantage as a means of determining the activity of the Na^+/H^+ antiporter compared to the more straightforward measurement of changes in intracellular Na^+ concentration, namely a much improved time resolution. This allows the lag phase, which may vary in length from 15 to 40 s, to be distinguished from the subsequent increase in rate of transport following addition of catecholamine and also gives better definition of the initial rate of H^+ transport, since this may only last 20–30 s before it slows down. Measurement of the initial rate following stimulation with maximal stimulatory doses of agonist, in cells with a large inwardly directed concentration gradient for Na^+ (i.e. low $[Na^+]_i$ and high $[Na^+]_e$), thus provides a viable measure of the transport capacity of the antiporter. These conditions are met after 2 h incubation of washed erythrocytes at 5°C when $[Na^+]_i$ approaches 10 mmol h^{-1} l^{-1} pcv. Interestingly, the pH-stat technique gave no indication of irregular pulses of proton ejections during the phase of declining efflux to match the pulses of Na^+ influx described by Borgese *et al.* (1987*a*). Perhaps the major disadvantage of the technique is that pH of the external medium is influenced not only by the Na^+/H^+ antiporter but also by other processes which lead to the movement of H^+, OH^- or HCO_3^- between the cell and the medium, and it is necessary to ensure that these interfering processes are minimized. This is largely achieved by adding DIDS to block the fast anion transporter (Cossins & Richardson, 1985; Romano & Passow, 1984) although this leads to a more transient efflux of H^+ than in the absence of DIDS (Baroin *et al.* 1984).

The application of the pH-stat technique to the determination of capacities of erythrocytic Na^+/H^+ exchange has allowed a number of interesting features of the system to be clarified. The exchange mechanism appears to be rather labile since incubation of washed erythrocyte suspensions at 20°C leads to a rapid loss of activity. Incubation at low temperatures maintained exchange activity at high levels for 24 h and occasionally for up to 36 h. The rapid loss of transport activity observed following bleeding may represent the effects of the rapid desensitization of the antiporter by external Na^+ described recently by Garcia-Romeu *et al.* (1988). The isoproterenol-induced efflux of H^+ subsequently recovered and high transport activities were recorded within 2 h of bleeding. However, *in vitro* exposure to high doses of catecholamines did lead to an irreversible loss of transport capacity which was quite distinct from the transient desensitization which occurred during and immediately after stimulation. The implication of this sustained loss of exchange capacity is that continued exposure of erythrocytes to catecholamines may lead to attenuated adrenergic responses just as prolonged exposure of turkey erythrocytes to catecholamines leads to attenuated responses of the receptor-linked adenyl cyclase (Leftkowitz *et al.* 1983). This may account for the low and more variable exchange activities recorded in erythrocytes of fish from the trout farm. The induction of adrenergic responses as a result of bleeding may also lead to a loss of transport capacity though, judging from the relatively small increase in $[Na^+]_i$ in red cells immediately after bleeding, it seems likely that much

smaller doses of catecholamine are involved over a much shorter period than during the *in vitro* experiment and that the resulting loss of exchange capacity is small.

The appearance of DIDS-induced H^+ efflux and the low isoproterenol-stimulated activity in trout-farm fish often corresponded with stressful episodes at the fish farm, in the laboratory or during transportation; for example, a violent rain storm or a 5°C reduction in water temperature at the trout farm. However, blood samples obtained at the farm and immediately washed into saline also displayed the effect, suggesting that it was not simply induced by the stress of transportation of fish to the laboratory. Acute stress administered in the laboratory (deep hypoxia and sustained handling for 1·5 h) to otherwise undisturbed fish did not consistently lead to DIDS-induced H^+ efflux. It therefore appears that the effect is induced by extended periods of stress or discomfort experienced by fish at the farm or in the aquarium. The mechanism underlying the DIDS-induced efflux of H^+ is not clear, but the inhibitory effects of amiloride indicate that the Na^+/H^+ exchanger is operative in the absence of adrenergic stimulation.

We describe here a seasonal rhythm of Na^+/H^+ transport capacity which has been recorded over a period of 24 months. The spring recovery has been separately recorded in four batches of fish over three cycles and the low values in February–March observed in four batches of fish. Seasonal variations in adrenergic responsiveness have been noted in other systems, notably in the gills (Peyraud-Waitzenegger *et al.* 1980) and heart of the eel (Pennec & Peyraud, 1983) as well as in the vasculature of the rainbow trout (Part *et al.* 1982). Nikinmaa & Jensen (1986) found a complete absence of responsiveness in erythrocytes of the trout during the winter months in Finland, as judged by the lack of cell swelling or shift in pHi following adrenergic stimulation. Tetens *et al.* (1988) found that adrenoceptor affinities for adrenaline and noradrenaline were largely unaffected by season. Although they obtained a clear-cut adrenergic response in winter fish it was not quantitatively compared to that in the summer response. The distinction between the attenuated winter responses in the present study and the apparently complete absence of responses in the Finnish fish is probably not due to the different techniques employed, but may be related to the more extended and more extreme conditions that occur during the winter in Finland.

The water temperature in the aquarium was significantly less extreme and variable than at the trout farm and it is reasonable to suggest that these conditions were responsible for the earlier increase in exchange capacity observed in fish held in the aquarium for the previous 3 months (Fig. 8A, curve *A*). The importance of temperature experience of the fish is also suggested by the early spring increase in the particularly mild winter and spring of 1988–89. Nevertheless, the increase in transport capacity during spring 1988 in batch 3 occurred some months after the fish had been transferred from the colder waters of the trout farm (4°C) to the warmer water in the aquarium (10°C), suggesting that water temperature was not the only important influence. This lag may indicate that photoperiod is also

involved in the regulation of the spring increase in transport capacity, perhaps by interacting with temperature.

This demonstration of a seasonal rhythm of exchange capacity raises a number of questions. For example, it is not clear from the present studies why the exchange capacity becomes reduced during the winter months; that is, whether there is a change in affinity of the β-adrenergic receptor, a change in the intracellular signalling apparatus or perhaps a decline in the number of exchange sites or their turnover number. In view of the use of maximally saturating doses of agonist in this study and the observations of Tetens *et al.* (1988) regarding adrenoceptor affinities during the winter and summer, it seems that the first possibility is unlikely. The high $[Na^+]_i$ following adrenergic stimulation largely represents a balance between the rate of Na^+ entry *via* the exchange mechanism and the rate of Na^+ efflux *via* the Na^+ pump. Cold-acclimation of trout does lead to a small increase in erythrocyte Na^+ pump capacity (Raynard, 1988) so it seems likely that the lower steady-state $[Na^+]_i$ of stimulated erythrocytes in winter fish represents an even greater reduction in Na^+/H^+ exchange capacity.

Another possibility is that erythropoiesis effectively stops during the winter months so that reticulocytes are not being added to the erythrocyte population. The age structure of the population would then progressively increase so that the cells would display properties more characteristic of fully matured or aged erythrocytes (Lane *et al.* 1982). The spring increase may be caused by the liberation into the circulation of large numbers of immature erythrocytes. Provided the transport properties of the cells alter with maturity or age then blockage of erythropoiesis will affect the transport capacity of the circulating population of erythrocytes. To this end, R. S. Raynard, R. V. Kilbey & A. R. Cossins (in preparation) have recently shown that the Na^+/H^+ exchange capacity does vary with the buoyant density of trout erythrocytes in a Percoll density gradient. Since density is also closely related to the proportion of immature erythroid cells (R. S. Raynard, R. V Kilbey & A. R. Cossins, in preparation) this suggests that transport capacity is, indeed, related to cell maturity; increasing during maturation of reticulocytes and declining during ageing. This appears to be a specific property of the Na^+/H^+ exchange mechanism since Raynard (1988) failed to demonstrate any consistent relationship between density and the transport activity of the ouabain-sensitive Na^+ pump.

To our knowledge, seasonal variations in erythropoiesis in trout have not been demonstrated directly, but a number of studies have observed seasonal variations in haematology of rainbow trout. For example, Haider (1970) showed a gradual reduction in haematocrit from the beginning of the year to the end. Lane (1979) showed a gradual decline in haematocrit and erythrocyte number in rainbow trout from October to March. Denton & Yousef (1975) found that haematocrit was usually at the high value of 52–57 % except during March, when it fell to 35 %. DeWilde & Houston (1967) found that haematocrit increased during the autumn and winter months but fell during summer. Pickering (1986) also observed marked variations in erythrocyte numbers of brown trout during the winter months, with

sexually mature males showing a consistent increase in the number of circulating erythrocytes early in the spawning period (November to early January) followed by a decline.

The role of temperature and erythropoiesis in these seasonal rhythms is difficult to establish, although there is good circumstantial evidence for an important effect. Thus, Chudzik & Houston (1983) have shown that erythropoiesis in goldfish which have suffered a phenylhydrazine-induced anaemia was prevented at 7·5°C. Exposure of animals to graded increases in temperature resulted in increases in haematocrit (Houston *et al.* 1988). However, the rhythm of haematocrit variations described by Denton & Yousef (1975) were obtained in a stock held throughout the year at 11–13°C, so that temperature variations *per se* may not be necessary for expression of the rhythm. In any case, it is difficult to separate temperature from other influential factors which covary with temperature, including the stocking density of the fish, hypoxia, appetite, starvation, condition and photoperiod. Reproductive rhythms may be of major importance in this respect and differences in the spawning period of different stocks of trout may contribute to the different patterns of seasonal variation in haematocrit just described (Pickering, 1986).

Finally, the consequences of a reduction in transport capacity for the functional role of the adrenergic response of trout erythrocytes in the aetiology of stress remains to be established. In principle, a reduction in the rate constant of Na^+/H^+ exchange should reduce the half-time of the increase in $[Na^+]_i$ as well as the resulting steady-state $[Na^+]_i$. Because of the linkage between the transport of Na^+ and H^+, the net transport of H^+ would be reduced, leading to a smaller adrenergically stimulated increase in pHi (Cossins & Richardson, 1985). The precise functional significance of adrenergic stimulation of the exchanger is not clear, although Nikinmaa (1983) and Cossins & Richardson (1985) have suggested it influences the oxygen-binding affinity and capacity of haemoglobin. Whatever turns out to be the functional role, the obvious inference is that the erythrocytes of winter fish are less responsive than erythrocytes of summer fish.

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