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THALLIUM AND THE SODIUM PUMP IN HUMAN RED CELLS

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SUMMARY

1. Thallium (Tl) inhibits the ouabain-sensitive K influx in human red cells in high-Na medium. At 1 mM external K concentration [K_o], the ouabain-sensitive K influx decreases steadily with increasing Tl concentration, up to 0·9 mM outside; at 0·17 mM-K_o, however, Tl stimulates the ouabain-sensitive K influx below 0·1 mM-Tl_o and inhibits it at higher concentrations.

2. In a K-free medium in which all except 5 mM-Na is replaced by choline, and into which red cells show zero control ouabain-sensitive Na efflux, Tl is able to support ouabain-sensitive Na efflux up to 2·1 m-mole/l. cells.hr following a sigmoid activation curve which is half-maximal between 0·03 and 0·05 mM-Tl_o and that follows two-site kinetics up to 0·1 mM-Tl_o. Beyond 0·15 mM-Tl_o, the Tl-activated ouabain-sensitive Na efflux attained is inhibited slightly.

3. When the ouabain-sensitive Na efflux is measured at 5 mM-Na_o and 5 mM-K_o, increasing concentrations of Tl have little effect on it, 0·9 mM-Tl_o inhibiting by some 14 %; in similar conditions, the ouabain-sensitive K influx is inhibited by about 40 %.

4. The dependence of ouabain-sensitive K influx on external K concentration at 5 mM-Na_o, which follows a slightly sigmoid curve in the absence of Tl, changes to hyperbolic at 0·06 mM-Tl_o at the same time that ouabain-sensitive K influx is inhibited. The fitted V_{max} values for ouabain-sensitive K influx are the same in the presence and in the absence of 0·06 mM-Tl_o.

5. In high-Na cells, loaded by nystatin treatment, the ouabain-sensitive K influx measured at 0·2 mM-Na_o follows a hyperbolic curve between 0·05 and 0·4 mM-K_o, and is inhibited by Tl in a strictly competitive fashion.

6. The effects of Tl on ouabain-sensitive Na efflux and ouabain-sensitive K influx are interpreted in terms of a high-affinity substitution for K at the external K sites of the Na pump and suggest that in human red cells Tl can be actively transported inwards in exchange for internal Na.

7. Thallium can inhibit about 25 % of the ouabain-insensitive Na efflux into 5 mM-Na_o and part of this inhibition occurs with a high Tl-affinity; the ouabain-insensitive K influx is inhibited by Tl both in high-Na and in 5 mM-Na medium, but with a different concentration dependence than the ouabain-insensitive Na efflux.

INTRODUCTION

Thallium has been reported to substitute for K with a higher affinity than this ion, in the activation of the (Na-K)ATPase of rabbit kidney (Britten & Blank, 1968) and in the K-activated phosphatase from beef brain (Inturrisi, 1969), as well as in activating other K-requiring enzymes (Kayne, 1971). Rapid Tl fluxes have been observed in rabbit erythrocytes (Gehring & Hammond, 1964) and recently in human red cells (Skulskii, Manninen & Järnefelt, 1973) but there are not conclusive reports about their relevance to the Na pump.

The experiments presented in this paper were aimed at deciding whether the Tl-activation of the membrane (Na-K)ATPase and K-activated phosphatase correlated with an interaction of Tl with the Na efflux and K influx in human red cells. The results indicated that Tl can effectively replace K, with a higher affinity, in the activation of the ouabain-sensitive Na efflux, and that it can inhibit the ouabain-sensitive K influx, changing the kinetics of the process in a way that implies that K and Tl can bind simultaneously to the outwards-facing aspect of the Na pump.

METHODS

Solutions. Three basic media were used in these experiments: a 'high-Na medium' that contained (mM): Na 140·5; Mg 2; Tris (pH 7·9 at 20°) 15; Cl 153·5; and glucose 10; a '5 mM-Na medium' and a '0·1 mM-Na medium', both prepared by replacing equivalent amounts of choline chloride for NaCl in the 'high-Na medium'. For some of the washes, a 'Mg-Tris solution' containing (mM): Mg 106; Tris (pH 7·4 at 20° C) 10 and Cl 213, was used.

Because of the low solubility of TlCl, preliminary experiments were conducted in media in which sulphate was the only anion, using sulphate-loaded cells, but that led to low values for the ouabain-sensitive potassium influx. Since the solubility of TlCl at 25° C is 2·68 mM in 200 mM-NaCl and 8·71 mM in 25 mM-NaCl, and is reduced only 1·6 times at 0° C (Mellor, 1942), all other experiments were done in 153·5 mM-Cl media with Tl at a maximal concentration of 1 mM. In order to avoid precipitation of TlCl, solid Tl_2SO_4 was first dissolved in a small volume of distilled water (not greater than 1 % of the final volume of the solution) and this solution was added dropwise on to the bulk of the high-chloride medium while stirring, and at room temperature, making it up to the final volume with more chloride medium.

Na efflux experiments. Blood was drawn with a heparinized syringe and plasma and buffy coat removed by centrifugation. Red cells were washed by centrifugation

(3000 *g*, 5 min) four times with about 8 volumes of 'high-Na medium' and then loaded with ^{24}Na with glucose added, for 5 hours at 37° C. The ^{24}Na-loaded cells were spun and washed 5 times with the appropriate medium at 4–8° C (see legend to Figures for composition), resuspended in the same medium and kept in an ice-bath while being distributed into flasks or tubes. In the experiments in flasks, samples were withdrawn at 2 min (initials) and then at regular intervals up to 60 min and the cells spun out in 15 sec with an Eppendorf 3200 Centrifuge. Aliquots of the supernatant as well as aliquots of the cell suspension (to which 1 drop of Triton X-100 solution was added) were transferred to tubes and counted in a Packard Scintillation-Spectrometer. The 'initials' were subtracted from the counts remaining in the cells and from the total counts of the suspension, and the logarithm of the fraction of counts remaining in the cells was plotted against time; straight lines were fitted by least squares. As the Na efflux followed a single exponential under all conditions tested, the rate constants were obtained as the regression coefficients for the above linear fittings.

Subsequent experiments to test the effect of increasing Tl concentrations were done by incubating in tubes for 5 min (initials) and 45 min (finals). In this case, 0·2 ml. aliquots of a cold suspension of ^{24}Na-loaded cells were added in the cold into tubes containing 2·8 ml. of media (with Tl at different concentrations) and the contents mixed. The incubation was started by immersing the racks in the 37° C bath and stopped by replacing them in the ice-bath. After 5 min the tubes were spun at 4–8° C, transferred back to the ice-bath and the supernatants were rapidly sampled for Čerenkov counting with Oxford pipettes. For sampling the cell suspension, 0·2 ml. aliquots were taken and added while stirring into tubes containing 2·8 ml. 5 % TCA, the precipitate was spun down and aliquots of the clear supernatant were drawn for Čerenkov counting in a Packard TriCarb Liquid Scintillation Spectrometer; 5 % TCA did not affect the counting efficiency. Rate constants were calculated in this case as

$$^{0}k_{Na} = -\tfrac{3}{2}\ln \text{ (fraction of counts in cells after 40 min) } (hr^{-1})$$

once the 'initials' had been subtracted as in the former case.

A portion of the ^{24}Na-loaded cell suspension was removed in each case while the efflux was about half-way through and washed 7 times in 8 volumes of cold 'Mg-Tris solution'. The washed cells were finally suspended in more 'Mg-Tris solution' and analysed for Na, K and haemoglobin. Fluxes were calculated by multiplying the rate constants by the Na content per litre cells.

Potassium influx experiments. Either fresh cells washed 3 times with 'high-Na medium' or cation-loaded cells were spun and washed at least once with the medium convenient for the particular experiment (see legend to Figures). The cells were then resuspended in the wash solutions, divided into several equal portions in centrifuge tubes and spun. Each lot was washed once with medium containing Tl at a particular concentration (ranging from 0 to 1 mM-Tl) and resuspended in the respective Tl solution. In some cases, the suspensions were made up to 5–10 % haematocrit and allowed to equilibrate with Tl up to 3 hr at 37° C, spun and resuspended in more Tl-containing medium. The final haematocrit was adjusted to a value between 0·3 and 2·0 % and the suspensions cooled. Portions of 4·7 or 4·8 ml. of each cell suspension were dispensed with Krogh-type syringes into tubes containing 0·3 or 0·2 ml. ^{42}KCl solutions at different K concentrations ± ouabain, all made up in the basic medium and cooled. Aliquots of each suspension were saved for haemoglobin determination, cell Na and K and cell water determinations. The racks were transferred to the 37° C bath and replaced in the ice-bath after up to 1 hr incubation.

After 5 min in the cold, the cells were spun and the supernatants sampled for determining Na and K concentrations. The cells were finally washed 3 times with 50 volumes of cold 'Mg-Tris solution' and extracted with 7 or 10 ml. 5% TCA. The precipitate was packed by spinning and the whole of the supernatant was counted for Čerenkov radiation. K influx was calculated as

$$\text{K influx (m-mole/l.cells.hr)} = \frac{\text{counts in cells after 1 hr}}{\text{ext.sp.act.}\left[\frac{\text{counts}}{\mu\text{mole } K_o}\right] \times \text{haematocrit} \times \text{vol. of suspension (ml.)}}.$$

Cation loading of the cells. The nystatin method of Cass & Dalmark (1973) was used. Fresh packed red cells were washed twice with 'Mg-Tris solution' and then twice with the loading solution, that contained (mM): NaCl + KCl + choline chloride 150; K_2HPO_4 0·9; NaH_2PO_4 0·6 (pH 6·7 at 20° C). Nystatin was dissolved in methanol at 5 mg/ml. and the solution cleared by centrifugation. A 1% in volume of this solution was added to a volume of loading solution (usually 90 ml.) already supplemented with 27 mM sucrose. This nystatin loading solution (50 μg nystatin/ml.) was mixed with the packed washed cells at about 3·5% haematocrit and allowed to stand 30 min (expt. Fig. 6) or 90 min (expt. Fig. 7) at room temperature. The cells were then spun, washed 4 times with loading solution (nystatin-free), resuspended in the same solution and stored at 4° C overnight. There was only negligible lysis during the loading incubation and not at all during the washes.

Na and K determinations. Both elements were determined by either absorption with a Pye-Unicam SP90 Atomic Absorption Spectrophotometer or emission with an 'EEL' Flame Photometer (Evans Electroselenium Ltd., Halstead, Essex). Samples and standards for supernatant K concentration were prepared by dilution in the appropriate ('high Na', '5 mM-Na' or '0·1 mM-Na') medium. Standards for supernatant Na were prepared in an all-choline medium; the readings for low supernatant Na were corrected for enhancement due to varying K. Cell Na and K were referred either to the cell mass as estimated from the haemoglobin content of an aliquot of the same washed cell suspension, or to the cell water volume, determined separately. Thallium did not interfere with Na and K determinations.

Haemoglobin. It was determined either as oxyhaemoglobin and read at 540 nm (the extinction coefficient of packed red cells was taken as 284 at that wave-length) or as cyanmethaemoglobin and read at the same wave-length.

Cell water determinations. Red cell suspensions adjusted to about 10% haematocrit were spun for exactly 1 min in an Eppendorf 3200 Centrifuge, and the supernatant and upper layer of cells sucked off. Samples of packed red cells were quickly weighed in tared vials and consequently dried overnight at 110° C and weighed again. The per cent (v/v) water content (% X) was calculated as

$$\% X = 100\left(d_c - (t + d_c)\frac{W_d}{W_w}\right),$$

where W_d and W_w are the dry and wet weights respectively, d_c is the density of packed red cells (taken as 1·0964; Ruch & Fulton, 1960) and t is the trapped extracellular volume expressed as fraction of the cell volume. The magnitude of t was assessed independently in cell pellets obtained in the same conditions of the assay, as the distribution space of ^{60}CoEDTA prepared by the method of Brading & Jones (1969) (mean t = 0·0804 at 60 sec).

Source of materials. ^{24}NaCl and ^{42}KCl were obtained as sterile isotonic solutions from the Radiochemical Centre, Amersham. Thallous sulphate was purchased from

Hopkin & Williams, Ltd (Chadwell Heath, Essex) and nystatin (mycostatin) from Sigma London. 'Aculute' cyanmethaemoglobin reagent was purchased from Ortho Pharmaceutical Ltd (Saunderton, Bucks.) and cyanmethaemoglobin standard from C. Davis Keeler Ltd (London). Choline chloride was from BDH Biochemicals (BDH Chemicals Ltd, Poole, Dorset) and was recrystallized from hot ethanol. All other reagents, including ethanol, were AnalaR grade.

Fitting of curves. Fittings were made by the method of least squares. Non-linear functions were fitted to the data using the 'Simplex' method of Nelder & Mead (1965) or by a maximum likelihood method (available as library subroutines on ICL 4·70, Rothamsted Experimental Station). The linear regression of Fig. 2 was unweighted.

The linear transformation S/v *vs.* S was used in Fig. 8 to replot the data of Fig. 7, since the variable S/v presents a more uniform distribution of the variance along the abscissa than do the usual Lineweaver-Burk and Eadie-Hofstee plots. The weighted linear regression applied and the standard errors of the mean calculated for the reciprocal data were according to Wilkinson (1961). No refinement of the calculated parameters was made.

RESULTS

Effect of Tl on the ouabain-sensitive K influx in high-Na medium. In order to avoid net Tl movements during the measurement of K influx at different Tl concentrations in high-Na medium, for the K influx experiments shown in Figs. 1 and 5 the red cells were pre-incubated in the corresponding Tl media in the absence of ^{42}K. Preliminary experiments to measure ^{204}Tl fluxes in human red cells had revealed that the cells equilibrated completely with external Tl within 30 min at 37° C, although at 5 mM-Na_o, ^{204}Tl equilibrated considerably faster. The pre-equilibration was therefore omitted in later K influx experiments and, for convenience, in Na efflux experiments.

Since Tl can replace K in activating membrane (Na-K)ATPase (Britten & Blank, 1968) and K-activated phosphatase (Inturrisi, 1969), one would expect it to inhibit the ouabain-sensitive K influx in red cells. Moreover, if the thallous ion can be translocated by the pump machinery, replacing partially for K, there should be an activation of the ouabain-sensitive K influx by low concentrations of Tl when the measurements are made at high external Na and low external potassium concentrations, as predicted by the two-site model of Sachs & Welt (1967) for K influx. Fig. 1 shows the result of one of three similar experiments to test this possibility. It can be seen that while at 0·17 mM external K concentration, Tl first stimulates the ouabain-sensitive K influx with a maximum at 0·1 mM-Tl, at 1·0 mM external K, Tl only inhibits monotonically. It should be noted that although the maximal activation of ouabain-sensitive K influx by Tl is in these conditions about 60 % of the control influx, it amounts to only 0·042 m-mole/l.cells. hr, i.e. about 4 % of the estimated maximal rate. In another of these three experiments in high Na medium (not

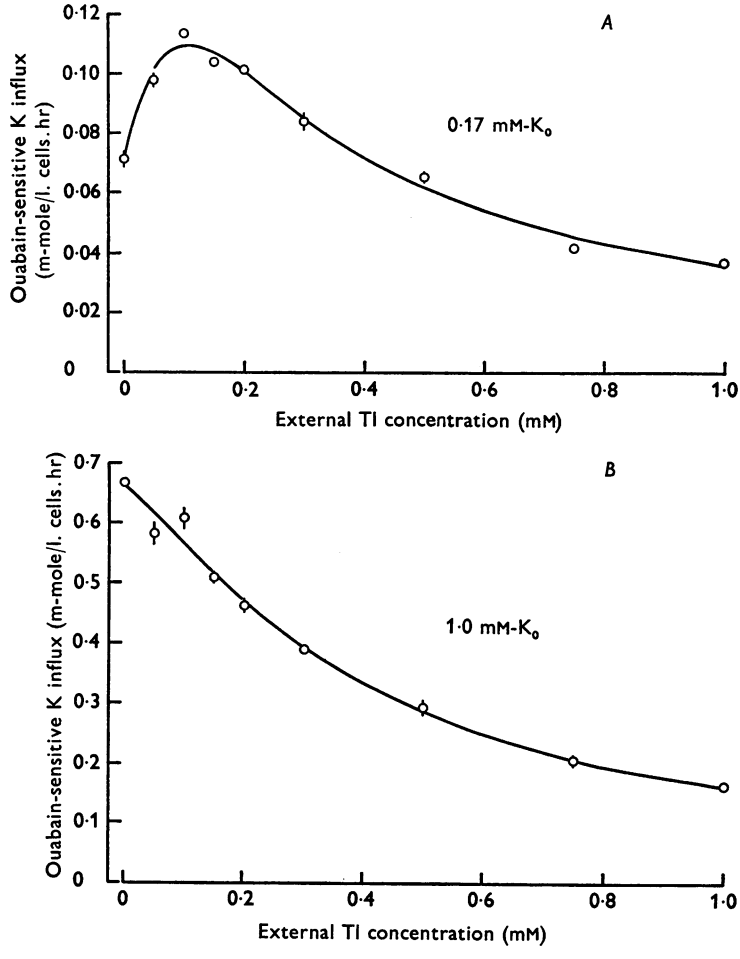

Fig. 1. The effect of Tl on the ouabain-sensitive K influx in high-Na medium. Nine lots of washed red cells were pre-incubated for 3 hr at 37° C in 'high-Na medium' (see Methods for composition) with Tl ranging from 0 to 1 mM, washed and resuspended in fresh portions of the same media in the cold. ^{42}K influx was measured in triplicate $\pm 10^{-4}$ M ouabain for 1 hr at 37° C in aliquots of these suspensions, at two different external K concentrations and at an average haematocrit of 1·6%. *A*, 0·17 mM external K concentration. The curve drawn represents the equation

$$v = \frac{A + B[Tl_o]}{1 + C[Tl_o] + D[Tl_o]^2},$$

fitted by least squares with $A = 0{\cdot}0709$, $B = 1{\cdot}11$, $C = 3{\cdot}82$ and $D = 29{\cdot}0$. *B*, 1·0 mM external K concentration. The curve represents the same equation as above, with parameters $A = 0{\cdot}661$, $B = 1{\cdot}33$, $C = 3{\cdot}23$ and $D = 7{\cdot}94$. The vertical bars indicate $\pm$ 1 S.E. of mean.

shown) thallium concentrations in the range 0·01–0·1 mM were used, and the ouabain-sensitive K influx increased linearly with external Tl concentration, both at 0·17 and at 0·40 mM external K.

Effect of Tl on the ouabain-sensitive sodium efflux at 5 mM external Na. This group of experiments was designed to investigate the ability of Tl to support ouabain-sensitive Na efflux from red cells into a K-free medium. Although Tl might substitute effectively for K at the external sites of the pump, it was quite likely that at low Tl concentrations, a fraction of the ouabain-sensitive Na efflux into K-free high-Na medium could represent Na:Na exchange. Na efflux was therefore measured at 5 mM external Na,

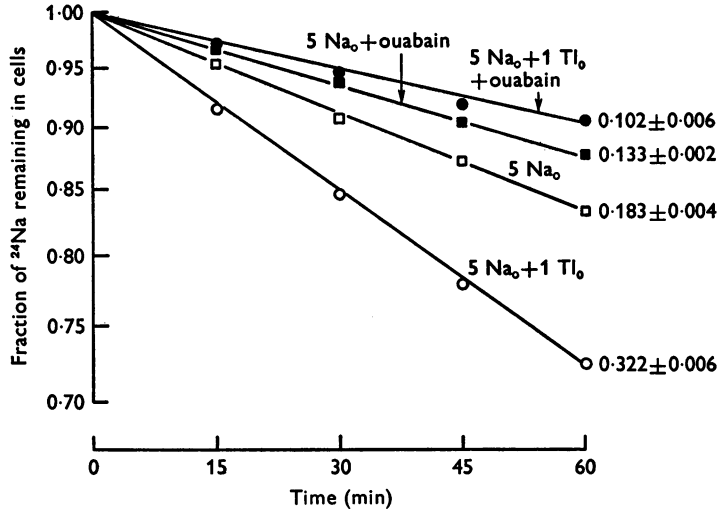

Fig. 2. Effect of 1 mM-Tl and 10^{-4} M ouabain on the Na efflux from red cells into K-free 5 mM-Na medium. ^{24}Na-loaded cells were washed with and finally resuspended in the cold (at 40 × the final haematocrit) in '5 mM-Na medium'. The ^{24}Na efflux was measured for periods up to 1 hr at 37° C and 0·5 % haematocrit in a medium of the same composition ± 1 mM-Tl ± 10^{-4} M ouabain. □, *5 mM-Na$_o$*; ○, *5 mM-Na$_o$ + 1 mM-Tl$_o$*; ■, *5 mM-Na$_o$ + ouabain*; ●, *5 mM-Na$_o$ + 1 mM-Tl$_o$ + ouabain*. The straight lines represent the least squares regression lines on the experimental points and the numbers are the rate constants for the efflux (regression coefficients) ± S.E. of means. The linear correlation coefficients were all better than 0·995. The intracellular Na content was 7·4 m-mole/l. cells.

since it is known that both the ouabain-sensitive Na:Na exchange and the uncoupled ouabain-sensitive Na extrusion become very small at 5 mM Na outside (Garrahan & Glynn, 1967*a*; Lew, Hardy & Ellory, 1973). The result of a time-course experiment is shown in Fig. 2. Clearly, 1 mM-Tl is able to support an ouabain-sensitive Na efflux into this medium

(1·631 ± 0·065 m-mole/l.cells.hr, mean ± S.E. of mean), while in the absence of Tl the ouabain-sensitive Na efflux is small (0·375 ± 0·033 m-mole/l.cells.hr). It is also clear that the Na efflux in the presence of 1 mM-Tl follows a single exponential, that goes through the origin, although for at least part of the first 15 min, net Tl influx must have occurred in this type of experiment. An inhibition of the ouabain-insensitive component of the Na efflux in the presence of Tl is apparent, an effect that will be dealt with later.

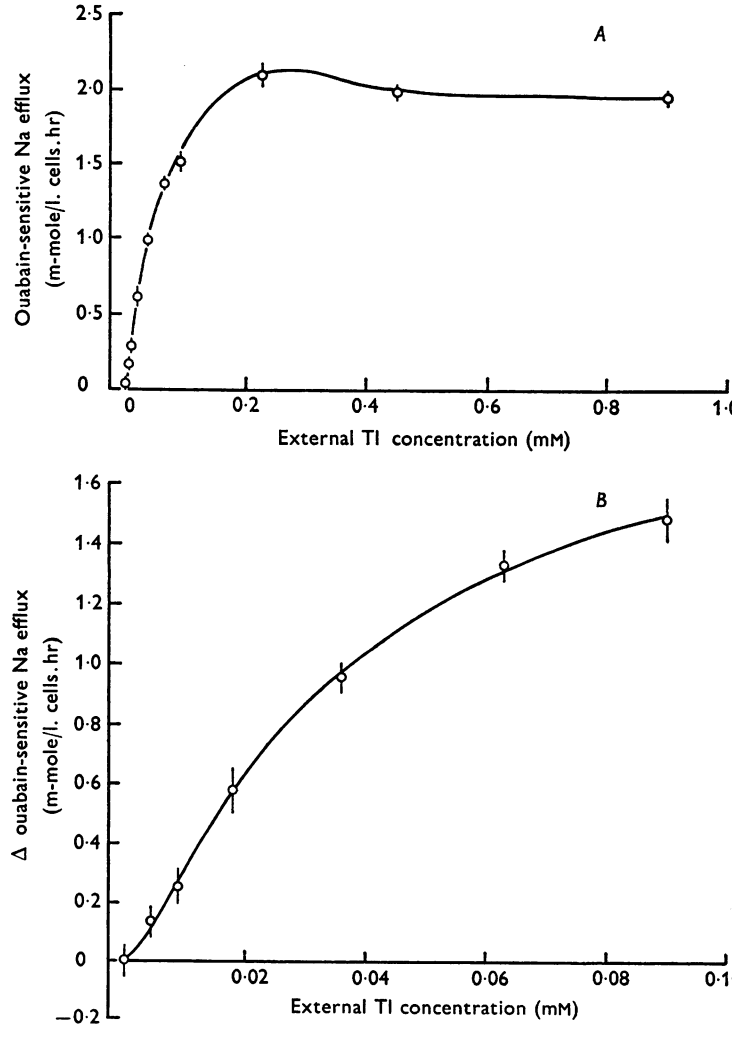

Fig. 3. For legend see facing page.

Fig. 3 shows the result of an experiment to determine the dependence of the ouabain-sensitive Na efflux on Tl concentration in the medium. It shows that Tl is able to activate the pump with an affinity that is considerably higher than that of K under the same conditions (Garrahan & Glynn, 1967*b*), the Na efflux being half-maximal between 0·03 and 0·05 mM external Tl. Up to 0·10 mM external Tl, the Tl-dependent part of the ouabain-sensitive Na efflux fits well with a two-site model for the combination of Tl with the pump. Besides, these cells (in contrast to the cells in Fig. 2 and from a different donor) show essentially no ouabain-sensitive Na efflux into K-free Tl-free 5 mM-Na medium. As the concentration of Tl is increased beyond 0·1 mM, however (Fig. 3*A*), it tends to inhibit the ouabain-sensitive Na efflux.

An experiment was then carried out to see how K and Tl act together in supporting the outward Na movement through the pump. Fig. 4 shows the effect of increasing Tl concentrations on the ouabain-sensitive Na efflux from red cells into a 5 mM-Na (choline) medium and *in the presence* of 5 mM external K. The filled symbol close to the ordinate represents the control ouabain-sensitive Na efflux in the same basic medium but at 10 mM-K_o, showing that the pump is almost completely saturated by 5 mM-K_o at the external K sites. The results indicate that Tl does not increase the ouabain-sensitive Na efflux beyond the maximal rate attained with saturating K_o concentration and, considering the activation of the pump by Tl shown in Fig. 3, suggests strongly that Tl substitutes for K stoichiometrically at the external activation sites, keeping the ouabain-sensitive Na efflux and hence pump turnover almost unchanged. To test this point further, the effect of 0·94 mM-Tl on the ouabain-sensitive K influx

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Fig. 3. The effect of Tl on the ouabain-sensitive Na efflux from fresh red cells into K-free 5 mM-Na medium. The ^{24}Na-loaded cells were washed with and resuspended in '5 mM-Na medium' (at 15 × the final haematocrit and in the cold). The ^{24}Na efflux was measured in triplicate for 5 and 45 min at 37° C and at a final haematocrit below 1 %, in the same K-free 5 mM-Na medium and containing varying concentrations of Tl ± 10^{-4} M ouabain. *A*, dependence of the ouabain-sensitive sodium efflux on Tl concentration up to 1 mM-Tl_o. The curve has been drawn by eye. *B*, magnification of the onset of the curve. The small remaining ouabain-sensitive Na efflux in the absence of Tl has been deducted from all values and new standard errors calculated. The curve has been drawn for the equation

$$\Delta v = \frac{2{\cdot}187}{1 + \frac{0{\cdot}03923}{[Tl_o]} + \frac{0{\cdot}0002130}{[Tl_o]^2}}$$

fitted by least squares to the experimental differences up to 0·1 mM-Tl_o. All vertical bars indicate ± 1 S.E. of mean.

was measured in another experiment at K_o = 5 mM, Na_o = 5 mM, i.e. the same external conditions as those of Fig. 4. The results showed that the ouabain-sensitive K influx was inhibited by 40·2 ± 2·9 % at 5 mM-K_o (77·5 ± 2·8 % at 1 mM-K_o), whilst in the experiment of Fig. 4, the ouabain-sensitive Na efflux at 0·9 mM-Tl was inhibited by 13·7 ± 4·4 %. This suggests that 0·9 mM-Tl supports about 30 % of the ouabain-sensitive Na efflux in the experiment of Fig. 4, in substitution for external K.

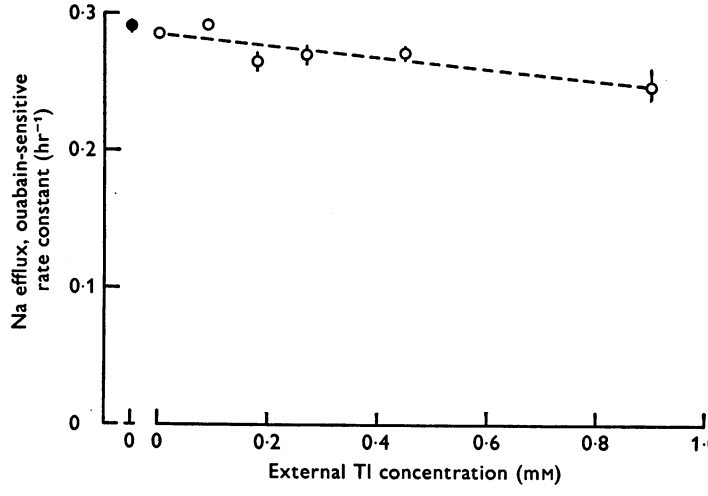

Fig. 4. The effect of Tl on the ouabain-sensitive Na efflux from fresh red cells into 5 mM-K 5 mM-Na medium. ^{24}Na-loaded red cells were washed with and resuspended (in the cold and at 15 × the final haematocrit) in a medium containing (mM): Na 5, K 5, choline 130·5, Mg 2, Tris (pH 7·9 at 20° C) 15, Cl 153·5 and glucose 10. The efflux of ^{24}Na was measured in triplicate for 5 and 45 min at 37° C and at a final haematocrit of 0·95 % in the same medium as above and containing varying concentrations of Tl ± 10^{-4} M ouabain. The filled symbol shows the value of the efflux when measured in 10 mM-K 5 mM-Na medium in the absence of Tl. The vertical bars represent ± 1 S.E. of mean.

Effect of Tl on the ouabain-sensitive K influx at low external Na. Since the affinity of the pump for K outside is greatly increased by reducing the external Na concentration (Post, Merritt, Kingsolving & Albright, 1960; Garrahan & Glynn, 1967*b*) one would expect increasing concentrations of Tl_o to produce little or no stimulation of the ouabain-sensitive K influx at 5 mM external Na and low external K, but instead to inhibit it steadily in a way that paralleled the Tl activation of ouabain-sensitive Na efflux as shown in Fig. 3.

The effect of Tl on ouabain-sensitive K influx at 5 mM external Na was

then investigated at varying K_o concentrations; the results are shown in Fig. 5. In the absence of Tl (upper curve) the ouabain-sensitive K influx curve is slightly sigmoid (see Garrahan & Glynn, 1967*b*) and seems in the present conditions adequately described (with $V_{max} = 0{\cdot}468 \pm 0{\cdot}026$ m-mole/l.cells.hr) by a quadratic equation similar to that used for Tl in Fig. 3*B* and derived for a model that implies that two K ions must bind to the outside of the pump for ouabain-sensitive K influx to occur (Sachs & Welt, 1967). At 0·06 mM-Tl_o it is evident that a sigmoid-to-hyperbolic transition has taken place. In this case, by changing the fitted hyperbola into the basic Michaelis expression (Dixon & Webb, 1964*a*), one can obtain $0{\cdot}444 \pm 0{\cdot}189$ m-mole/l.cells.hr for V^{max} and $0{\cdot}426 \pm 0{\cdot}155$ mM for the apparent $K_{\frac{1}{2}}$ for external K. This V^{max} value is not different from that in the absence of Tl, whereas the apparent affinity for K_o is evidently decreased, these two features being characteristic of competitive inhibition. At 0·02 mM external Tl, the ouabain-sensitive K influx can be fitted by a more general equation which can also fit the data for 0·06 mM-Tl_o (the parameters A and D then adopting values not significantly different from zero, see legend to Fig. 5); the expression left in this case is a hyperbola closely similar to that already fitted to the same data, and its parameters give 0·468 m-mole/l.cells.hr for V_{max} and 0·451 mM for the apparent $K_{\frac{1}{2}}$. The low values for the Na content of the cells (measured when the final incubation was half-way through) must have mostly originated during the pre-incubation in 5 mM-Na medium at 37° C. The reduced ouabain-sensitive K influx values in this experiment are consistent with this low cell Na, since the K-dependent ouabain-sensitive Na efflux does not become saturated until cell Na reaches nearly 35 m-mole/l.cells (Sachs, 1970; Garay & Garrahan, 1973).

The ouabain-sensitive K influx was then measured at 5 mM external Na with increasing Tl concentrations (0–0·2 mM) and at two external K concentrations; the washed cells were pre-incubated with Tl up to 3 hr at 37° C. Two experiments (not presented), one of them in all-chloride media and the other in all-sulphate media (using sulphate-loaded cells), gave apparently hyperbolic curves for inhibition by Tl, with a similar concentration-dependence as the activation of ouabain-sensitive Na efflux in similar conditions (Fig. 3); however, Dixon replots ($1/v$ *vs.* the inhibitor concentration) yielded straight lines that when fitted met *on* the horizontal axis, a feature of fully *non*-competitive inhibition (Dixon & Webb, 1964*b*). Since from the Na efflux experiments, it seemed unlikely that Tl inhibited strongly at the internal sites of the pump, we investigated the possibility that the non-competitive type of inhibition could be an artifact arising in the variations in cell Na due to preincubation at varying Tl concentrations in 5 mM-Na medium (up to 20 % of the control cell Na

content). To avoid significant effects of changes in the cell Na occurring during an experiment, the concentration was adjusted to about 90 m-mole/l. cell water, using the nystatin method (Cass & Dalmark, 1973). This concentration allows enough margin for a displacement of Na_c by 3–15 m-mole/l. cell water not to affect the ouabain-sensitive Na efflux significantly at the K_c used (see Garay & Garrahan, 1973, Figs. 8*a* and 9) and does not increase the Na loss excessively; similarly, cell K was adjusted to about 35 m-mole/l. cell water to ensure a sufficient increase of the affinity for Na_c at the internal Na sites, without decreasing V^{max} for Na:K exchange (see Garay & Garrahan, 1973, Figs. 8*a*, 9 and 10). The preincubation of the cells with Tl at 37° C was omitted.

The result of one experiment to measure the initial region of the ouabain-sensitive K influx curve of cation-loaded cells at 5 mM external Na and different Tl concentrations, is shown in Fig. 6. It can be observed that although the flux is quite large, the sigmoid shape of the ouabain-sensitive K influx curve at 5 mM-Na_o is maintained. Tl, however, fails in these conditions to transform the ouabain-sensitive K influx curve to a hyperbola, causing instead a slight stimulation of the ouabain-sensitive K influx at low concentrations of external Tl and K. The cell Na and K

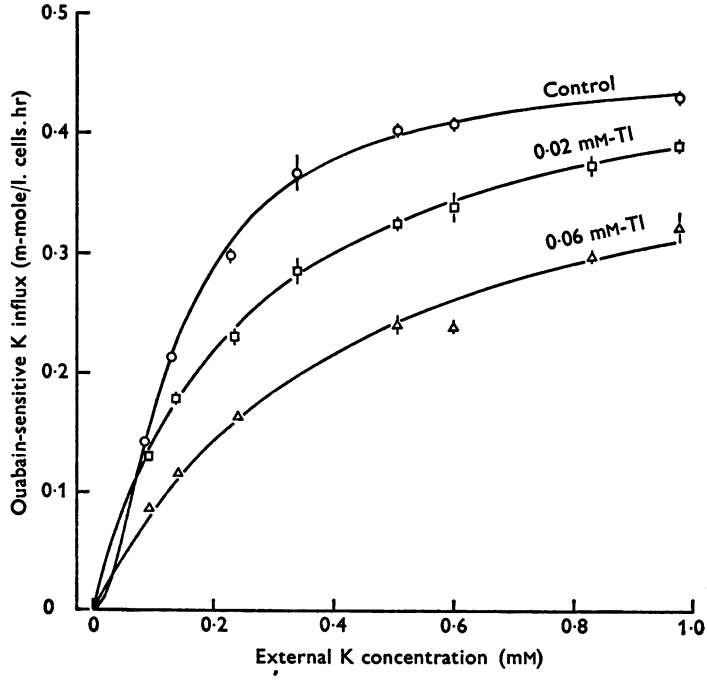

Fig. 5. For legend see facing page.

concentrations at the beginning and at the end of the incubation are given in Table 1*a*, and it can be seen that thallium does not induce variations in the intracellular Na and K concentrations, except at 1 mM.

As the curves obtained were not ready amenable to kinetic analysis, the experiment was repeated at very low external Na. When the medium is nominally Na-free, the ouabain-sensitive K influx curve becomes quasi-hyperbolic (Garrahan & Glynn, 1967*b*); however, since even at low haematocrits one can expect a build-up of Na in the external solution when dealing with high-Na cells, we set the initial Na_o to 0·1 mM in order to buffer external variations, and reduced the incubation period to 40 min to minimize changes in the internal Na and K concentrations. The result of such an experiment is shown in Fig. 7. It can now be seen that all four sets of results can be fitted well by hyperbolic equations. The reciprocal

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Fig. 5. Effect of Tl on the K influx curve in 5 mM-Na medium. Three separate lots of washed red cells were pre-incubated at 37° C and 10% haematocrit for 30 min, in '5 mM-Na medium' plus 0 or 0·02 or 0·06 mM-Tl. After a wash, they were pre-incubated for a further 60 min in the same media and conditions. After a final wash and resuspension in the same media, ^{42}K influx was measured in triplicate at the different external K concentrations shown ± 10^{-4} M ouabain, for 1 hr at 37° C and at an average haematocrit of 0·4%. ○, *Control*: the curve represents the equation

$$v = \frac{A}{1 + \frac{B}{[K_o]} + \frac{C}{[K_o]^2}},$$

fitted to the data by least squares with $A = 0{\cdot}468 \pm 0{\cdot}026$ (mean ± S.E. of mean), $B = 0{\cdot}0683 \pm 0{\cdot}0402$ and $C = 0{\cdot}0115 \pm 0{\cdot}0038$. □, *0·02 mM-$Tl_o$*; the curve is drawn for the equation

$$v = \frac{A + B[K_o]}{1 + C[K_o] + \frac{D}{[K_o]}},$$

fitted by the same procedure with $A = 0{\cdot}267 \pm 0{\cdot}097$, $B = 0{\cdot}817 \pm 0{\cdot}249$, $C = 1{\cdot}66 \pm 0{\cdot}54$ and $D = 0{\cdot}129 \pm 0{\cdot}067$. △, *0·06 mM-$Tl_o$*; the curve is drawn for the equation

$$v = \frac{A[K_o]}{1 + B[K_o]},$$

fitted by least squares with $A = 1{\cdot}04 \pm 0{\cdot}23$ and $B = 2{\cdot}34 \pm 0{\cdot}85$; the data can also be fitted by the same function as the middle curve with $A = -0{\cdot}044 \pm 0{\cdot}129$, $B = 1{\cdot}04 \pm 0{\cdot}14$, $C = 2{\cdot}21 \pm 0{\cdot}14$ and $D = -0{\cdot}057 \pm 0{\cdot}131$. The vertical bars indicate ± 1 S.E. of mean. Duplicate determinations of the cell Na content gave (m-mole/l. cells): 5·3 and 5·2 for the control cells, 5·8 and 5·0 for the 0·02 mM-Tl_o cells and 4·2 and 4·2 for the 0·06 mM-Tl_o cells; the duplicates for the K contents were (m-mole/l. cells): 94 and 90 for the control cells, 90 and 88 for the 0·02 mM-Tl_o cells and 80 and 83 for the 0·06 mM-Tl_o cells.

(S/v *vs.* S) plot is shown in Fig. 8. The straight lines have been fitted by weighted least-squares linear regression (Wilkinson, 1961) and their regression coefficients do not differ significantly. The lines are parallel and hence the inhibition of ouabain-sensitive K influx by Tl is strictly

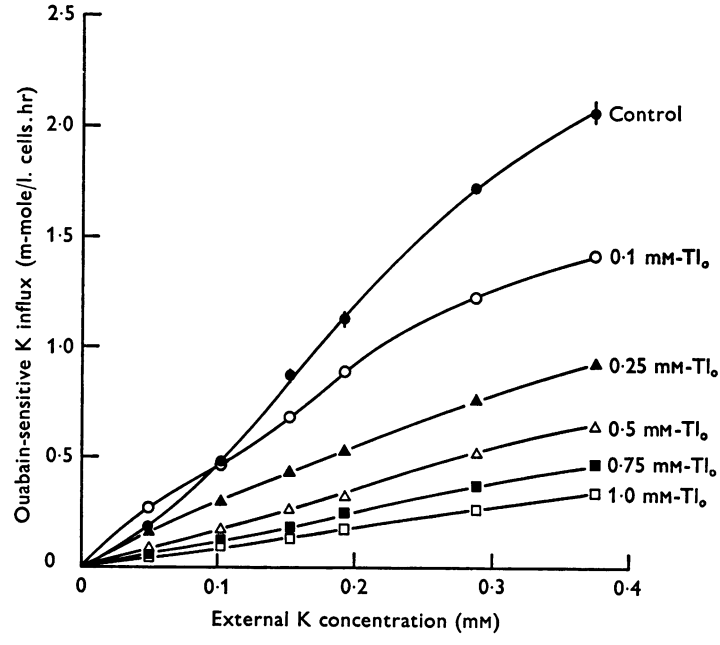

Fig. 6. The rising part of the K influx curve of high-Na cells in 5 mM-Na medium and the effect of Tl.

Washed red cells were loaded for 30 min as described in Methods. The cations in the loading solution were (mM) Na 90·6, K 31·8 and choline 30. After four washes with the loading solution (nystatin-free), the cells were resuspended in the same solution and stored overnight at 4° C. The cells were then spun down and washed twice with cold 'high-Na medium' and divided into six lots of which two were resuspended in more 'high-Na medium' and kept in the cold; the other four were given one wash with cold '5 mM-Na medium' and the cells resuspended in cold solutions containing 0, 0·25, 0·50 and 0·75 mM-Tl respectively in '5 mM-Na medium'. After a final wash and resuspension in the same cold solutions, ^{42}K influx was measured in triplicate $\pm$ 10^{-4} M ouabain at varying external K concentrations. The incubation was for 60 min at 37° C and at an average haematocrit of 0·26 %. Meanwhile, and after standing 1 hr in the cold, the two other lots of cells were given similar treatment (at 0·1 and 1·0 mM-Tl) and the ^{42}K influx was measured consequently. (●), *Control*; (○), *0·10 mM-Tl$_o$* (▲), *0·25 mM-Tl$_o$*; (△), *0·50 mM-Tl$_o$*; (■), *0·75 mM-Tl$_o$*; (□), *1·00 mM-Tl$_o$*. The vertical bars represent $\pm$ 1 S.E. of mean. The curves have been drawn by eye. Cell Na and K and external K concentration were determined at the beginning and at the end of the 37° C incubation; cell Na and K data are given in Table 1*a*.

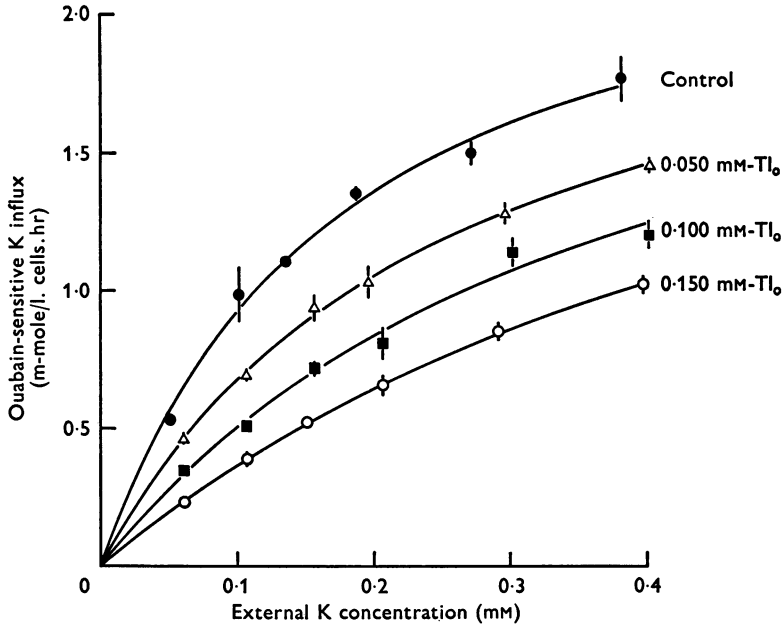

Fig. 7. The inhibition by Tl of the rising part of the K influx curve for high-Na cells in low Na medium.

Red cells were equilibrated as described in Methods, for 90 min, against (mM) Na 90·6, K 36·8 and choline 25. After 4 washes with the loading solution (nystatin-free) the cells were resuspended in more of the same solution and stored overnight at 4° C. The cells were then spun down and washed twice in cold 'high-Na medium', divided in four lots and then washed 3 times with '0·1 mM-Na medium'. The cells were resuspended in cold solutions containing 0, 0·05, 0·10 and 0·15 mM-Tl in '0·1 mM-Na medium'; after one wash and resuspension in the same respective solutions, ^{42}K influx was measured in triplicate at the different K concentrations shown $\pm 10^{-4}$ M ouabain. The incubation was for 40 min at 37° C and at an average final haematocrit of 0·33 %.

Na and K concentrations were determined in the supernatants after spinning the cells at the end of the 40 min incubation at 37° C. (●), *Control*; 0·25 mM-Na$_o$, average; (△), *0·05 mM-Tl$_o$*, 0·205 mM-Na$_o$, av.; (■), *0·10 mM-Tl$_o$*, 0·195 mM-Na$_o$ av.; (○), *0·15 mM-Tl$_o$*, 0·225 mM-Na$_o$, av. The bars represent ± S.E. of mean. The curves represent the hyperbola

$$v = \frac{V^{max}}{1 + \frac{K'_i}{[K_o]}}$$

calculated for V^{max} (m-mole/l.cells.hr) and K'_i (mM): 2·561 ± 0·170 and 0·1745 ± 0·0186 (control); 2·361 ± 0·068 and 0·2443 ± 0·0116 (0·05 mM-Tl$_o$); 2·413 ± 0·331 and 0·3716 ± 0·0629 (0·10 mM-Tl$_o$) and 2·582 ± 0·067 and 0·5958 ± 0·0287 (0·15 mM-Tl$_o$). These parameters were obtained from the weighted linear regressions in Fig. 8. Cell Na and K were determined at the beginning and the end of the 37° C incubation period and are shown in Table 1*b*.

competitive. However, the secondary plot of the intercepts with the abscissa (apparent $K_{\frac{1}{2}}$ for K_o) against Tl concentrations (not shown) gives a parabolic curve instead of the straight line that would have been expected for apparently one-site kinetics; this suggests that more than one Tl must

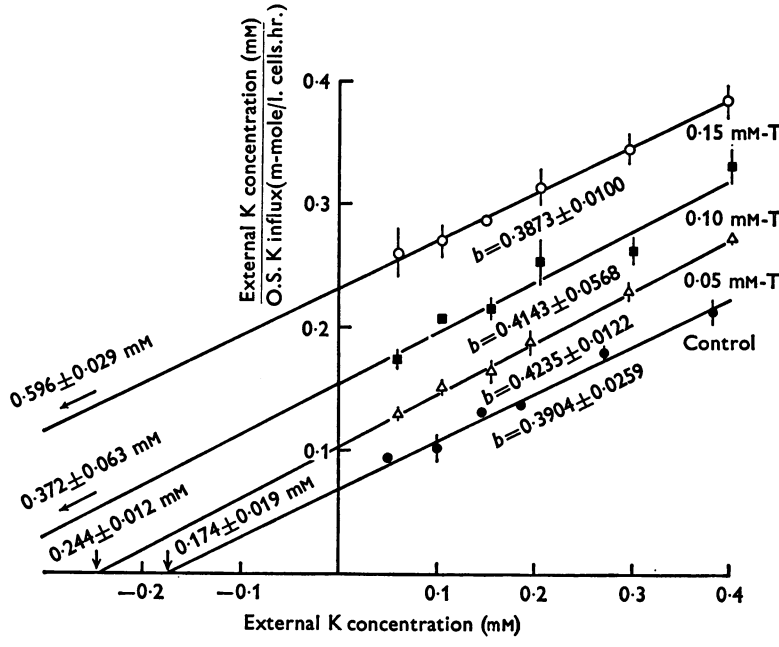

Fig. 8. Reciprocal plot of the data of Fig. 7. The straight lines represent the equation

$$\frac{[K_o]}{v} = a + b\,[K_o]$$

fitted by the weighted least squares regression of Wilkinson (1961). (●), *Control*, $a = 0{\cdot}0681 \pm 0{\cdot}0057$, $b = 0{\cdot}3904 \pm 0{\cdot}0259$, $r = +0{\cdot}996$; (△), *0·05 mM-Tl$_o$*, $a = 0{\cdot}1035 \pm 0{\cdot}0030$, $b = 0{\cdot}4235 \pm 0{\cdot}0122$, $r = +0{\cdot}9999$; (■), *0·10 mM-Tl$_o$*, $a = 0{\cdot}1540 \pm 0{\cdot}0153$, $b = 0{\cdot}4143 \pm 0{\cdot}0568$, $r = +0{\cdot}997$; (○), *0·15 mM-Tl$_o$*, $a = 0{\cdot}2308 \pm 0{\cdot}0028$, $b = 0{\cdot}3873 \pm 0{\cdot}0100$, $r = +0{\cdot}99996$.
All vertical bars indicate ± 1 S.E. of mean on the data, calculated as described in the above mentioned article.

bind for the competitive inhibition to occur. Table 1*b* shows the cell Na and K concentrations in the experiment of Fig. 7. The magnitude of the variations of Na_c are within the limits to keep the Na-loading sites fully saturated and those of K_c to leave V^{max} of ouabain-sensitive Na efflux and hence pump turnover unaffected (see Garay & Garrahan, 1973; Sachs, 1970); and variations in Na_o concentration are small enough not to shift

per se $K_{\frac{1}{2}}$ of external K for ouabain-sensitive K influx (Garrahan & Glynn, 1967*b*). Although the mean Na_c at 0·39 mM-K_o drops from 94 m-mole/l. cell water in the control cells, to 81 m-mole/l. cell water in the 0·15 mM-Tl_o cells, this does not affect V^{max} for ouabain-sensitive K influx (see legend to Fig. 7). Incidentally, Figs. 6 and 7 and Table 1 show that the gross characteristics of the (control) ouabain-sensitive K influx curve are not changed after the nystatin treatment and that the cells are not unduly leaky to Na or K.

TABLE 1*a*. Cell Na and K contents in experiment of Fig. 6

A portion of each cell suspension was washed 5 times in cold 'Mg-Tris solution' at the moment that the flux incubation began (initials), while other aliquots (finals) were incubated at 37 °C in parallel with the flux tubes at both the lowest and the highest K_o used; after cooling, the cells were washed as for the initials. Cell water content was determined in separate aliquots of the suspensions

	Na_c and K_c (m-mole/l. cell water)					
			Finals			
	Initials		0·05 mM-K_o		0·37 mM-K_o	
	Na_c	K_c	Na_c	K_c	Na_c	K_c
Control	95	37	92	34	86	37
0·10 mM-Tl_o	94	34	87	34	89	35
0·25 mM-Tl_o	92	35	81	34	82	35
0·50 mM-Tl_o	93	36	84	36	87	36
0·75 mM-Tl_o	95	35	81	33	88	44
1·00 mM-Tl_o	94	33	79	30	76	30

TABLE 1*b*. Cell Na and K contents in experiment of Fig. 7. The same procedure described for 1*a* was used.

	Na_c and K_c (m-mole/l. cell water)					
			Finals			
	Initials		0·06 mM-K_o		0·39 mM-K_o	
	Na_c	K_c	Na_c	K_c	Na_c	K_c
Control	98	35	95	33	90	35
0·05 mM-Tl_o	98	34	95	35	87	38
0·10 mM-Tl_o	84	30	88	35	88	37
0·15 mM-Tl_o	84	30	83	32	77	34

Effect of Tl on ouabain-insensitive fluxes. The experiment of Fig. 2 showed that at the same time that Tl activated ouabain-sensitive Na efflux into a K-free 5 mM-Na medium, it decreased significantly the Na efflux in the presence of 10^{-4}M ouabain. In Fig. 9, ouabain-insensitive

Na efflux into K-free 5 mM-Na medium is plotted as a function of external Tl concentration. The figure shows that 0·9 mM-Tl_o can inhibit up to 25 % of ouabain-insensitive Na efflux. About half of this inhibition occurs below 0·02 mM-Tl_o whilst for the other half, Tl has a very low affinity of the same order of magnitude as that for the partial inhibition of ouabain-sensitive Na efflux (see Fig. 3).

The effect of Tl on ouabain-insensitive K influx in both high-Na and 5 mM-Na media is shown in Fig. 10. Curves 10*A* show that Tl inhibits ouabain-insensitive K influx in high-Na medium; Fig. 10*B*, covering a smaller Tl_o concentration range, demonstrates that the inhibition of ouabain-insensitive K influx by Tl is comparable whether in high-Na or in 5 mM-Na media.

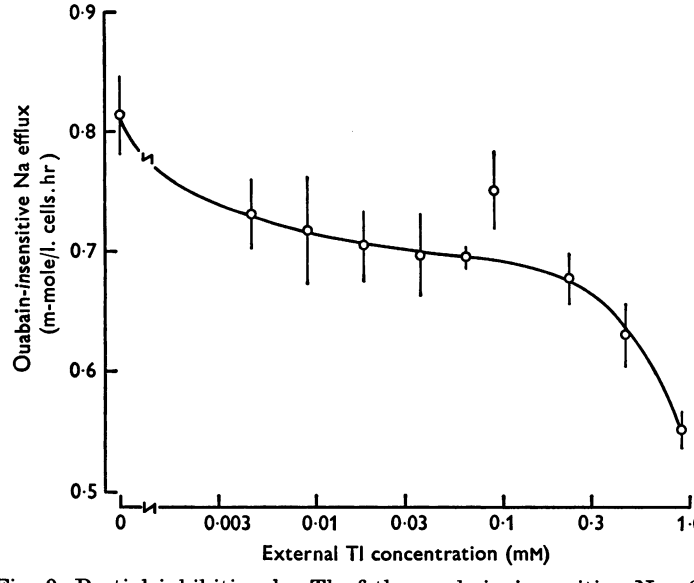

Fig. 9. Partial inhibition by Tl of the ouabain-*in*sensitive Na efflux into 5 mM-Na medium. Na efflux values measured in the presence of 10^{-4} M ouabain, corresponding to the experiment of Fig. 3. The logarithmic scale was chosen simply for convenience. The bars indicate ± 1 S.E. of mean. The curve has been drawn by eye.

DISCUSSION

The experiments reported here allow us to conclude that Tl can substitute for K at the external K sites of the Na pump supporting ouabain-sensitive Na efflux and inhibiting the ouabain-sensitive K influx competitively.

In conditions of negligible Na:Na exchange through the pump, Tl can support an ouabain-sensitive Na efflux from human red cells with a greater

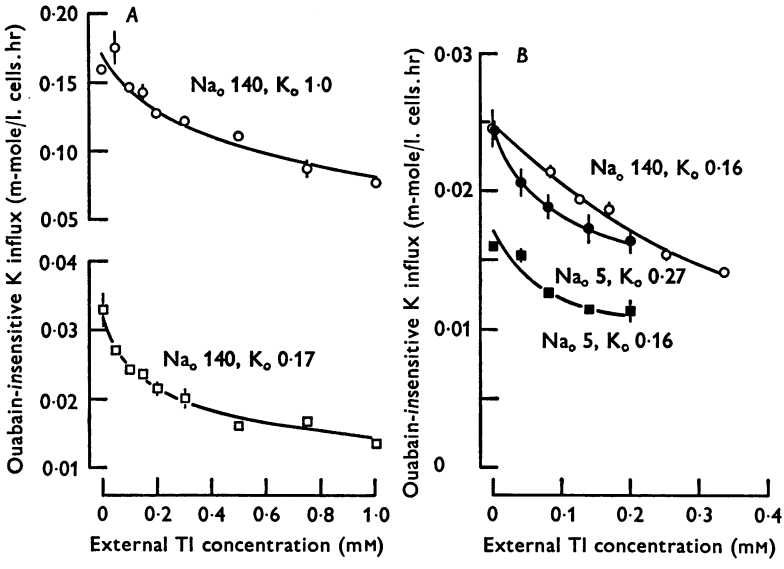

Fig. 10. Inhibition by Tl of the ouabain-*in*sensitive K influx at 5 and 140·5 mM external Na. All K influxes shown were measured in the presence of 10^{-4} M ouabain. *A*, ouabain-insensitive K influx from 140·5 mM-Na medium corresponding to the experiment of Fig. 1. (□), *0·17 mM-K$_o$*; (○), *1·0 mM-K$_o$*. *B*, comparison of the Tl-dependence of the ouabain-insensitive K influx from 140·5 and 5 mM-Na media. (○), *140·5 mM-Na and 0·16 mM-K$_o$ medium*, experiment made in conditions similar to those in Fig. 1; (●), *'5 mM-Na medium' and 0·27 mM-K$_o$*; (■), *'5 mM-Na medium' and 0·16 mM-K$_o$*. The vertical bars indicate ± 1 S.E. of mean. The curves have been drawn by eye.

affinity than K and displaying two-site kinetics. Thallium distributes very rapidly across the red cell membrane (Gehring & Hammond, 1964) and, although we do not know the actual intracellular concentrations of Tl at equilibrium in the experiments shown, preliminary results of tracer distribution suggest that they are not greatly different from the external Tl concentrations. A Tl_i/Tl_o ratio near 1 has been reported recently for human red cells in sulphate medium (Skulskii, Manninen & Järnefelt, 1973). There seems to be little doubt, however, that the activation of ouabain-sensitive Na efflux occurs on the outside aspect of the pump, considering the effects of Tl on ouabain-sensitive K influx which are in line with the observed substitution of K for Tl in the (Na-K)ATPase and K-activated phosphatase, and with the ability of Tl to promote the splitting of a phosphorylated intermediate in guinea-pig kidney membrane (Post, Hegyvary & Kume, 1972), even more effectively than K. The slight inhibition of ouabain-sensitive Na efflux at the highest Tl_o concentrations (that can also be seen in Fig. 4), may be on a different locus

of the pump; this parallels well the activation–partial inhibition curve of kidney (Na-K)ATPase by Tl (Britten & Blank, 1968) measured in high-Na medium and that we have also observed with red cell ghost ATPase (not shown).

Since the turnover of the pump as judged from the value of ouabain-sensitive Na efflux remains essentially unchanged in the presence of increasing concentrations of Tl (Fig. 4) at the same time that ouabain-sensitive K influx is inhibited, it seems that Na:Tl exchange takes place through the pump. However, ouabain-sensitive Tl fluxes and hence the stoichiometry of Na:Tl exchange have not been measured so far, due to the rapid ouabain-insensitive Tl movements which occur across the red cell membrane.

The results of the Na efflux experiments of Figs. 3 and 4 seem to preclude the possibility that the inhibition of ouabain-sensitive K influx by Tl is due to an effect at the inside aspect of the Na pump (e.g. at the 'Na-loading sites'), since an external Tl concentration of 0·9 mM in 5 mM-Na medium can support ouabain-sensitive Na efflux of about 2 m-mole/l.cells.hr (Na_c = 7 m-mole/l.cells). In similar conditions this Tl concentration inhibits as much as 78 % of the ouabain-sensitive K influx at an external K concentration of 1 mM.

When the affinity for K outside is low (high-Na medium), Tl can actually stimulate the ouabain-sensitive K influx (Fig. 1). This fact together with the sigmoid-to-hyperbolic transition of the ouabain-sensitive K influx curve that occurs with low-Na cells in 5 mM-Na medium (Fig. 5), and the slight stimulation observed at 0·1 mM-Tl with high-Na cells (Fig. 6) show that K and Tl can attach simultaneously to the outside of the pump in at least one form that can promote active K influx (although not necessarily active Tl influx). A similar behaviour has been described by Sachs & Welt (1967) for Rb, Cs and Li in a high-Na medium.

It is apparent from the experiment of Fig. 5 that at concentrations which inhibit ouabain-sensitive K influx by about 20 %, Tl has already transformed the shape of the ouabain-sensitive K influx curve from sigmoid to hyperbolic. It seems then that in these conditions (Na_c = 5 m-mole/l.cells), Na inhibition at the outside of the pump can be relieved by Tl with a very high affinity (apparently higher than in high-Na medium), while the ouabain-sensitive K influx is left following Michaelis–Menten kinetics and is amenable in turn to competitive Tl inhibition. This is consistent with an interaction of Tl with the outside of the pump that implies binding to at least two sites and with different affinities. The cause for the restricted effect of Tl on ouabain-sensitive K influx in the experiment of Fig. 6, as compared with Fig. 5, is not clear: a possible explanation based on a change of the dissociation constant for Tl binding

at the outside of the pump caused by the change in the ionic conditions *inside* the cell, seems ruled out by evidence for independence of the dissociation constants at each side of the Na pump from the ionic conditions at the other side, presented by Hoffman & Tosteson (1971) and Garay & Garrahan (1973). Certainly the nystatin treatment seems not to have affected either the ouabain-sensitive K influx (which is of about the expected magnitude given the internal Na and K concentrations) or the kinetic behaviour of the pump.

At 0·25 mM-Na_o, however, the ouabain-sensitive K influx curves of Na-loaded cells are hyperbolic down to 0·05 mM-K_o and here Tl behaves unequivocally as a competitive inhibitor; hence, the non-competitive inhibition pattern of ouabain-sensitive K influx observed with non-loaded cells at 5 mM-Na_o may be due to changes in the cell Na concentration upon pre-incubation with Tl at 37° C. The need to bind more than one Tl to bring about the competitive inhibition at 0·25 mM-Na_o (where Michaelis–Menten kinetics seem to describe the ouabain-sensitive K influx curves satisfactorily) may be explained by recalling that even at Na_o concentrations as low as 15 μM, an inflexion of the ouabain-sensitive K influx curve can be found, at about 15 μM-K_o (Garrahan & Glynn, 1967*b*). This may mean that the reduction of the external Na concentration to nearly zero does not dispense with the need for K to bind externally at two sites, but simply that the dissociation constant for K_o at one of the binding sites is considerably decreased.

The high relative affinity of the outside of the Na pump for Tl deserves some comments. The corrected hydrated radius of thallous ion is 3·30 A°, essentially the same as those reported for K and Rb (Nightingale, 1959), whereas its crystal radius is 1·44 A° and lies between those of K and Rb (Robinson & Stokes, 1970). Eisenman's selectivity sequence III (Diamond & Wright, 1969) has been reported to apply for the external K sites of the Na pump of human erythrocytes (Sachs, 1967). From Fig. 3 and the control curve in Fig. 5 a value of 3–5 can be visually assessed for the Tl/K affinity ratio in these sites. Since sequence III is closer to the lyotropic series for alkali metals than to the sequence of non-hydrated radii, the participation of non-Coulomb forces seems necessary for the Tl-binding. Thallium stimulates rabbit kidney (Na-K)ATPase at concentrations about 5 times lower than K (Britten & Blank, 1968) and K-stimulated phosphatase from beef brain at 1/10 of the K concentrations (Inturrisi, 1969), throughout the concentration range. Similar 'displacements' of Tl from its expected position in the selectivity sequence occur in other systems as with goldfish intestine mucosal permeability, sequence IV (Ellory, Nibelle & Smith, 1973), and K-channel permeability in nerve axon, sequence IV (Hille, 1973), in both of which Tl shows the highest

permeability; and in the axonal Na-channel, sequence X or XI, where the anomalously high position of Tl has been interpreted in terms of polarization of the thallous ion by the 'site' (Hille, 1972).

Thallium can also inhibit ouabain-insensitive fluxes in human red cells. It is well known that some of the ouabain-insensitive Na efflux represents a saturable component and not merely passive diffusion (Hoffman & Kregenow, 1966; Sachs, 1971). In the presence of 10^{-4} M ouabain, Tl inhibits part of the Na efflux into a K-free medium (Fig. 9), and nearly half of this inhibition occurs at quite low Tl_o concentrations. External K has been reported to inhibit the Na efflux in the presence of ouabain (Sachs, 1971), an action which is not fully understood. Although at concentrations several hundred times lower than K, it is conceivable that Tl is acting on ouabain-insensitive Na efflux simply as a K substitute. On the other hand, Tl can inhibit part of the K influx in the presence of ouabain (Fig. 10). Garrahan & Glynn (1967*c*) have shown a saturable behaviour of the ouabain-insensitive K influx in high-Na medium, whereas in 2·3 mM-Na (choline) medium, the ouabain-insensitive K influx is linear with external K concentration (Glynn, Lew & Lüthi, 1970). In our experiments, however, the inhibition by Tl of the ouabain-insensitive K influx seems not confined to a 'saturable component', since our data for the K influx at 5 mM-Na_o in the presence of ouabain show a linear dependence on external K concentration (not shown) and yet part of this ouabain-resistant influx can be inhibited by Tl (Fig. 10*B*). As the dependence on Tl_o concentration is different for ouabain-insensitive Na efflux and ouabain-insensitive K influx, it is unlikely that both of these effects are related through the same mechanism. In any case, the possibility that the inhibitions of the ouabain-resistant fluxes occur due to Tl acting on the inside of the cell membrane cannot be excluded.

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