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SODIUM AND POTASSIUM TRANSPORT IN TROUT (*SALMO GAIRDNERI*) ERYTHROCYTES

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SUMMARY

1. The principal pathways of Na^+ and K^+ transport in trout erythrocytes have been characterized.

2. Approximately 50 % of K^+ influx in steady-state erythrocytes was inhibited by ouabain (1 mM) and 46 % by furosemide (1 mM). Furosemide-sensitive K^+ influx was a saturable function of external K^+ concentration with a K_m of 25 mM. This flux component was also inhibited by SITS (4-acetamido-4′-isothiocyanatostilbene-2′2-disulphonate) (concentration required for 50 % inhibition, $I_{50} = 7{\cdot}6 \times 10^{-6}$ M) and by the removal of external Cl^-. An increase in cell volume stimulated furosemide-sensitive K^+ influx and cell shrinkage inhibited this flux.

3. K^+ efflux was mainly furosemide-sensitive (64 % of total). This pathway was unaffected by variations in extracellular K^+ concentration and is therefore not exchange diffusion. However, it was affected by variations in cell volume in a similar way to the furosemide-sensitive K^+ influx.

4. Na^+ influx was only slightly sensitive to furosemide (13 % of total) but this component was very sensitive to changes in cell volume; decreased cell volume increased Na^+ influx whilst increased cell volume inhibited Na^+ influx.

5. Furosemide-sensitive K^+ influx was unaffected by variations in external Na^+ concentration. Similarly, furosemide-sensitive Na^+ influx was unaffected by variations in external K^+ concentration. This indicates that the passive influxes of Na^+ and K^+ were not coupled, in contrast to the situation in avian erythrocytes.

6. The opposite effects of cell volume upon passive Na^+ and K^+ fluxes are in good agreement with the net movements of these cations during volume regulation in erythrocytes of the flounder (Cala, 1977) and the toadfish (Lauf, 1982).

INTRODUCTION

The plasma osmolarity of fish, though under homeostatic control, varies somewhat more widely than in mammals, mainly as a result of the more intimate contact between their blood and the external osmotic medium. This is particularly true in those species that migrate between fresh water and sea water on a diurnal or seasonal time scale. Bath & Eddy (1979*a*, *b*) have found that on sudden immersion of sea-water-adapted trout into fresh water, plasma osmolarity drops within a few hours

by 10–15 %. The plasma osmolarity of sea-water- and fresh-water-adapted trout may differ by as much as 20–40 mosmol kg^{-1} (Fugelli, 1967; Leray, Colin & Florentz, 1981; P. K. Bourne & A. R. Cossins, unpublished observations). These shock and steady-state variations in plasma osmolarity must have significant effects upon the volume of their constituent cells unless regulatory processes intervene at the cellular level. Fugelli (1967), Cala (1977) and Lauf (1982) have demonstrated volume regulatory responses in erythrocytes of the european flounder, the winter flounder and the toadfish, respectively. Lauf (1982) has shown that the response to cellular swelling in toadfish erythrocytes is primarily by the activation of an ouabain-insensitive, Cl^--dependent transport of K^+ which is sensitive to furosemide. Similar volume regulatory responses have been observed in avian erythrocytes (McManus & Schmidt, 1978; Kregenow, 1981).

With respect to the routes and mechanisms of ion permeation, the erythrocytes of fish have been largely ignored and it is not known if they conform to the pattern that has been established for the higher vertebrates. Erythrocytes of fish are somewhat unusual in that they possess both nuclei and mitochondria, they consume oxygen and are able to incorporate radio-labelled amino acids into acid-precipitable protein (Greaney & Powers, 1978; P. Christmas & A. R. Cossins, unpublished observations). Furthermore, erythrocytes of carp and trout, in common with those of some avian species, increase volume and passive K^+ fluxes substantially in the presence of low concentrations of catecholamine hormones (Riddick, Kregenow & Orloff, 1971; Bourne & Cossins, 1982).

We present here a detailed study of the principal routes of K^+ and Na^+ flux in trout erythrocytes and how they are affected by cell swelling and shrinkage. Although these mechanisms show many similarities to those of birds and mammals, they also show some substantial differences.

METHODS

Animals

Rainbow trout (*Salmo gairdneri*, 0·25–0·75 kg) were obtained from a commercial source and were maintained in large fresh-water aquaria for at least 28 days before bleeding. Water temperature varied between 8 and 14 °C during this period, and photoperiod was maintained constant at 16Light:8Dark. Fish were fed twice daily to excess with commercial trout food (B.P. Nutrition Ltd).

Chemicals

Inorganic compounds, *d*-glucose, choline chloride, dibutyl phthalate, trichloroacetic acid, SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate) and Tris were obtained from BDH Chemicals Ltd. (Poole, Dorset) and were of analytical reagent grade. Bovine serum albumin (fraction V) and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) were obtained from Sigma Chemicals (Poole, Dorset). Imidazole was obtained from Koch-Light Laboratories (Sharnworth, Beds.). Furosemide was a gift from Hoescht Pharmaceuticals (UK) Ltd. $^{86}RbCl$ was obtained from Amersham International (UK) Ltd. $^{24}NaHCO_3$ and $^{42}K_2CO_3$ were provided by the Universities Research Reactor (Warrington, Cheshire).

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 needle). Clotting was prevented by gently shaking the blood in a heparinized tube. Samples from each fish were pooled and stored on ice. Plasma osmolarity was measured using a freezing point depression osmometer (Advanced Instruments, MA, U.S.A.) and the osmolarity of the trout saline was adjusted to that of the plasma by mixing appropriate volumes

of salines containing 120 or 170 mM-NaCl (other constituents were 6 mM-KCl, 5 mM-glucose, 15 mM-imidazole-HCl, pH 7·60 at approximately 20 °C).

Cells were washed at least four times by centrifugation (5 min at 1000 *g*) followed by resuspension with ice-cold isosmotic saline. The buffy coat and supernatant were discarded. Erythrocytes were finally suspended in isosmotic saline at a similar haematocrit to the original blood sample and left for 12–18 h at 10 °C. This ensured that cells were fully equilibrated and were not in a catecholamine-stimulated condition (Bourne & Cossins, 1983).

Measurement of K^+ and Na^+ influx

Unidirectional K^+ fluxes were determined from the uptake of $^{86}Rb^+$. This tracer will provide reliable estimates of K^+ fluxes only if the behaviour of both $^{86}Rb^+$ and $^{42}K^+$ are identical. This was confirmed in preliminary experiments in the presence of ouabain and furosemide (Table 1). Furthermore, Fig. 1*A* shows that the ouabain inhibition curve was identical when estimated using either $^{86}Rb^+$ or $^{42}K^+$.

Aliquots (50 μl) of the washed cell suspension were added to reaction tubes containing 0·9 ml of isosmotic trout saline pre-equilibrated to 10 ± 0·1 °C. The final haematocrit was 2–5 %. After exactly 10 min, the influx measurement was started by the addition of 50 μl $^{86}Rb^+$ in isosmotic saline (final specific activity, 0·5 μCi ml^{-1}) or 50 μl $^{24}Na^+$ in isosmotic saline (final specific activity, 5 μCi ml^{-1}). During incubation all reaction tubes were agitated at frequent intervals. The reaction was stopped by rapidly washing the cells in ice-cold isotonic $MgCl_2$ solution (100–200 mM-$MgCl_2$, 10 mM-Tris-HCl, pH 7·60 at approximately 20 °C) using an Eppendorf micro-centrifuge (5 s at 10000 *g*). Cells were washed four 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) trichloroacetic acid. The protein was removed by centrifugation (2 min at 10000 *g*) and the radioactivity of the supernatant determined by Cerenkov counting.

The influx was measured in duplicate samples which agreed within 5 %. Samples were taken usually at 6 and 12 min after addition of the tracer. Values are given as mmol l^{-1} p.c.v. h^{-1} (p.c.v. = packed cell volume). The haematocrit (Gelman-Hawksley microhaematocrit centrifuge, 8 min at 10000 *g*) was routinely monitored during the course of the experiment. Experiments with [^{3}H]inulin demonstrated that approximately 4–5 % of the packed cell volume was extracellular fluid. Components of the K^+ and Na^+ influx were estimated by including ouabain and/or furosemide in the reaction medium at their maximal inhibitory concentrations of 1 mM. Ouabain-sensitive and furosemide-sensitive influxes were calculated by subtracting the influx determined in the presence of the inhibitor from that determined in its absence.

Measurement of K^+ efflux

$^{86}RbCl$ was added (12–15 μCi ml^{-1} p.c.v.) to washed erythrocytes in a trout saline containing 1 % bovine serum albumin and then loaded for 15–18 h at 4 °C. The bovine serum albumin was found to prevent the small amount of haemolysis which occasionally occurred during this incubation. Immediately before the experiment, the cells were again washed four times with isosmotic saline (3 min at 1200 *g*) to remove the radioactive supernatant and then resuspended at a haematocrit of approximately 40 %. Aliquots (50 μl) of the suspension were added to reaction tubes containing 0·95 ml isosmotic saline, pre-equilibrated to 10 °C to give a final haematocrit of 2–3 %. At specific times reaction tubes were rapidly centrifuged (5 s at 10000 *g*) and 0·85 ml supernatant removed. Bovine serum albumin was precipitated by adding 0·5 ml 5 % (w/v) trichloracetic acid and removed by centrifugation (2 min at 10000 *g*). The radioactivity in the supernatant was determined by Cerenkov counting. As with the influx experiments, the components of K^+ efflux were determined in the presence of 1 mM-ouabain and/or furosemide. All efflux determinations were triplicated and efflux was calculated from three time points.

Internal cell radioactivity was determined immediately before and following the experiment by the re-suspension of replicate 50 μl aliquots of cells in 0·5 ml isosmotic saline layered on 0·5 ml dibutyl phthalate. Centrifugation (4 min at 10000 *g*) isolated the cell pellet from the supernatant. The pellet was lysed with 0·5 ml Triton X-100 and deproteinized with 0·5 ml trichloroacetic acid as described previously. The precipitate was removed by centrifugation (2 min at 10000 *g*) and the radioactivity of the supernatant was determined by Cerenkov counting. The internal K^+ concentration of the cells was determined from this final supernatant by flame spectrophotometry. Efflux was calculated on the basis of haematocrit and internal K^+ concentration and is expressed as mmol l^{-1} p.c.v. h^{-1}.

RESULTS

K^+ influx

The dose–response curve for the effect of ouabain upon K^+ influx is presented in Fig. 1*A*. The concentration of ouabain for 50 % inhibition of ouabain-sensitive influx (I_{50}) was $5{\cdot}0 \times 10^{-6}$ M with a medium K^+ concentration of 6 mM. Ouabain at 10^{-3} M produced maximal inhibition and was used at this concentration for all subsequent experiments. Ouabain-sensitive K^+ influx accounted for approximately 50 % of the total influx in steady-state erythrocytes (Table 2).

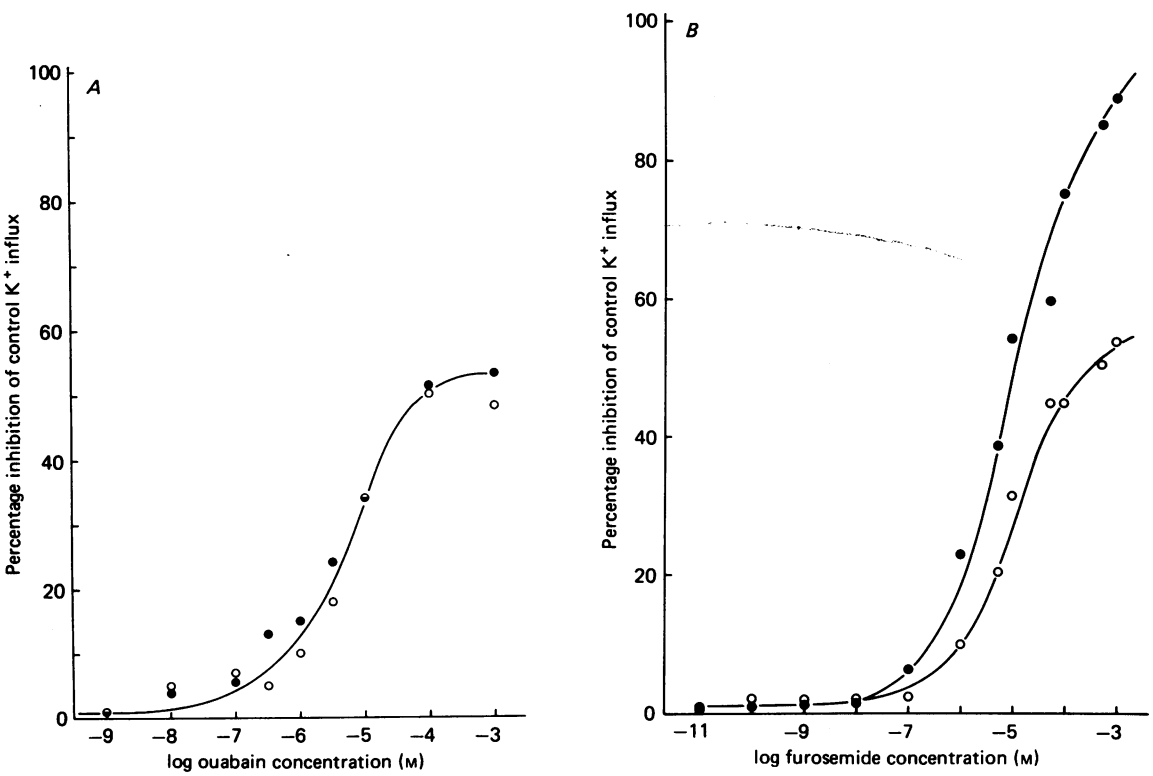

Fig. 1. *A*, the dose–response curves for ouabain inhibition of K^+ influx into trout erythrocytes. The values represent the percentage inhibition of K^+ influx compared to the control value (i.e. with no inhibitor). Tracer was either ^{86}Rb (●) or ^{42}K (○). *B*, dose–response curves for furosemide inhibition of K^+ influx in the absence and presence of ouabain. (○), percentage inhibition of total influx (i.e. no inhibitors). (●), percentage inhibition of ouabain-insensitive influx (i.e. in the presence of 1 mM-ouabain). Similar results were obtained in two other experiments.

The effect of furosemide upon the total and the ouabain-insensitive K^+ influx is presented in Fig. 1*B*. The curves were essentially identical both in their respective I_{50} values ($2{\cdot}0 \times 10^{-5}$ M) and in the proportion of the total K^+ influx that was inhibited (not shown). The curves failed to reach a convincing plateau at the higher furosemide concentrations, though there are other reasons for believing that inhibition was

essentially maximal at 10^{-3} M and above (see Discussion). In all subsequent experiments furosemide was used at 10^{-3} M, when it inhibited approximately 46% of the total influx (Table 2).

The K^+ influx in the presence of both ouabain and furosemide, the residual flux, comprised only 5% of the total influx (Table 1). The sum of the ouabain-sensitive, furosemide-sensitive and residual influxes was usually not different from the total influx determined in the absence of inhibitors.

TABLE 1. A comparison of the K^+ influx in trout erythrocytes as determined using ^{42}K or ^{86}Rb as tracer

	Influx (mmol l^{-1} p.c.v. h^{-1})	
	^{42}K	^{86}Rb
Total influx	13·99 ± 0·34	14·40 ± 0·50†
Ouabain-resistant influx	8·03 ± 0·27	8·19 ± 0·32†
Furosemide-resistant influx	5·35 ± 0·41	5·51 ± 0·17†
Residual influx*	1·92 ± 0·17	1·73 ± 0·08†

Values represent the mean ± S.E. of the mean for four flux determinations on the same preparation of erythrocytes. Ouabain and furosemide were present at a final concentration of 1 mM. † Probability > 0·1 as calculated with the Student's *t* test. * Residual influx was measured in the presence of both ouabain and furosemide at 10^{-3} M (final concentration).

TABLE 2. Components of Na^+ and K^+ influx and of K^+ efflux in trout erythrocytes

		Ouabain-sensitive		Furosemide-sensitive		Residual activity	
	Control flux	Flux	% of control	Flux	% of control	Flux	% of control
K^+ influx (7)	17·1 ± 2·2	8·6 ± 1·2	49·6 ± 4·4	7·6 ± 1·1	45·6 ± 4·6	0·7 ± 0·1	4·3 ± 0·6
K^+ efflux (7)	16·7 ± 1·7	1·3 ± 0·6	8·0 ± 3·6	11·2 ± 1·7	63·7 ± 6·9		
Na^+ influx (4)	16·7 ± 0·5	0·9 ± 0·6	5·1 ± 3·0	2·4 ± 1·2	13·9 ± 6·5		
Na^+ influx (10)	21·0 ± 0·5			2·6 ± 0·2	13·1 ± 0·84		

Values represent the mean ± S.E. of the mean (mmol l^{-1} p.c.v. h^{-1}). Numbers in brackets refer to the number of determinations. Residual activity refers to the influx in the presence of both ouabain (1 mM) and furosemide (1 mM). Na^+ influx was measured in two separate series of experiments.

The effects of varying the concentration of K^+ in the external medium on K^+ influx are illustrated in Fig. 2. The curve for the ouabain-resistant influx represents the sum of a saturable component (K_m = 25 mM, V_{max} = 60 mmol l^{-1} p.c.v. h^{-1}), and a linear component with a rate constant of 0·08 h^{-1}. The curve for the furosemide-resistant influx was fitted by a similar equation with K_m = 1·5 mM, V_{max} = 13 mmol l^{-1} p.c.v. h^{-1} and with the same non-saturable component. The agreement was good and supports the involvement of specific, saturable mechanisms in both cases. The curve fitted to the data points for total K^+ influx (i.e. with no inhibitors) was calculated by summing the influx through the two saturable and one non-saturable routes, again with good agreement.

Equilibration of trout erythrocytes in media with any of several different anions caused a reduction in ouabain-insensitive K^+ influx (Table 3). Br^- and I^- acted as

partial substitutes in that the ouabain-insensitive influx was greater than the residual influx. On the other hand, NO_3^- reduced the K^+ influx to a value that was close to the residual influx, whilst acetate reduced it still further. A substantial fraction of the ouabain-insensitive K^+ influx was dependent in a nearly linear manner on the external concentration of Cl^- (Fig. 3) when NO_3^- was used as the replacement anion.

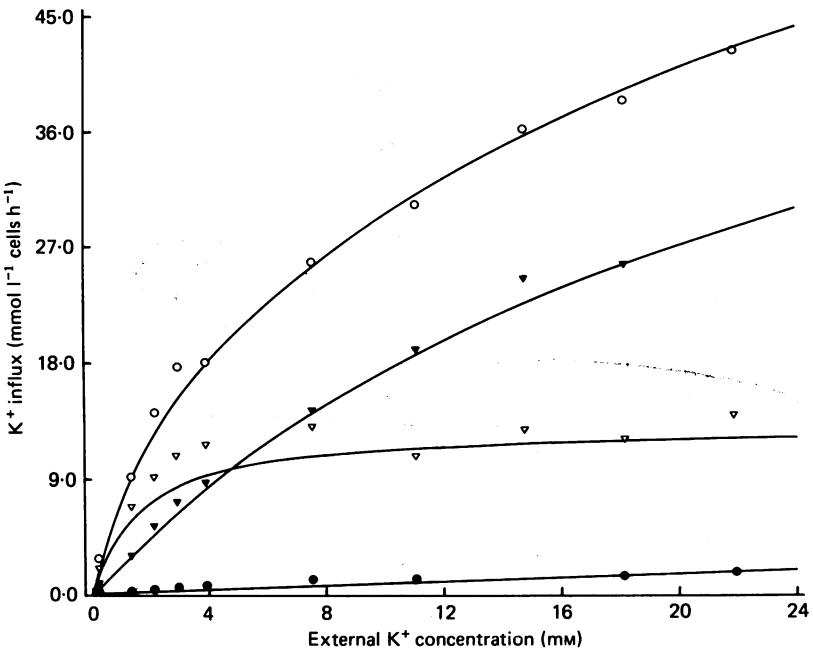

Fig. 2. The effect of the K^+ concentration in the external medium on K^+ influx into trout erythrocytes. (○), total K^+ influx; (▼), K^+ influx in the presence of ouabain (1 mM); (▽), K^+ influx in the presence of furosemide (1 mM); (●), K^+ influx with both ouabain and furosemide (both 1 mM). External K^+ concentration was adjusted by mixing appropriate volumes of isosmotic salines containing 0 and 24 mM-KCl, respectively. The replacement cation was Na^+. The lines fitted to the data points were calculated as described in the text. Similar results were obtained in one other experiment.

Following Chipperfield (1981) and others, these results are interpreted as a dependence of K^+ influx upon external Cl^- rather than NO_3^- inhibition. Addition of furosemide reduced K^+ influx at all external Cl^- concentrations to levels similar to that observed in the absence of external Cl^-. This suggests that the Cl^--dependent and furosemide-sensitive mechanisms are the same.

SITS, a well known inhibitor of the anion exchange system of mammalian erythrocytes, inhibited a substantial fraction of the ouabain-insensitive K^+ influx with an I_{50} of $7{\cdot}6 \times 10^{-6}$ M (Fig. 4). SITS at 1 mM reduced the ouabain-insensitive influx to a level that was similar to that observed both in the presence of furosemide or in the absence of Cl^- (Fig. 3). The presence of both SITS and furosemide produced no further inhibition of K^+ influx. SITS and furosemide, therefore, inhibit the same mechanism of K^+ influx.

The effects of cell volume upon K^+ influx was studied by suspending erythrocytes

TABLE 3. K^+ influx into trout erythrocytes equilibrated in media containing various anions

Principal anion	Treatment	K^+ influx (mmol l^{-1} p.c.v. h^{-1})
Cl^-	—	18·59*
Cl^-	Ouabain	12·15±0·69
Cl^-	Ouabain + furosemide	1·79*
Br^-	Ouabain	9·15±0·37
I^-	Ouabain	3·18±0·6
NO_3^-	Ouabain	2·04±0·32
Acetate	Ouabain	0·81±0·08

The media were buffered at pH = 7·6 with HEPES/Tris (room temperature) and the Na^+ concentration of each solution was adjusted to give an osmolarity of 300±2 mosM. Erythrocytes were washed three times in the appropriate saline, incubated at room temperature for 40 min and finally washed two more times before influx was determined. ^{86}Rb was added as tracer in the appropriate anion-substituted saline. * $n = 2$, otherwise $n = 4$ where n represents the number of separate determinations on a single sample of erythrocytes.

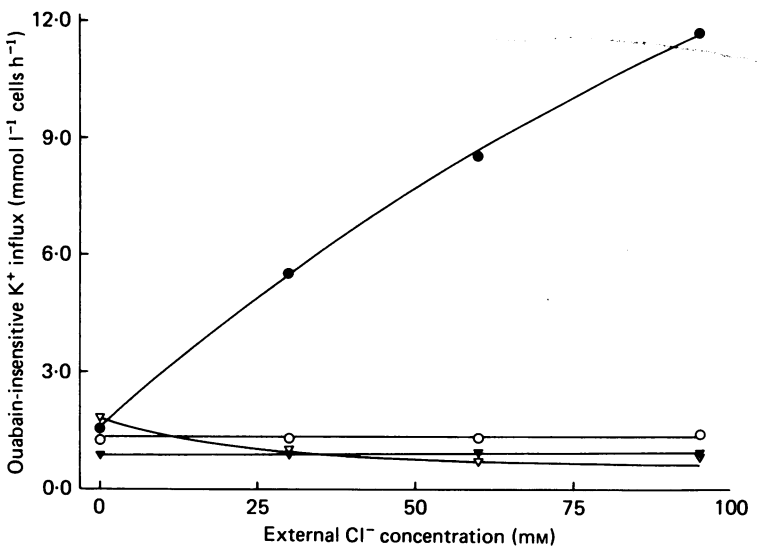

Fig. 3. The effect of external Cl^- concentration on the ouabain-insensitive K^+ influx in trout erythrocytes. (●), K^+ influx with ouabain (1 mM); (▼), K^+ influx with ouabain and furosemide (1 mM); (○), K^+ influx with ouabain and SITS (1 mM); (▽), K^+ influx with ouabain, SITS and furosemide (all at 1 mM). External Cl^- was adjusted by mixing appropriate volumes of isosmotic salines containing either Cl^- or NO_3^- as the principal anion.

in different reaction media with osmolalities between 278 and 376 mosmol kg^{-1} (made by varying NaCl concentration). The resulting cell volumes (0·85–1·15 of their original value) were calculated assuming that the cells were perfect osmometers. The ouabain-sensitive K^+ influx and the residual influx were unaffected by variations in cell volume. The furosemide-sensitive K^+ influx, on the other hand, was approximately doubled by a 15 % increase in cell volume and reduced by a third for a 15 % reduction in cell volume (Fig. 5).

K^+ efflux

The K^+ efflux was unaffected by ouabain (Table 2) whilst furosemide caused a reduction of about 64%. The ouabain and furosemide-insensitive efflux, which comprised the remaining 36%, was somewhat higher than the corresponding (residual) influx.

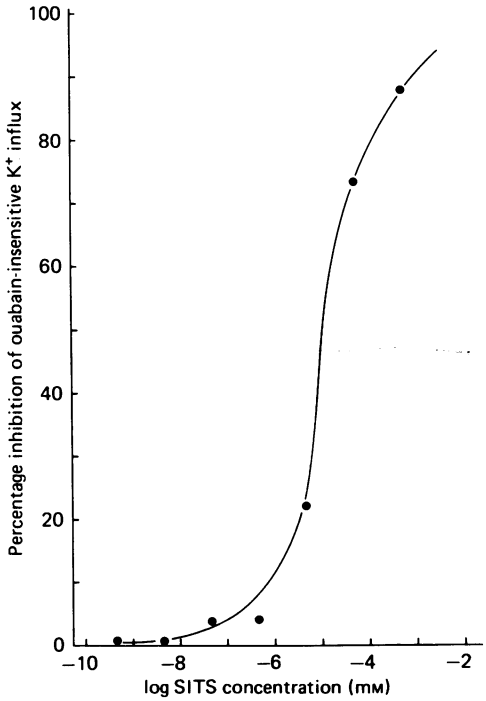

Fig. 4. The dose–response curve for SITS in the presence of ouabain (1 mM) upon K^+ influx into trout erythrocytes. Values represent the percentage inhibition of ouabain-insensitive K^+ influx compared to that measured in the absence of SITS.

Fig. 6 illustrates the effect of variations in cell volume upon K^+ efflux. Furosemide-sensitive K^+ efflux was almost halved by a 15% cell shrinkage but increased by approximately 40% for a 15% cell swelling in qualitatively the same way as K^+ influx was affected. Furosemide-insensitive K^+ efflux was unaffected by variations in cell volume.

In view of the similar effects of cell volume on both K^+ influx and efflux and the fact that the volume-sensitive components of both influx and efflux were inhibited by furosemide, the possible coupling between these unidirectional fluxes was investigated (Fig. 7). Both the furosemide-sensitive and insensitive fractions of K^+ efflux were unaffected by variations in external K^+ between 0 and 24 mM.

Na^+ influx

Na^+ influx was only marginally inhibited by ouabain though furosemide caused some inhibition (Table 2). The dependence of Na^+ influx upon external Na^+

concentration is illustrated in Fig. 8. The graph for the total Na^+ influx was curvilinear, whilst the furosemide-sensitive fraction was approximately linear, although the low flux makes this conclusion less certain. The lines for the total influx were calculated assuming the presence of a saturable component and two non-saturable components, one of which corresponded to the furosemide-sensitive component. The agreement of the calculated line with the data points was good.

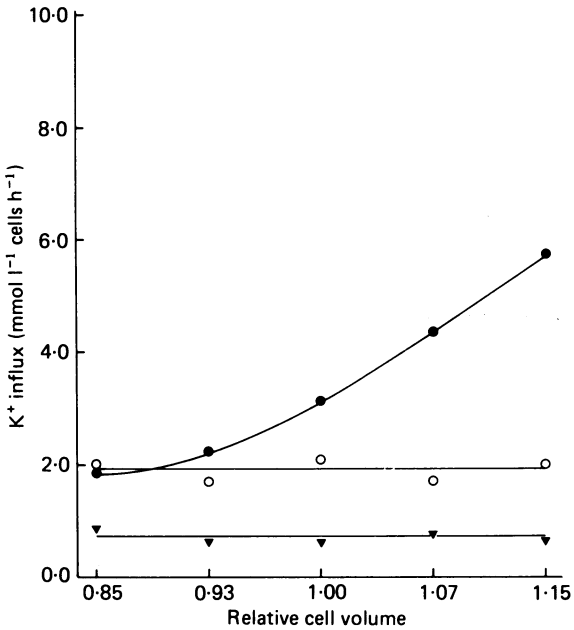

Fig. 5. The effects of variations in cell volume on the components of K^+ influx into trout erythrocytes. (●), furosemide-sensitive influx; (○), ouabain-sensitive flux; (▼), ouabain- and furosemide-insensitive flux. Cell volume was adjusted by altering the osmolality of the external medium by mixing appropriate volumes of salines containing 120 and 170 mM-NaCl, respectively (for other constituents see Methods). Relative cell volume was calculated assuming the cells were perfect osmometers. Similar results were obtained in one other experiment.

Furosemide-sensitive Na^+ influx was very sensitive to variations in cell volume (Fig. 9), whilst the furosemide-insensitive fraction was completely unaffected (not shown). However, in contrast to the effects of cell volume on K^+ flux, a reduction in cell volume stimulated Na^+ influx whilst an increase in cell volume inhibited Na^+ influx. Indeed, a 15% reduction in cell volume almost completely abolished Na^+ influx.

Interactions between Na^+ and K^+ fluxes

The possible coupling of the ouabain-insensitive fluxes of Na^+ and K^+ in trout erythrocytes was investigated by examining the effects of external Na^+ concentration on K^+ influx. Furosemide-sensitive K^+ influx was completely unaffected by variations in external Na^+ concentration between 0 and 150 mM although ouabain-sensitive

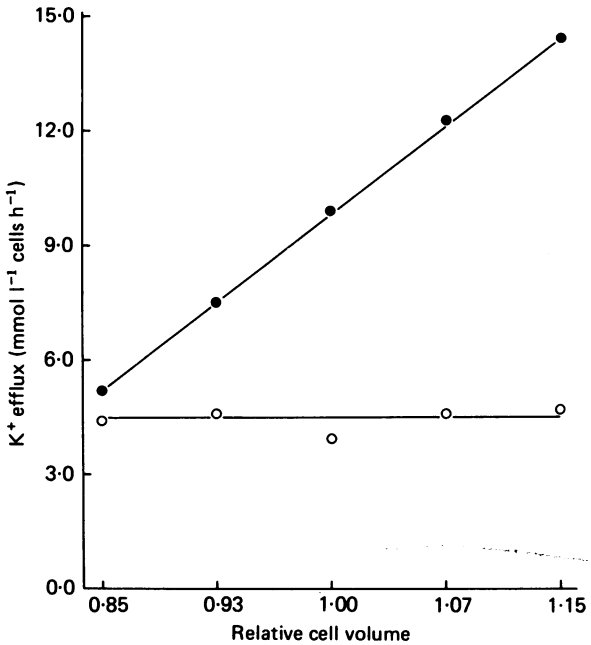

Fig. 6. The effect of cell volume on the components of K^+ efflux from trout erythrocytes. (●), furosemide-sensitive efflux; (○), ouabain-sensitive efflux. Cell volume was altered as described in the legend to Fig. 5. Similar results were obtained in four other experiments.

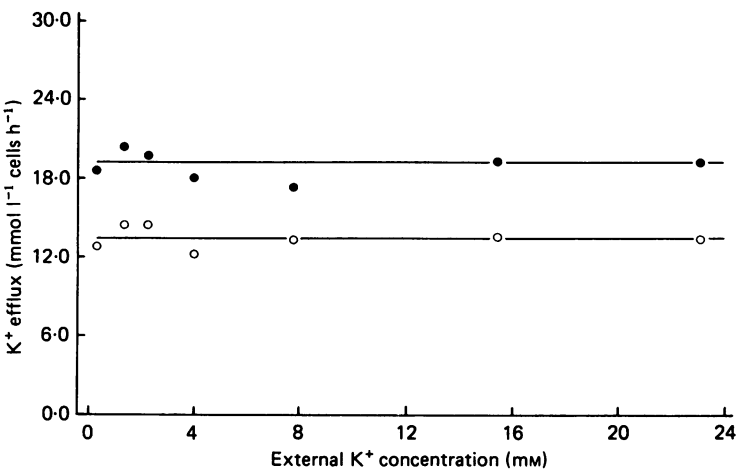

Fig. 7. The effect of external K^+ concentration on K^+ efflux from trout erythrocytes. (●), total K^+ efflux; (○), furosemide-sensitive flux. External K^+ concentration was adjusted as described in the legend to Fig. 2. Similar results were obtained in one other experiment.

influx showed a small but reproducible decrease as the external Na^+ concentration was reduced below 30 mM. This latter effect was presumably due to the effects of a reduced cell concentration of Na^+ upon the activity of the Na^+–K^+ pump. The converse experiment, namely the effect of external K^+ concentration (0–23 mM) upon both the total and furosemide-sensitive Na^+ influx also showed a similar independence.

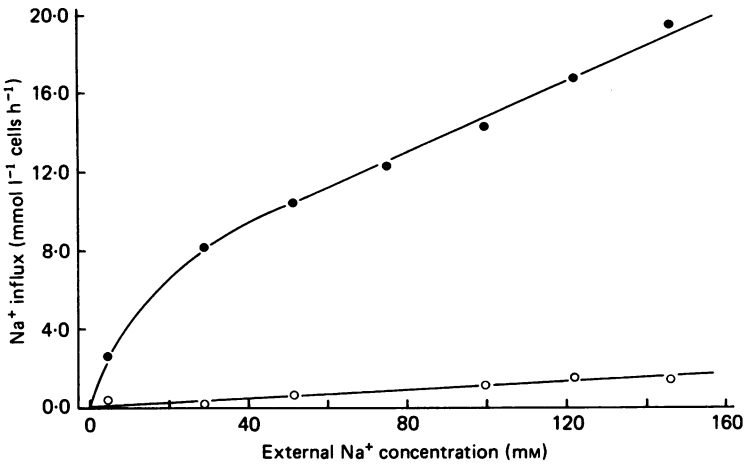

Fig. 8. The effect of external Na^+ concentration on Na^+ influx into trout erythrocytes. (●), total influx; (○), furosemide-sensitive flux. The external Na^+ concentration was adjusted by mixing appropriate volumes of isosmotic salines containing either Na^+ or choline as the principal cation. The erythrocytes used at 0 mM-external Na^+ concentration were washed three times in the choline-containing saline before K^+ influx was determined.

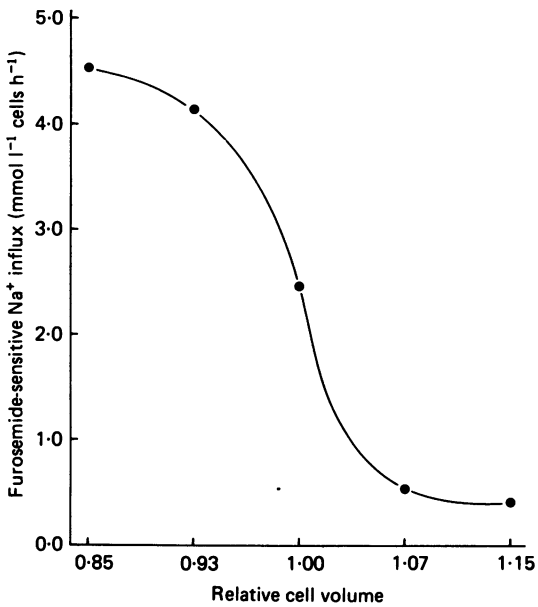

Fig. 9. The effects of cell volume on the furosemide-sensitive Na^+ influx into trout erythrocytes. Cell volume was adjusted as described in the legend to Fig. 5. Similar results were obtained in two other experiments.

DISCUSSION

Except where cell volume and the composition of the external medium was altered, the observations reported here are on cells in the 'lower steady state'; that is, the erythrocytes had been equilibrated by incubation in a saline for 15–18 h at 10 °C and were not catecholamine-stimulated (Riddick *et al.* 1971; Bourne & Cossins, 1982).

As in studies with other cells the inhibitors ouabain and furosemide provided a convenient means of distinguishing between the different routes of cation flux across cellular membranes. The ouabain-sensitive K^+ flux is apparently quite separate from the furosemide-sensitive K^+ mechanism (Karlish, Ellory & Lew, 1981) since the former is neither influenced by furosemide itself, by variations in cell volume nor by the concentration of Cl^- in the external medium. The degree of furosemide-inhibition and its I_{50} are not affected by the presence of ouabain. Finally, the furosemide-sensitive K^+ influx conforms to saturation kinetics with respect to the external K^+ concentration with a substantially higher V_{max} and K_m than the corresponding ouabain-sensitive influx.

Although the dose–response curve for furosemide failed to reach a convincing plateau at the higher inhibitor concentrations (10^{-3} M), there is evidence to suggest that the residual influx in the presence of both ouabain and furosemide (both at 10^{-3} M) is also a quite separate route of permeation. First, residual influx of K^+ was apparently linearly related to the external concentration of K^+. Secondly, it was totally unaffected by variations in cell volume and, thirdly, it was unaffected by variations in external Cl^- concentration. Thus, it seems reasonable to conclude that K^+ influx in trout erythrocytes occurs by at least three kinetically distinct mechanisms; the ouabain-sensitive Na^+–K^+ pump, the specific furosemide-sensitive route and a third route which has the characteristics of passive, non-specific permeability. In this respect trout erythrocytes seem little different to those of other vertebrates.

K^+ efflux is largely furosemide-sensitive and this presumably occurs through the same or a similar mechanism as the furosemide-sensitive influx of K^+, for in addition to their furosemide-sensitivity, they are both affected by cell volume in the same way. However, the absence of any effect of external K^+ concentration upon K^+ efflux rules out the coupling of influx and efflux in any obligatory manner. Haas, Schmidt & McManus, (1982) have also noted that Na^+–K^+ co-transport in avian erythrocytes has no *trans* requirement. The furosemide-insensitive, passive K^+ efflux in trout erythrocytes presumably represents, like the residual influx, passive electrochemical diffusion. The residual efflux is somewhat greater than the corresponding residual influx (Table 2) probably because of the outwardly directed K^+ concentration gradient. This net residual efflux, which together with the net furosemide-sensitive K^+ efflux roughly balances the ouabain-sensitive K^+ uptake.

The Cl^--dependence of passive K^+ influx seen in trout erythrocytes has been widely observed in other cell types (human erythrocytes, Funder & Wieth, 1967; Dunham, Stuart & Ellory, 1980; Chipperfield, 1981; sheep erythrocytes, Dunham & Ellory, 1981; cardiac cells, Carmeliet & Verdonck, 1977; avian erythrocytes, Schmidt & McManus, 1977*b*; Kregenow & Caryk, 1979; cultured cells, Bakker-Grunwald & Sinensky, 1979; Aiton, Chipperfield, Lamb, Ogden & Simmons, 1981; Bakker-

Grunwald, Ogden & Lamb, 1982). However, the furosemide-sensitive K^+ influx in trout erythrocytes is clearly rather different from that described for the erythrocytes of higher vertebrates in that it is completely blocked by SITS. This unusual observation is supported by Lauf (1982) who has also noted that DIDS (4,4-diisothiocyanostilbene-2,2′-disulphonate) prevented K^+ loss and volume regulation in toadfish erythrocytes. Classically, SITS-sensitivity is interpreted as indicating an important role for the anion transporter, although it is not possible to rule out a non-specific effect of SITS on the furosemide-sensitive K^+ flux mechanism. In view of these uncertainties no firm conclusions can be made at present concerning the site of inhibition of cation flux by SITS and furosemide. However, it is worth pointing out that the I_{50} for SITS inhibition of K^+ influx in trout erythrocytes is only slightly higher than the I_{50} for anion self-exchange in ox erythrocytes (Motais, 1977).

On the basis of the effects of varying external Na^+ concentration, Na^+ influx in trout erythrocytes appears to comprise two and possibly three kinetically distinct components. The predominant furosemide-insensitive flux appears to consist of a saturable and a non-saturable component but their properties are not known. Cala (1980) has reported an amiloride-sensitive Na^+ permeability in *Amphiuma* erythrocytes. It seems that Na^+ influx was not normally sensitive to amiloride in the steady state but that cell shrinkage induced an amiloride-sensitive Na^+ flux. Similarly, for *Ehrlich* cells in the steady state, there is very little co-transport of sodium chloride into cells (Sjoholm, Hoffman & Simonsen, 1981). However, in trout erythrocytes in the steady state, all components of Na^+ influx are expressed. Furthermore, variations in cell volume induced changes in the furosemide-sensitive flux of K^+ and Na^+, but not in the furosemide-insensitive component.

The linked flux of Na^+ and K^+ by a ouabain-insensitive mechanism is a feature of a number of cell types including human erythrocytes (Wiley & Cooper, 1974), avian erythrocytes (Schmidt & McManus, 1977*a*; McManus & Schmidt, 1978; Kregenow, 1981; Haas *et al.* 1982), *Ehrlich* cells (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980) and the Madin-Darby canine kidney cell line (Rindler, McRoberts & Saier, 1982) although in trout cells this does not seem to be the case. The evidence for this is 3-fold. First, Na^+ influx is independent of variations in the external concentrations of K^+. Secondly, K^+ is unaffected by variations in external Na^+ concentration. Thirdly, variations in cell volume induce opposite effects upon furosemide-sensitive influxes of Na^+ and K^+. Thus cell swelling led to an increased K^+ influx and efflux and to a decreased Na^+ influx, whilst cell shrinkage led to the opposite effects. In both cases, the selective increase in cation flux would oppose the induced volume change in a volume regulatory manner. The independence of furosemide-sensitive K^+ and Na^+ fluxes observed in trout erythrocytes and their differing responses to variations in cell volume, resemble the properties of cat and dog erythrocytes (Parker, 1977) and correlate well with those of Cala (1977) on volume regulation in erythrocytes of the winter flounder where regulatory volume increase was associated mainly with an increased cell Na^+ content.

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