ON THE INSTABILITY OF K+ INFLUX IN ERYTHROCYTES OF THE RAINBOW TROUT, *SALMO GAIRDNERI*, AND THE ROLE OF CATECHOLAMINE HORMONES IN MAINTAINING *IN VIVO* INFLUX ACTIVITY

BY P. K. BOURNE AND A. R. COSSINS

*Department of Zoology, University of Liverpool, P.O. Box 147,
Liverpool L69 3BX*

SUMMARY

The rate of K+ influx in washed trout erythrocytes was not stable and declined by 60% over a 6 h period, but decreased only slowly thereafter. During this initial period the cells shrank, although $[K^+]_i$ was maintained constant. Both ouabain-sensitive and furosemide-sensitive K+ influx were reduced by approximately equal amounts.

Extensive modification of the saline composition produced no diminution of the loss of K+ influx activity, making it unlikely that the response was due to deficiencies in saline composition.

The rate of K+ influx in unwashed cells was affected only slightly by noradrenaline and adrenaline. By contrast, K+ influx in cells which had been washed and incubated overnight before assay was stimulated 100–250% by these hormones. Half-maximal stimulation was at 2×10^{-6} and 8×10^{-6} M respectively. Replacement of plasma by a saline containing 5×10^{-6} M adrenaline and noradrenaline resulted in high and stable K+ influx rates over a 6 h period.

These results support the hypothesis that K+ influx in erythrocytes of freshly drawn blood is high as a result of stimulation by catecholamine hormones. Removal of the normal hormonal milieu, by washing of the cells, results in the rapid loss of K+ influx activity.

INTRODUCTION

Mammalian erythrocytes have proved to be a valuable source of information concerning the ability of cells to transport organic and inorganic material across cellular membranes, and the techniques used in such studies are comparatively well developed (Ellory & Lew, 1977; Lew & Beaugé, 1979; Sarkadi & Tosteson, 1979). A major assumption is that transport properties of isolated cells are stable for an extended period of time and remain so when the cells are washed and resuspended in a physiological saline. This appears to be the case in mammalian erythrocytes, but is rarely demonstrated in the literature.

In our preliminary studies of ion transport in erythrocytes from fish we noted that

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the K^+ influx activity of washed cells declined progressively over a 4–5 h period. This inconvenient property severely impeded our efforts to study the *in vivo* adaptation of erythrocyte function to environmental variations in temperature and salinity. Consequently, we have examined the loss of K^+ influx activity of washed cells in some detail to determine its cause and to develop possible ways of prevention. We have found that the loss of K^+ influx activity in washed cells was not related to deficiencies in the resuspension medium but to the removal of a plasma-borne factor which stimulated K^+ influx activity *in vivo*. We present evidence that this factor is a catecholamine.

MATERIALS AND METHODS

Animals

Rainbow trout (*Salmo gairdneri*, 0·25–0·75 kg) were obtained from a commercial source and were maintained in large freshwater aquaria. Water temperature varied between 8 and 14°C during the experimental period, and photoperiod was maintained constant at 16L:8D. Fish were fed twice daily to excess with commercial trout food (B.P. Nutrition Ltd).

Chemicals

Inorganic compounds, D-glucose, D-sucrose, trichloroacetic acid, Tris-HCl* and MOPS were obtained from BDH Chemicals Ltd (Poole, Dorset) and were analytical reagent grade. D-Fructose, D-erythrose, D-ribose, inosine, HEPES, Triton X-100, bovine serum albumin (fraction V), ouabain, L-epinephrine hydrochloride (adrenaline), arterenol hydrochloride (noradrenaline) were obtained from Sigma Chemicals Ltd (Poole, Dorset). Imidazole was obtained from Koch–Light Laboratories (Sharnworth, Beds.) and furosemide was donated by Hoechst Pharmaceuticals (U.K.) Ltd. ^{86}RbCl and [^{3}H]inulin were obtained from Amersham International (U.K.), Ltd.

Blood removal and preparation

Fish were stunned by a sharp blow to the head and blood was removed from the caudal vein by hypodermic syringe (21 gauge). Clotting was prevented by gently shaking the blood in a heparinized blood-tube. Samples from an individual fish were pooled and stored on ice. Plasma osmolarity was measured using a freezing-point depression osmometer (Advanced Instruments, Massachusetts, U.S.A.) and the osmolarity of the trout saline was adjusted to that of the plasma by mixing appropriate volumes of salines containing 160 mM-NaCl and 120 mM-NaCl. (Other saline constituents: 6 mM-KCl; 5 mM glucose; 15 mM imidazole-HCl, pH 7·60 at 20 °C.)

Cells were washed at least four times by centrifugation (5 min at 1000 g) and resuspension with isosmotic saline. The supernatant and buffy coat were discarded. Erythrocytes were finally suspended in isosmotic saline at a similar haematocrit (packed cell volume) to the original blood sample.

* Abbreviations: Tris-HCl = [2-amino-2-(hydroxyethyl)propane-1, 3 diol (tris)], MOPS = 3-(N-morpholino) propanesulphonic acid, HEPES = (N-2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid, ATP = Adenosine-5'-triphosphate.

Measurement of K+ influx rates

Unidirectional K+ influx was determined from the uptake of ⁸⁶Rb. ⁸⁶Rb serves as a faithful analogue of K+ fluxes in most cell types (Beaugé & Adragna, 1971; Mills & Tupper, 1975) and this is assumed in the present study.

Aliquots (50 μl) of blood or washed cell suspension were added to reaction tubes containing 0·9 ml of isotonic trout saline pre-equilibrated to 10 ± 0·1 °C. The final reaction haematocrit was 2–5 %. After exactly 10 min, the reaction was started by adding 50 μl ⁸⁶Rb+ in isotonic saline (final specific activity of 0·5 μCi ml⁻¹). During incubation all reaction tubes were agitated at intervals. The reaction was stopped by rapidly washing the cells in ice-cold isotonic $MgCl_2$ solution (100–120 mM-$MgCl_2$, 10 mM Tris-HCl, pH 7·60 at 20 °C) using an Eppendorf micro-centrifuge (5 s at 10 000 g). Cells were washed 4 times. The final cell pellet was lysed with 0·5 ml 0·05 % (v/v) Triton X-100 and the protein precipitated with 0·5 ml 5 % (w/v) tri-chloroacetic acid. Protein was removed by centrifugation (2 min at 10 000 g) and the radioactivity of the supernatant determined by Cérenkov counting. Components of the K+ influx were estimated by including ouabain and/or furosemide in the reaction tube at their maximal inhibitory concentrations of 0·1 and 1·0 mM (final), respectively. All influx determinations were duplicated. Ouabain-sensitive and furosemide-sensitive activity was calculated by subtracting the influx rate determined in the presence of inhibitor from that determined in its absence. Rates of K+ influx were calculated on the basis of haematocrit and are presented as mmol.l pcv⁻¹ (packed cell volume). Haematocrit was determined at intervals throughout experiments using a Gelman–Hawksley microhaematocrit centrifuge (8 min at 10 000 g). Experiments with [³H]-inulin demonstrated that approximately 4–5 % of the packed cell volume was extra-cellular fluid.

Influx rates were determined from two time points, the rate being linear, or nearly so, during this period.

Determination of cellular cation concentration

Aliquots (50 μl) of blood or washed cells were rapidly washed four times with ice-cold isotonic $MgCl_2$ solution. The cell pellet was lysed with 0·5 ml 0·05 % (v/v) Triton X-100 solution. Protein was precipitated with 0·5 ml 5 % (w/v) trichloro-acetic acid and the precipitate removed by centrifugation (2 min at 10 000 g). Super-natant concentrations of Na+ and K+ were determined by flame spectrophotometry.

RESULTS

Loss of K+ influx activity in washed erythrocytes

The rate of K+ influx of washed rainbow trout erythrocytes showed a progressive reduction with time (Fig. 1). The rate of loss of K+ influx activity was rapid over the first 2–3 h of incubation but plateaued after 5–6 h of incubation at approximately 30–40 % of its original activity. By contrast, the rate of K+ influx of erythrocytes in blood remained constant over a 6 h period.

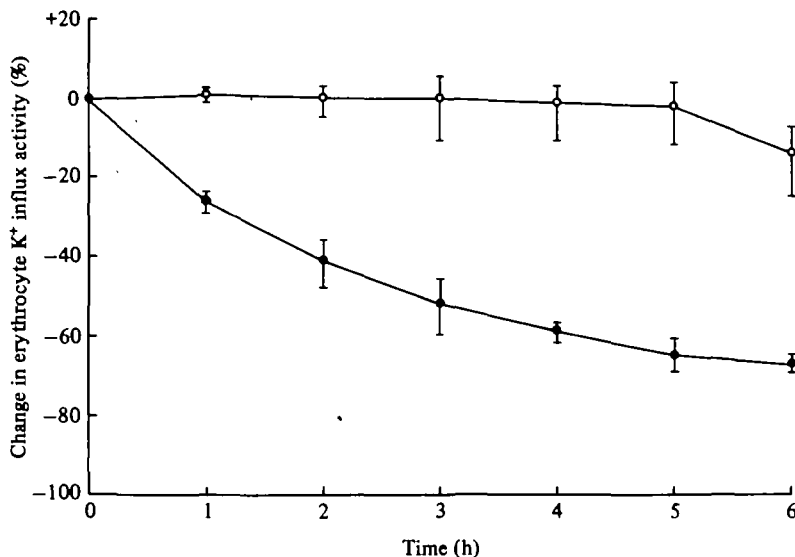The loss of K+ influx activity in washed cells was associated with a shrinkage of the

Fig. 1. The change of K$^+$ influx in rainbow-trout erythrocytes. O, erythrocytes added to reaction tubes as blood (i.e. unwashed cells); ●, erythrocytes were washed four times and resuspended in isosmotic trout saline. Each data point represents the mean of eight determinations and the vertical bars represent the 95 % confidence interval for percentage data (Zar, 1974).

cells by approximately 4–5% over the 6 h period (Fig. 2a). During this time the internal concentrations of K$^+$ and Na$^+$ remained constant (Fig. 2b) at approximately 68 mmol.1 pcv^{-1} ($n = 8$) and 13 mmol.1 pcv^{-1} ($n = 8$), respectively. The cation concentration of erythrocytes in blood also remained constant over the 6 h period at the slightly higher levels of 78 and 20 mmol.1 pcv^{-1} ($n = 10$), respectively. Erythrocytes in blood also shrank by approximately 7–8% over a 6 h incubation period.

In Fig. 3 we have compared the effects of plasma dilution on K$^+$ influx activity with the effects of extensive cell washing followed by resuspension in saline/plasma media. In the former case (Fig. 3a), the loss of activity was small even when the plasma was diluted to 5%. By contrast, the loss of K$^+$ influx activity was relatively large in cells that had been washed but resuspended at equivalent plasma dilutions (Fig. 3b). Thus the process of washing is a necessary factor in the loss of K$^+$ influx activity.

Modification to trout salines

Originally it was felt that the dramatic loss of K$^+$ influx activity was due to some omission or imbalance in the composition of the trout saline used for washing the erythrocytes and as the reaction media. Consequently, we have carefully modified the composition of the saline in the following ways and repeated the experiment shown in Fig. 1.

(i) Substrate: D-glucose, D-sucrose, D-erythrose, D-ribose, D-fructose and inosine were included at 5 and 10 mM.

(ii) Ca^{2+} (1, 2 and 5 mM), phosphate (2 and 5 mM) and Mg^{2+} (1 and 2 mM).

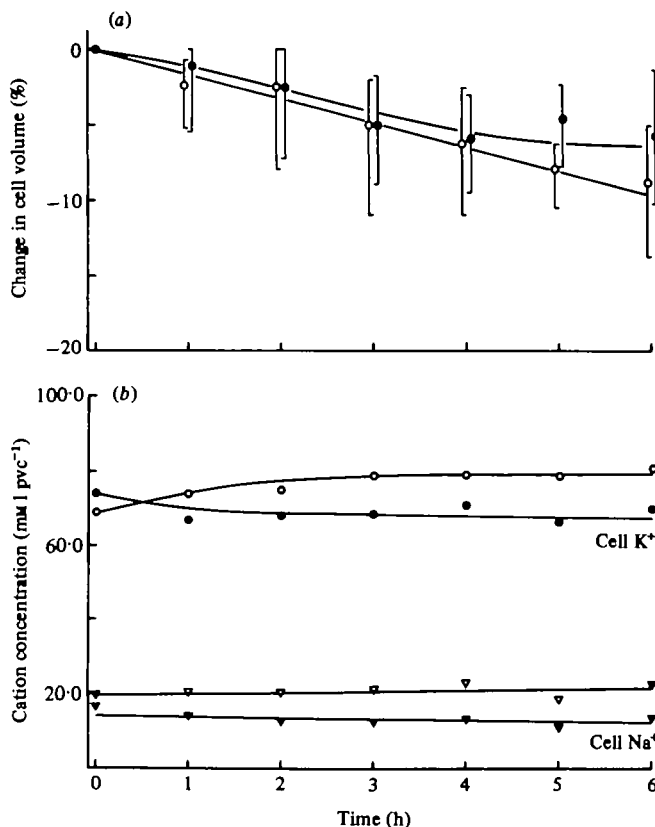(iii) Various buffer constituents: Tris-HCl; imidazole; HEPES; MOPS; and bicarbonate (at 10 and 20 mM).

Fig. 2. The effects of incubation of rainbow-trout erythrocytes. (*a*) The change in cell volume (i.e. haematocrit) with time; ○, blood; ●, washed cells. Mean ± 95 % c.i. (*b*) The change in cell $[K^+]_i$ and $[Na^+]_i$ with time; ○, ▽, blood; ●,▼, washed cells. Mean ± s.e.m. (the s.e.m. falls within the symbol for all values recorded).

(iv) Bovine serum albumin and polyvinylpyrrolidone were included at 1 % and 2 % (w/v) to provide colloid osmotic pressure.

(v) Blood and washed erythrocytes were pre-equilibrated with pure nitrogen or oxygen to examine the effect of oxygenation on K^+ influx activity.

In no case was there any significant or consistent reduction in the loss of K^+ influx activity of washed erythrocytes. It was, therefore, concluded that the loss of activity was not due to deficiencies in saline composition.

Effect of catecholamine hormones on K^+ influx activity

A number of workers have noted that ion transport in erythrocytes of various species is sensitive to the presence of hormones, notably noradrenaline (Furukawa, Bilezikian & Loeb, 1980; Gardner *et al.* 1973; Kregenow, 1971, 1973; Schmidt & McManus, 1977). If trout erythrocytes are sensitive to circulating hormones and substantial plasma concentrations of these hormones exist in trout, then their removal by washing might cause a further change in ion transport properties. If such is the case, then washed and incubated erythrocytes should firstly display an enhanced ■nsitivity of K^+ influx kinetics to the inclusion of the hormone in the influx medium,
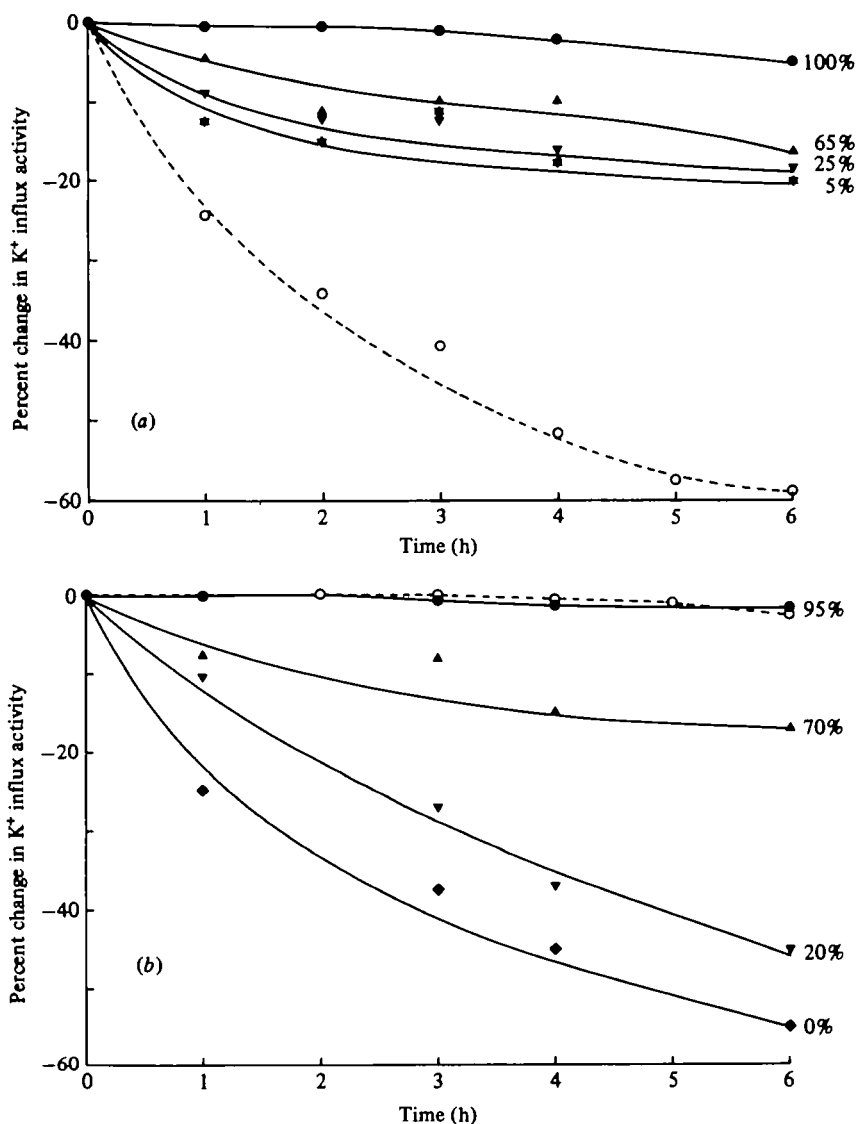
Fig. 3. A comparison of the effects of (a) plasma dilution and (b) washing followed by plasma supplementation upon the loss of K^+ influx activity with time. In (a) the blood plasma was replaced with equivalent volumes of saline. Values are expressed as percent plasma remaining in the sample (●, 100%, ▲, 65%, ▼, 25%, ★, 5%; ○, control cells washed four times and resuspended in isoosmotic saline). In (b) erythrocytes were washed four times and then resuspended in trout saline containing various concentrations (v/v) of supplemented plasma. Again values expressed as percent plasma in sample (●, 95%; ▲, 70%, ▼, 20%; ◆, 0%; ○, blood, unwashed cells).

and secondly the rate of loss of K^+ influx activity in washed cells should be attenuated by inclusion of the hormone in the trout saline during incubation.

The dose–response curves for adrenaline and noradrenaline on K^+ influx activity are illustrated in Fig. 4(a) and (b), for washed and unwashed erythrocytes, respectively. The K^+ influx activity of blood was increased at most by only 20–35% at hormone concentrations up to 10^{-3} M. By contrast, the K^+ influx activity of trout erythrocytes
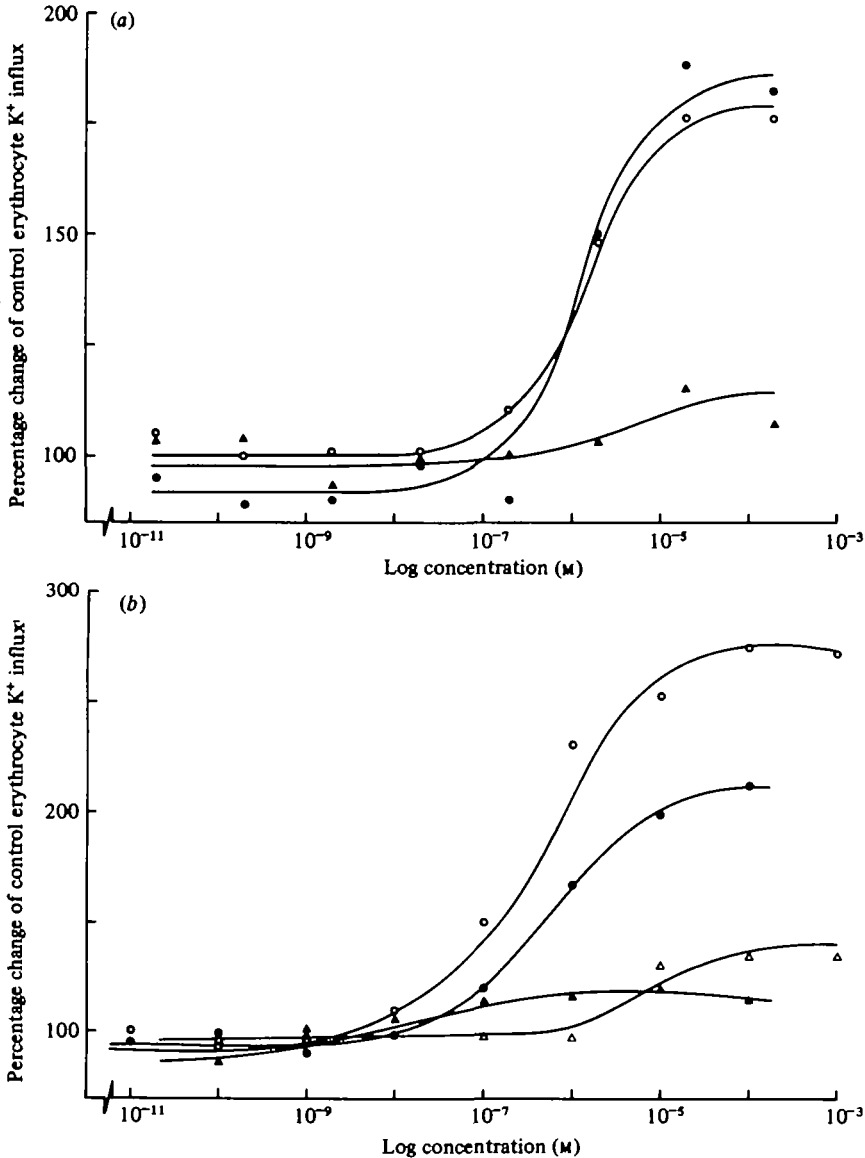
Fig. 4. The dose–response curves for adrenaline (*a*) and noradrenaline (*b*) on the rate of K^+ influx in trout and carp erythrocytes. Erythrocytes were added to a reaction medium containing hormone either as blood (▲, carp; △, trout) or as washed cells in isosmotic saline (●, carp; ○, trout). Both had been incubated overnight at 5 °C prior to influx measurement. Values represented are the increase of K^+ influx in the presence of hormone to that in its absence (control). Hormones were prepared and diluted in deoxygenated salines to prevent degradation.

that had been washed and incubated in isosmotic saline for 12–15 h was stimulated by 160% for adrenaline and 280% for noradrenaline. The concentration for half-maximal stimulation was 8×10^{-7} and 2×10^{-6} M, respectively. Fig. 4 also illustrates identical experiments performed on carp erythrocytes (*Cyprinus carpio*) with essentially the same results.

Trout erythrocytes were washed and resuspended in saline containing adrenaline and noradrenaline at two concentrations, 5×10^{-6} and 5×10^{-8} M, which correspond approximately to the *in vivo* range of concentrations observed in rainbow trout (see Discussion). The change in K^+ influx activity was then monitored over a 6 h period. The presence of either hormone at 5×10^{-6} M resulted in only minor reductions in K^+ influx activity which were essentially identical to the slight loss of activity observed with unwashed erythrocytes. The presence of either hormone at 5×10^{-8} M, however, resulted in a reduction in activity by 60%, comparable to that found with cells that had been washed and resuspended in a trout saline lacking hormone.

The stimulation of K^+ influx by noradrenaline occurred within 10 min (Fig. 6) and remained constant for at least 6 h (Figs. 5(*b*), 6). The K^+ influx activity that was insensitive to both ouabain and furosemide-inhibition was unaffected by noradrenaline stimulation (Fig. 6). Of the remaining influx activity in unstimulated erythrocytes, 38% was ouabain-sensitive and 58% was furosemide-sensitive. In noradrenaline-stimulated cells these values became 42% and 53%, respectively (Fig. 6).

DISCUSSION

The changes in cation transport properties of washed trout erythrocytes were characterized by a progressive shrinkage together with a substantial loss of K^+ influx activity. The constancy of the internal K^+ concentration during this period suggests that these time-dependent effects form part of a controlled cellular response to the washing procedure, rather than resulting from a general loss of viability. In a negative way, our failure to maintain K^+ influx activity at a constant level in washed cells by exhaustive tests with modified trout salines supports this view.

Freshly isolated erythrocytes were also observed to shrink during incubation over a 6 h period, although in this case K^+ influx activity was maintained constant. Fish erythrocytes appear to swell or shrink by up to 30%, depending upon their state of oxygenation both *in vitro* and *in vivo* (Soivio, Westman & Nyholm, 1974; Soivio & Nikinmaa, 1981). It seems likely that the frequent mixing of blood prior to each K^+ influx assay increased its oxygen content sufficiently to cause a general cellular shrinkage. These observations have practical importance not only for the condition of erythrocytes isolated from anoxic fish and for the need to control the state of oxygenation of erythrocytes *in vitro*, but also for the use of blood haemoglobin content as a measure of packed cell volume. Clearly this relationship changes progressively with time in a manner that depends upon the treatment of the sample. For this reason we have routinely monitored the haematocrit of blood and erythrocyte suspensions throughout each experiment and have expressd K^+ influx rates accordingly.

The idea that the changes in cell transport properties result from the withdrawal of hormone stimulation is supported, firstly, by the great sensitivity of washed, stabilized erythrocytes to catecholamine hormones. Unwashed cells by contrast were only slightly sensitive in similar experiments. Secondly, the effects of high concentrations of catecholamines on K^+ influx activity were of the same magnitude as the loss of activity that occurs in washed cells. Thirdly, the presence of catecholamine
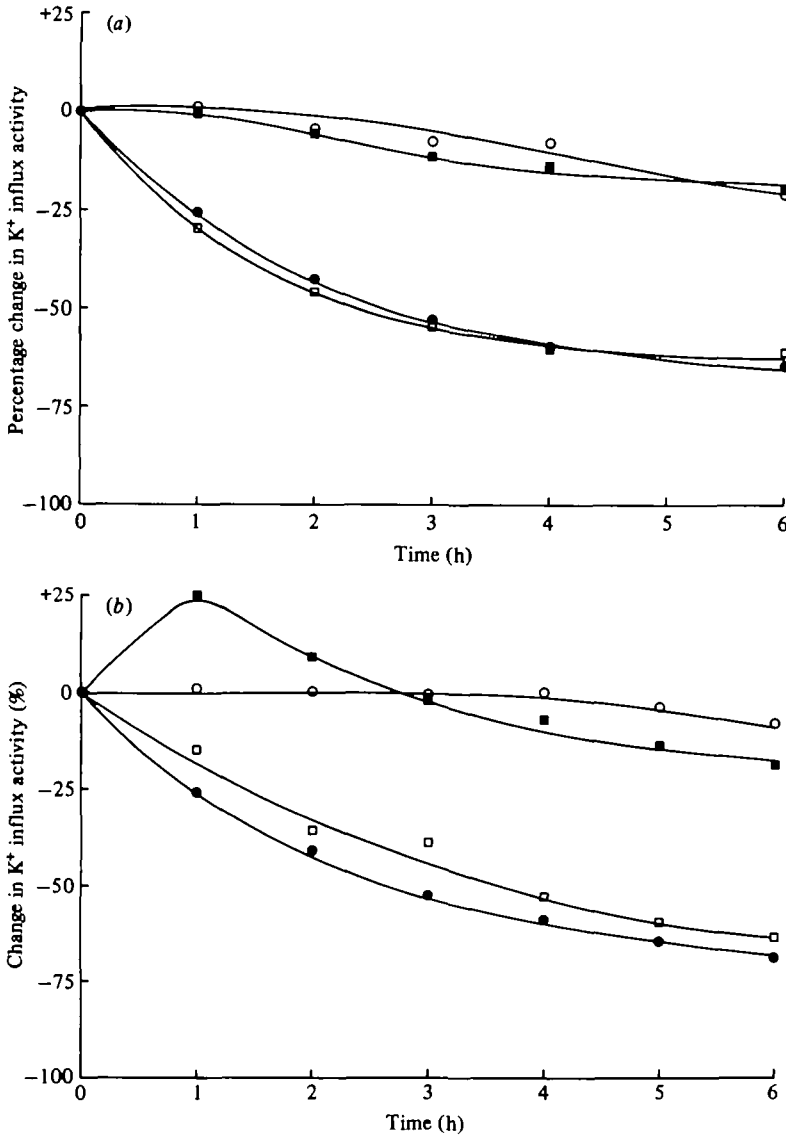
Fig. 5. The protection of K⁺ influx activity in washed trout erythrocytes by adrenaline (*a*) and noradrenaline (*b*). Cells were washed with isosmotic trout saline and incubated in saline with either 5×10^{-4} M hormone (■), 5×10^{-8} M hormone (□), or in the absence of hormone (●). In one series, cells were added directly as blood to the reaction tubes containing no hormone (○).

hormones at 5×10^{-6} M in the resuspension medium resulted in some preservation of K⁺ influx activity of washed cells. Finally, the changes observed in washed trout erythrocytes are entirely consistent with the known effects of adrenaline and nor-adrenaline on volume regulation and cation transport in avian erythrocytes. Thus Riddick, Kregenow & Orloff (1971) have also noted a similar shrinkage of washed duck erythrocytes which could be prevented by the incorporation of noradrenaline (5×10^{-6} M) in the resuspension medium.
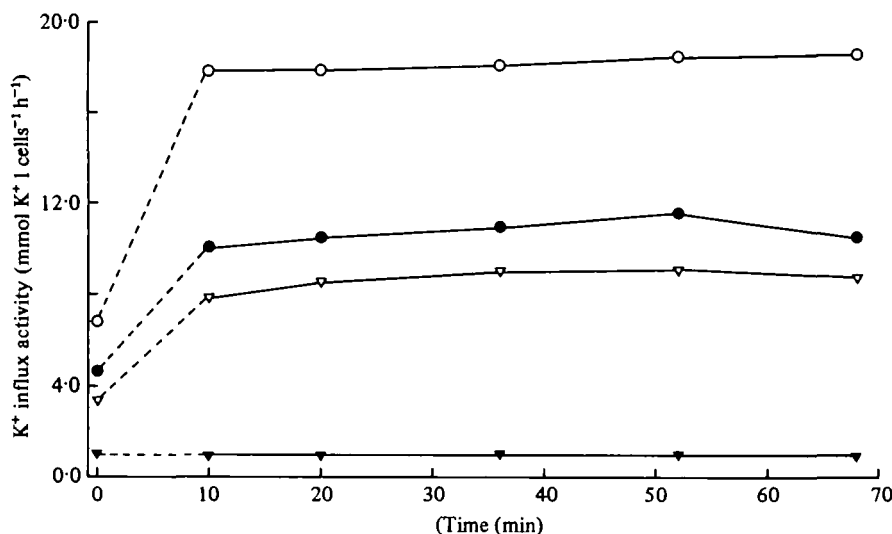
Fig. 6. The time-course of noradrenaline-stimulation of K^+ influx activity in trout erythrocytes. Cells were washed in trout saline and incubated overnight at 5 °C. The concentration of noradrenaline was 5×10^{-6} M. ○, Control influx; ●, ouabain-resistant influx (0·1 mM final); ▽, furosemide-resistant influx (1·0 mM final); ▼, ouabain and furosemide combined-resistant influx.

The fact that freshly-drawn trout and avian blood is under the influence of catecholamine hormones has important implications for their use in studies of volume regulation and cation transport. Firstly, it is not possible to use freshly washed erythrocytes in experiments that take some time to complete, since the rate of K^+ influx is not stable. Secondly, one might expect that the normal volume regulatory and cation transport properties would be perturbed or overridden by the stimulatory effects of catecholamines. In any event, these properties probably would not reflect the normal *in vivo* condition of the erythrocytes. In other studies we have observed only a slight effect of cell volume on furosemide-sensitive K^+ influx in whole blood, but much larger effects in cells which had been washed and incubated in trout saline for 12–15 h (P. K. Bourne & A. R. Cossins, unpublished observations).

Two important and related questions follow from these observations. Firstly, what is the physiological and adaptive significance of the catecholamine-stimulated cation transport and erythrocyte swelling? It is not clear whether the significance of the response is through the regulation of cellular cation content or of cell volume, the regulation of plasma cation concentration (Rudolf & Lefkowitz, 1978), or the adjustment of some haemodynamic properties of the blood. Another possibility is that cellular swelling reduces the cellular concentration of ATP, which in addition to its normal role in cellular energy metabolism, is the principal organic phosphate modulator of haemoglobin–oxygen affinity in fish. ATP affects the oxygen-binding affinity of haemoglobin by two mechanisms: a specific binding to the haemoglobin molecule and by influencing the intracellular pH. The decrease in ATP concentration during swelling may therefore serve to increase the oxygen affinity of haemoglobin (Soivio & Nikinmaa, 1981).

The second question concerns the status of the catecholamine-stimulated K^+

Influx in undisturbed or unstressed animals. Very low concentrations of adrenaline (3×10^{-9} M) and noradrenaline (2×10^{-9} M) have been recorded in rainbow trout kept in small, darkened boxes and sampled by means of an aortic cannula (Ristori, Rehm & Laurent, 1979). During stress this concentration may increase up to $0.5 - 1.5 \times 10^{-6}$ M (reviewed by Mazeaud & Mazeaud, 1981). It appears, therefore, that the *in vivo* variations in catecholamine concentrations in rainbow trout are quite sufficient to elicit the responses observed in these present studies. In trout, large amounts of noradrenaline are released into the blood stream within a few minutes of the fish being subjected to stress (Nakano & Tomlinson, 1967) and we have found the catecholamine stimulation of K+ influx to be very rapid (< 10 min).

A pertinent observation in this respect is that of DeVries & Ellory (1982), who obtained blood by caudal vien cannulation of two Antarctic fish species, *Dissostycus mawsoni* and *Notothenia angustata*. The rate of ouabain-inhibited K+ influx was stimulated by approximately 300–400% in blood obtained after a period of forced activity and excitement compared to blood withdrawn from the quiescent animal. This observation indicates that catecholamine stimulation occurs during stressful occasions *in vivo* and points to some role of the response in the aetiology of stress.

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