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FACTORS AFFECTING THE RELATIVE MAGNITUDES OF THE OUABAIN-SENSITIVE AND THE OUABAIN-INSENSITIVE FLUXES OF THALLIUM ION IN ERYTHROCYTES

I.A. SKULSKII [a], V. MANNINEN [b] and J. JÄRNEFELT [c]

[a] *Institute of Evolutionary Physiology and Biochemistry Academy of Science, Leningrad 194223 (U.S.S.R.),* [b] *Wihuri Research Institute, SF-00140 Helsinki 14, and* [c] *Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17 (Finland)*

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Summary

A maximal rate of the ouabain-sensitive ^{204}Tl influx in human erythrocytes can be attained at trace concentrations of Tl^+ in Mg^{2+} isotonic media free of K^+ and Na^+. The maximal influx of Tl^+ from isotonic $Mg(NO_3)_2$ at 20°C and pH 7.4 was 0.45 mM · l^{-1} · h^{-1} with a K_m of 0.025 mM. In contrast to the active influx of Tl^+, the passive Tl^+ fluxes were neither saturated nor influenced by external cations in the range of concentrations of Tl^+ and K^+ studied. The rate constants of Tl^+ passive fluxes in human and cat erythrocytes can be related to pH by the equation $\log k_{in(out)} = -A + B \cdot pH$, where A and B are empirical constants for particular conditions. The apparent activation energy was 16 and 11 kcal/mol in sulphate and nitrate media, respectively. Tl^+ and the alkali metal cations seem to overcome a common barrier in the erythrocyte membrane. Nevertheless, the rate of the passive penetration of Tl^+ is about two orders of magnitude faster than those of K^+ or Rb^+. An extra non-Coulombic interaction between Tl^+ and membrane ligands appears to be involved providing an accumulation of Tl^+ somewhere in the vicinity of the membrane barrier and increasing the diffusion fluxes of Tl^+ in both directions.

Introduction

Tl^+ has been found to substitute for K^+ in activating both the $(Na^+ + K^+)$-ATPase in human and rabbit erythrocytes [1—3] and the ouabain-sensitive Na^+ outward transport in human erythrocytes [2—5]. An accurate measurement of the ouabain-sensitive Tl^+ influx presumably coupled to the efflux of Na^+ proved to be a difficult task because of an extremely high passive permeability of the

erythrocyte membrane to Tl^+ compared to active Tl^+ influx [2,3,6]. The purpose of this investigation was to throw further light on the mechanism of the active and passive movement of Tl^+ across the erythrocyte membrane and to explore the conditions in which the relative proportion of the ouabain-sensitive flux would be highest.

Methods

Fresh heparinized blood was centrifuged at 3000 × *g* for 10 min to separate erythrocytes from plasma and buffy coat. The cells were then washed 2—3 times with two volumes of the buffered saline described below, and the cells were subsequently resuspended in the same saline solution. The composition of the incubation media was that of Ringer-type buffered saline in which Cl^- was replaced by SO_4^{2-} or NO_3^- in order to prevent the precipitation of TlCl at a high chloride concentration. The media are referred to as Ringer-SO_4 and Ringer-NO_3. The composition of the base medium was as follows (as mmol/l): Na_2SO_4, 80/$MgSO_4$, 1.0/$CaSO_4$, 0.5/K_2SO_4, 2.5; or: $NaNO_3$, 140/$Mg(NO_3)_2$, 1.0/$Ca(NO_3)_2$, 0.5/KNO_3, 5.0. Appropriate amounts of acetate, phthalate or Tris buffer were added in order to adjust the pH of the media from approx. 5 to approx. 9. The actual osmolarity of the incubation media measured from freezing point depression was approx. 290—300 mosM.

Thallium unidirectional fluxes were measured by means of the radioisotope ^{204}Tl [5]. The inward rate constants were calculated from the initial slope of the uptake curve during which ^{204}Tl accumulation was linearly related to time, i.e.

$$k_{in} = \frac{r_2 - r_1}{t_2 - t_1} \tag{1}$$

where r_1, r_2 = cell/medium distribution of ^{204}Tl at time t_1 and t_2, respectively. In some experiments the inward rate constants were calculated from the equation $k_{in} = k_{out} \cdot r_{ss}$, where r_{ss} = stationary state cell/medium distribution. At stationary state the tracer rate constants are related to the cell/medium distribution by the equation $k_{in}/k_{out} = r_{ss}$. The same procedure was used for the measurement of ^{86}Rb influx. The outward rate constants were determined from a semilogarithmic plot:

$$k_{out} = \frac{\ln A_2/A_1}{t_2 - t_1} \tag{2}$$

where A_1 and A_2 = cell radioactivity at time t_1 and t_2, respectively.

The influx of Tl^+ can be considered to consist of two components:

$$M_{in} = k_{in}[Tl]_0 = K_a[Tl]_0 + k_p[Tl]_0 \tag{3}$$

where $[Tl]_0$ = external concentration of Tl^+, k_a and k_p, the inward rate constants of the ouabain-sensitive (active) and ouabain-insensitive (passive) components of the Tl^+ influx, respectively. Accordingly, the ouabain-sensitive portion of the Tl^+ influx can be expressed as a ratio:

$$\frac{k_a}{k_{in}} = \frac{k_a}{k_a + k_p} \tag{4}$$

This ratio increases with increasing relative magnitude of the ouabain-sensitive influx of Tl^+. It should be noted that the rate constants of the saturable fluxes are concentration dependent.

Results

The effect of external cations of the ouabain-sensitive influx of Tl^+ and Rb^+

In an attempt to increase the ouabain-sensitive portion of the Tl^+ influx the uptake of Tl^+ in human erythrocytes was studied in the absence of external Na^+ and K^+. A typical experiment is shown in Fig. 1. A maximal rate of tracer ^{204}Tl influx was obtained when both Na^+ and K^+ were substituted by an isoosmotic concentration of Mg^{2+}. In the K^+-free $Mg(NO_3)_2$ medium the maximal ouabain-sensitive influx of Tl^+ at 20°C was approx. 0.45 mmol $\cdot$ l^{-1} $\cdot$ h^{-1} with a K_m as low as 0.025 mM (Fig. 2). Similar results (not presented here) were obtained in K^+-free media with Tris or choline as major cations. Both the replacement of Mg^{2+} by Na^+ or an addition of K^+ lead to a great reduction in the rate of the ouabain-sensitive influx of Tl^+ (Fig. 1 and Table I). The variability in r_{ss} observed at apparently identical conditions (Table I) seems to arise from an inevitable increase in the external concentration of K^+ lost by cells during the experiments. The K^+ concentration in the "K^+ free" media increased from 0.1 up to 0.3—0.5 mM during the incubation.

The behavior of Tl^+ was compared with that of Rb^+ in double label experiments. Table I shows that external cations affect the rates of the ouabain-sensitive influxes of ^{204}Tl and ^{86}Rb in a similar way.

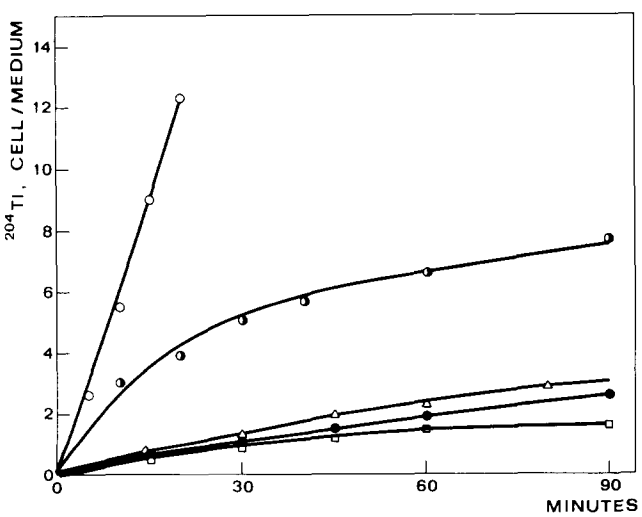

Fig. 1. Influx of $^{204}Tl^+$ tracer (about $1 \cdot 10^{-5}$ M) in human erythrocytes from media of different composition at 20°C. ○———○, K^+, Na^+-free isotonic $Mg(NO_3)_2$, 10 mM Tris/nitrate, pH 7.4; ◑———◑, K^+-free Ringer-NO_3: 140 mM Na^+, 0.5 mM Ca^{2+}, 1.0 mM Mg^{2+}, 10 mM Tris, pH 7.4; △———△, isotonic $Mg(NO_3)_2$, 10 mM KNO_3; ●———●, Ringer-NO_3, 5 mM KNO_3; □———□, Ringer-NO_3, 5 mM KNO_3, $5 \cdot 10^{-5}$ M ouabain. The effect of ouabain was independent of the composition of the medium.

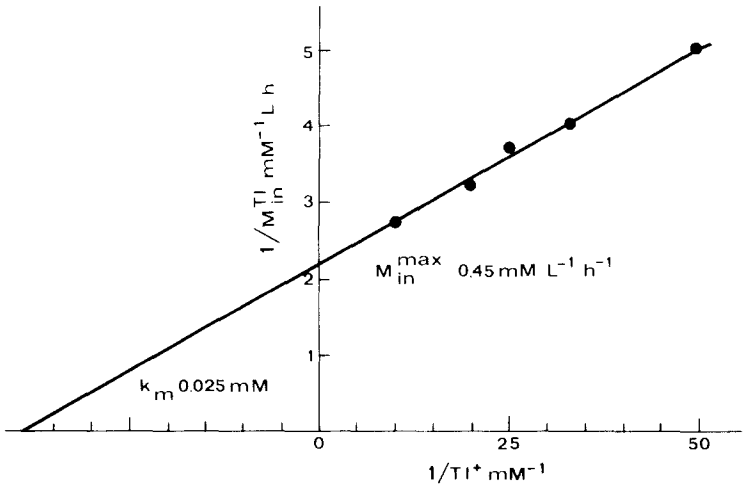

Fig. 2. Double reciprocal plot of the $^{204}Tl^{+}$ ouabain-sensitive influx in human erythrocytes at 20°C from the K^{+}, Na^{+}-free $Mg(NO_3)_2$ isotonic medium containing various concentrations of bulk $TlNO_3$. The actual K^{+} concentration in the medium was 0.1—0.3 mM increasing in the course of the experiment due to a loss of the cell K^{+}. K_m = 0.025 mM, rate of influx = 0.45 mM · l^{-1} · h^{-1}.

The ouabain-insensitive fluxes of Tl^{+}

The effect of external cations. In order to study passive fluxes of Tl^{+}, human erythrocytes were incubated in the presence of 10^{-5} M ouabain in influx experiments, while the efflux of Tl^{+} was insensitive to ouabain (Table I, 3rd column). In contrast to the ouabain-sensitive influx of Tl^{+}, its passive fluxes were found to be neither saturated by bulk Tl^{+}, nor influenced by external K^{+}

TABLE I

THE EFFECT OF EXTERNAL CATIONS ON THE ^{204}Tl AND ^{86}Rb FLUXES IN HUMAN ERYTROCYTES

Trace concentrations of the cations were approx. 10^{-6} M Tl^{+} and 10^{-5} M Rb^{+}, ouabain was 10^{-4} M. The experiments were made in triplicate, the extreme values of the stationary cell/medium ratio are shown in parentheses. 20°C, nitrate media, haematocrit 0.25.

Nitrate media composition (mM)	^{204}Tl					^{86}Rb
	$r_{ss} = [Tl]_i/[Tl]_o = k_{in}/k_{out}$	k_{out} (h^{-1})	$k_{in} = r_{ss} \cdot k_{out}$ (h^{-1})	k_a (h^{-1})	k_a/k_p (h^{-1})	k_{in} (h^{-1})
Na^{+} 145, K^{+} 5	2.0 (1.8—3.2)	0.9	1.80	0.45	0.33	0.062
Na^{+} 150, K^{+} 0.1—0.3	6.5 (4.2—7.1)	0.9	5.85	4.50	3.33	0.295
Na^{+} 150—140, K^{+} 0—10, + ouabain	1.5 (1.1—1.8)	0.9	1.35	0.00	0.00	0.009
Mg^{2+} 115, K^{+} 0.1—0.3	28 (16—42)	0.6	16.8	15.96	19.0	2.55
Mg^{2+} 105, K^{+} 10	3.6 (3.1—4.2)	0.6	2.16	1.32	1.57	0.124
Mg^{2+} 115, K^{+} 0.1—0.3, Tl^{+} 0.1	7.4 (5.9—8.1)	0.6	4.44	3.60	4.28	0.424
Mg^{2+} 115, K^{+} 0.1—0.3, Tl^{+} 1.0	2.1 (1.9—2.3)	0.6	1.26	0.42	0.50	0.046
Mg^{2+} 115, K^{+} 0.1—0.3, Tl^{+} 0—1, + ouabain	1.4 (1.1—1.9)	0.6	0.84	0.00	0.00	0.010

TABLE II

THE EFFECT OF TEMPERATURE AND MEDIUM COMPOSITION ON THE RATE CONSTANTS OF $^{204}Tl^{+}$ EFFLUX MEASURED FROM HUMAN ERTHROCYTES

Cells were loaded with trace concentrations of ^{204}Tl in cold for 12 h.

The apparent activation energy in nitrate medium is approx. 11 kcal/mol and in sulphate medium approx. 16 kcal/mol independent of the cation.

Temperature (°C)	$k_{out}(h^{-1})$				Ratios of the outward rate constants			
	$Mg(NO_3)2$	$NaNO_3$	$MgSO_4$	Na_2SO_4	Na^+/Mg^{2+}		Nitrate/Sulphate	
					Nitrate	Sulphate	Mg^{2+}	Na^+
0	0.147	0.250	0.054	0.121	1.47	2.24	2.72	2.07
20	0.593	0.900	0.435	1.190	1.51	2.73	1.36	0.75
38	1.77	3.02	2.34	4.17	1.70	1.78	0.76	0.72

in the range of concentrations studied (Table I, lines 3 and 8).

There was a decrease of the ouabain-insensitive influx of Tl^+ in $Mg(NO_3)_2$ media as compared to that in $NaNO_3$ (Table I, k_p = 1.35 and 0.84 h^{-1}, respectively). The inhibitory effect of Mg^{2+} on the passive permeability of Tl^+ could also be observed in efflux experiments (Table II). The rate constants of ^{204}Tl efflux from the erythrocytes preincubated in magnesium-containing media were about 60—70% of those observed in sodium-containing media. This difference was similar in both sulphate and nitrate solutions.

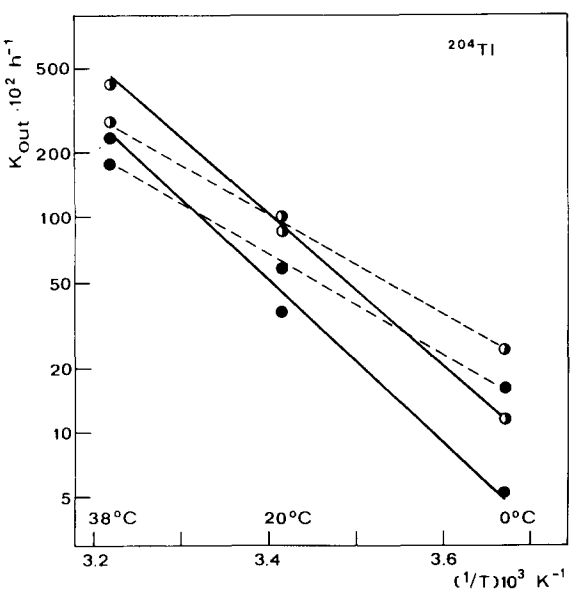

Fig. 3. Arrhenius plot of the outward rate constants of ^{204}Tl measured in human erythrocytes incubated in the following media: Ringer-NO_3 (◑- - - - -◑), isotonic $Mg(NO_3)_2$ (●- - - - -●), Ringer-SO_4 (◑———◑), isotonic $MgSO_4$ (●———●). Cells were loaded with ^{204}Tl in corresponding media during a preincubation in cold for approx. 12 h.

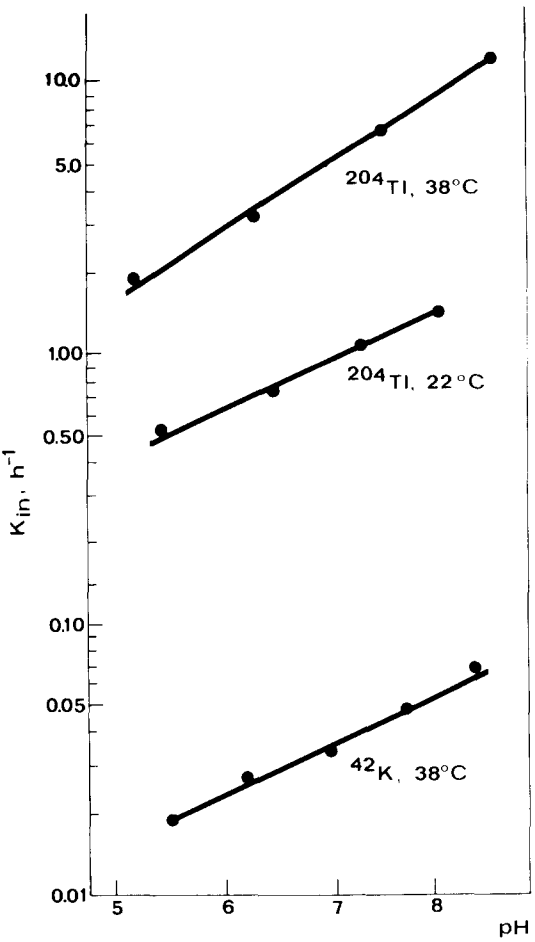

Fig. 4. pH dependence of inward rate constants of the ^{204}Tl and ^{42}K passive fluxes into cat erythrocytes incubated in Ringer-SO_4. No ouabain was added since there is practically no ouabain-sensitive ion transport in cat erythrocytes [7].

The effect of temperature. The apparent activation energies of Tl^+ efflux in nitrate and in sulphate media were 11 and 16 kcal/mol, respectively, and independent of the substitution of Na^+ by Mg^{2+} (Table II, Fig. 3). This difference in the activation energies in nitrate and sulphate media leads to a shift in the ratio of the Tl^+ outward rate constants in these media at various temperatures (Table II). For instance, at 0°C the efflux of Tl^+ in nitrate solution is faster than in sulphate solution. The reverse is true at 38°C.

The effect of pH. The influence of pH on the passive Tl^+ fluxes was studied in human erythrocytes treated with ouabain and in cat erythrocytes without ouabain. In the latter case there are practically no Na^+ and K^+ gradients and no ouabain-sensitive ion transport [7]. The rate of the ^{204}Tl passive influx was found to increase with increasing pH of the incubation medium (Fig. 4). The ^{42}K passive influx was also accelerated by increasing pH. The rate constants of

TABLE III

pH DEPENDENCE OF TRACER RATE CONSTANTS OF ^{204}Tl AND ^{42}K PASSIVE FLUXES IN CAT AND HUMAN ERYTHROCYTES INCUBATED IN K^+-FREE RINGER-SULPHATE MEDIUM

Cation	Concentration (mM)	Temperature (°C)	pH dependence of the log k (h^{-1})	Rate constants (h^{-1}) at pH 7.4
Cat erythrocytes				
^{204}Tl	trace	22	$\log k_{in} = -0.96 + 0.13$ pH	1.0
^{204}Tl	trace	38	$\log k_{in} = -1.09 \pm 0.26$ pH	6.76
^{204}Tl	trace	38	$\log k_{out} = -0.40 + 0.16$ pH	6.03
^{42}K	5.0	38	$\log k_{in} = -2.76 + 0.19$ pH	0.044
Human erythrocytes				
^{204}Tl	20.0	38	$\log k_{out} = -1.73 + 0.34$ pH	6.03
^{42}K *	a usual human erythrocyte concentration (about 100 mM)	27	$\log k_{out} = -3.66 + 0.59$ pH	4.90

* These outward rate constants were calculated by us from the data of by Passow [15] on human erythrocytes at a low ionic strength (8.3 mM NaCl in incubating medium).

Tl^+ and K^+ passive fluxes could be related to pH by the equation:

$$\log k_{in(out)} = -A + B \cdot \text{pH} \quad (5)$$

where A and B are empirical constants for the particular conditions (Table III). At a given pH the k_{in}/k_{out} ratio for Tl^+ seems to be mainly determined by an electric potential difference across the erythrocyte membrane. In accordance to Ussing's criterion [8,9]:

$$E = -RT/F \ln k_{in}/k_{out} \quad (6)$$

For example, the membrane potential of the cat erythrocyte incubated in Ringer-SO_4 at 38°C can be related to pH, using the equations of Table III:

$$E = -61.7 (\log k_{in} - \log k_{out}) = 42.5 - 6.17 \cdot \text{pH} \quad (7)$$

At pH 7.4 this gives $E = -3.1$ mV and at pH 6.88 $E = 0$.

Discussion

The results presented here can be basically accounted for by a model suggesting that Tl^+ influx consists of two components: the saturable ouabain-sensitive "active" influx of Tl^+ and the ouabain-insensitive, "passive" influx of Tl^+ which is much less saturable and is not influenced by K^+. An increase of the external K^+ concentration leads to a great decrease in the ouabain-sensitive component of the Tl^+ influx without affecting the passive Tl^+ influx. (Fig. 1, Table I). The inhibitory effect of K^+ can be attributed to a competitive interaction between K^+ and Tl^+ at the external sites of the Na^+ pump [4].

A further increase of the ouabain-sensitive inward rate constant of Tl^+ could be achieved by the replacement of external Na^+ by an isoosmotic concentration of Mg^{2+} (Fig. 1, Table I). This effect is consistent with the findings that Na^+

decreases the ability of Tl^+ to activate the ouabain-sensitive Na^+ efflux from human erythrocytes [5]. A similar interdependence between external Na^+ and K^+ has been described [10,11]. The similarity in affecting the ouabain-sensitive influxes of Tl^+ and K^+ by external cations can be further illustrated by the results obtained with ^{86}Rb and ^{204}Tl in the double label experiments (Table I). It is evident that the behavior of ^{86}Rb is similar to that of ^{204}Tl in spite of a great difference in the rate constants of the cations. The difference between Tl^+ and the alkali metal cations can to some extent arise from the difference in electronic structure of the cations. Being equal to Rb^+ in charge and size, Tl^+ unlike alkali metal cations possesses 6s electrons and is capable of a more appreciable association with anions and of complex formation [12]. A decrease in temperature caused a fall in both the active and passive fluxes of Tl^+ without changing significantly their relative magnitudes. The activation energies of both are of the same order (Q_{10} is about 2) [13].

The ouabain-insensitive fluxes of Tl^+ have also much in common with those of K^+. Both Tl^+ and K^+ passive fluxes have about the same temperature sensitivity. The apparent activation energy for the linear component of the K^+ influx in human erythrocytes is 15.6 kcal/mol [14]. The activation energy for the ouabain-insensitive efflux of Tl^+ ranged from 11 kcal/mol in nitrate media to 16 kcal/mol in sulfate solutions (Fig. 3, Table II). In the stationary state both the ^{204}Tl and ^{86}Rb rate constants could be related to the pH of the incubation media by equations of the same type (Table III).

At decreased ionic strength the loss of K^+ from human erythrocytes was also stimulated by increasing the pH of incubation media. The approximate outward rate constants calculated by us from the data of Passow [15] can be related to pH by a similar equation (Table III). Moreover [16], the ^{86}Rb passive flux in human erythrocytes increased 6—8 times when pH changed from 6.0 to 8.7 following a function of pH equivalent to that expressed by Eqn. 5.

The general conclusion is that Tl^+ and alkali metal cations seem to overcome the same membrane barrier which is temperature dependent and governed by pH. According to the fixed charge model [8,17] the erythrocyte membrane barrier contains positively charged $R\text{-}NH_3^+$ groups repelling cations. An increase of pH would thus decrease the number of positive fixed charges in the barrier facilitating cation penetration in both directions. At a given pH and temperature the k_{in}/k_{out} ratio appears to depend on the membrane potential. The stationary-state distribution of Tl^+, i.e. $r_{ss} = k_{in}/k_{out}$, in the ouabain-treated human erythrocytes ranged [1—6] from 1.2 to approx. 2 corresponding to membrane electric potential of 5—18 mV, negative inside. These values seem to be in a reasonable agreement with those determined in human erythrocytes [18]. In cat erythrocytes this value, calculated at pH 7.4 from the data of Table III, is as low as —3.1 mV. The difference between the two mammalian species is not surprising since the membrane potential may depend on various factors including cell ion composition, membrane permeability and haemoglobin acid-base properties. The erythrocyte membrane can thus be considered as a pH-dependent anion-exchanging barrier with a hydrophobic core. The penetration across the hydrophobic plane of the membrane seems to be a limiting step for both Tl^+ and K^+. The activation energy for K^+ fluxes across a synthetic phospholipid membrane (15 kcal/mol) [19] is about the same as for the K^+ and

Tl^+ passive fluxes through the erythrocyte membrane [14] (Fig. 3, Table II). The properties of an anion-exchanging barrier are expected to depend on the nature of anions neutralizing positively charged groups within the membrane. This could be an explanation of the effect of anions on the Tl^+ permeability and activation energy (Fig. 3, Table II). A decrease of the Tl^+ permeability after Mg^{2+} treatment might be accounted for by the interaction between Mg^{2+} and the acid groups of phospholipids [20]. The fixed charge hypothesis, providing a satisfactory explanation of the influence of pH, fails to explain the surprising difference between the rates of penetration of the Tl^+ and alkali cations. Tl^+ and K^+ or Rb^+ being close in their crystal and hydrated radii [13,21] could not conceivably be discriminated by pure electrostatic repulsion forces to a great extent. It must therefore be assumed that the high permeability of the erythrocyte membrane to Tl^+ is based upon specific and favourable interactions between Tl^+ and membrane ligands. Such interactions would to a greater extent than in the case of alkali cations increase the Tl^+ concentration in the vicinity of the membrane barrier, consequently increasing the diffusion fluxes of Tl^+ across the membrane. The well known increase of K^+ permeability caused by valinomycin is caused by such specific interaction between K^+ and the ionophore and it is interesting to note that gramicidin channels in artificial phospholipid membranes [22] show a high preference for Tl^+. The possibility of naturally existing sites interacting with Tl^+ in the erythrocyte membranes must be entertained.

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