Organotin-Mediated Exchange Diffusion of Anions in Human Red Cells

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ABSTRACT Organotin cations (R_3Sn^+) form electrically neutral ion pairs with monovalent anions. It is demonstrated that the tin derivatives induce exchange diffusion of chloride in red cells and resealed ghosts, without any detectable increase of membrane permeability to net movements of chloride ions. The obligatory anion exchange is believed to be due to the permeation of electroneutral ion pairs, whereas the organic cation (R_3Sn^+) has an extremely low membrane permeability. Exchange fluxes of chloride increased with the lipophilicity of the substituting group (R_3). At the same molar concentration of organotin, the relative potencies of the tin derivatives as anion carriers (with trimethyltin as a reference) were: methyl 1, ethyl 30, propyl = phenyl 1,000, and butyl 10,000. Tributyltin-mediated anion exchange was studied in detail. The organotin-induced anion transport increased through the sequence: $F^- \ll Cl^- < Br^- < I^- = SCN^- \ll OH^-$. Partitioning of tributyltin into red cell membranes was greater in iodide than in chloride media (partition coefficients 6.6 and 1.7×10^{-3} cm, respectively). Bicarbonate, fluoride, nitrate, phosphate, and sulphate did not exchange with chloride in the presence of tributyltin. Chloride exchange fluxes increased linearly with tributyltin concentrations up to 10^{-5} M, and with chloride concentrations up to at least 0.9 M. The apparent turnover number for tributyltin-mediated chloride exchange increased from 15 to 1,350 s^{-1} between 0 and 38°C. These figures are minimum turnover numbers, because it is not known what fraction of the organotin in the membrane exists as chloride ion pairs.

INTRODUCTION

This work deals with exchange diffusion of anions mediated by lipophilic organotin compounds. Many biological membranes possess transport systems which can perform an obligatory "self-exchange" of isotopes of one chemical ion species or a "heteroexchange" of ions of different chemical species. The phenomenon was named by Ussing (1948), who wrote: "in its ideal form such a mechanism will always take up one ion, when it gives off another, so that no net change in the ion concentration on either side of the membrane need take place. Such a diffusion we call exchange diffusion."

The above-mentioned criterion of ideality implies that the membrane permeability measured by isotope exchange is out of proportion to the low membrane conductance of the same ion, because the obligatory exchange mechanism cannot mediate a net flow of current through the membrane. This leads to the

paradox that an extremely large permeability to a tracer ion may be accompanied by a very low electrical conductance. The anion transport system in erythrocytes is a remarkable example. Measured as chloride self-exchange, the permeability to $^{36}Cl^-$ efflux at 38°C is 4×10^{-4} cm·s^{-1} (Brahm 1977), whereas the permeability to a net transfer of chloride ions through the same membrane is four orders of magnitude lower (Hunter 1971, 1977; Knauf et al., 1977). Electrically silent anion exchange has also been described in artificial membranes. Shean and Sollner (1966) demonstrated that certain lipophilic secondary amines can perform an obligatory exchange of anions through thick hydrophobic membranes, and it was later shown by Gutknecht et al. (1978) that the secondary amines are also capable of mediating exchange diffusion of bromide in bimolecular lecithin membranes, which maintained a high electrical resistance.

The purpose of the present work is to examine the exchange diffusion induced in red cell membranes by organotin compounds. Selwyn et al. (1970) showed that organotin derivatives can mediate chloride-hydroxyl exchange across the membranes of mitochondria, erythrocytes, and liposomes. These results were confirmed and extended in red blood cells by Aubert and Motais (1975). Stimulated by these observations we decided to examine if the organotin compounds mediate electrically silent exchange diffusion of anions in red cells and in lipid membranes. We have found that the trialkyltin derivatives can induce an anion exchange permeability, which is several orders of magnitude higher than the permeability to net anion movements deduced from indirect and direct measurements of membrane conductance in erythrocyte and artificial membranes. Brief reports of our work have been published previously (Tosteson et al., 1977; Wieth and Tosteson, 1977). During the preparation of this article, a report has confirmed that organotin compounds can mediate a self-exchange of chloride in red cells (Motais et al., 1977), but it has not been considered previously to which extent the membrane conductance of red cells is affected by the organometallic cations.

METHODS

Organotin Compounds

Trimethyltin chloride, triethyltin chloride, and triphenyltin chloride were obtained from Merck-Schuchardt (Darmstadt, West Germany), tripropyltin chloride and tributyltin chloride from BDH Chemical Ltd., Poole, England. The organotin salts were dissolved in ethanol (99% vol/vol) to give the desired concentration in the cell suspension, when diluted 700–1,000-fold with electrolyte medium. The ethanol concentration in the media was thus maximally 0.15% (vol/vol). The organotin was dissolved in the medium before the cells were injected. A constant rate of anion exchange was found to be established within the first few seconds of the experiments.

Preparation of Red Cells and Ghosts

Freshly drawn heparinized human blood was centrifuged, plasma and buffy coat were removed, and the cells were resuspended and washed in a 165 mM KCl solution. The preparation and labelling of resealed ghosts with isotopes followed the protocol given by Funder and Wieth (1976). The pH of the ghost preparations was adjusted before packing

the ghosts into nylon centrifuge tubes. Red cells were titrated to acid pH values with CO_2. When the desired pH value had been reached, the bicarbonate formed by the titration was removed by repeated washings of the cells in the bicarbonate-free electrolyte medium used for the subsequent experiment.

Inactivation of the Normal Anion Transport System

In most experiments anion transport mediated by the organotin compounds was studied in red cell membranes, whose natural anion transport mechanism had been inhibited irreversibly with the amino-group reagent 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a potent inhibitor of anion transport in red cells (Cabantchik and Rothstein, 1974). Chloride transport is inhibited >99% when binding is 1.1×10^6 DIDS molecules per cell (Lepke et al., 1976; Ship et al., 1977), and excess binding of DIDS causes no further reduction of anion transport (Funder et al., 1978). The DIDS treatment was carried out by incubating red cells or resealed ghosts for 45 min at 38°C in a medium containing $5–10 \times 10^6$ molecules per cell. The methods employed for preparation and purification of DIDS were recently reported by Funder et al., 1978.

Labelling with Isotopes

In order to label red cells or ghosts with radioactive anions, tracer was added to the cell suspension briefly before the addition of DIDS. Among the anions studied iodide has the slowest rate of exchange through the natural exchange system, but inasmuch as the $T_{1/2}$ of exchange is 2 s at 38°C (Dalmark and Wieth, 1972), complete equilibration was obtained in <15 s after addition of tracer, whereafter DIDS was added. The cells were separated from the radioactive medium by centrifuging the suspension in nylon tubes (i.d. 3 mm) for 10 min at 50,000 *g*. The amount of extracellular fluid trapped was 2% (wt/wt) in a column of packed erythrocytes, and 8–12% (wt/wt) between packed resealed ghosts (Funder and Wieth, 1976). The packed cells or ghosts were isolated from the radioactive medium by cutting the tube 1 mm below the interface between cells and medium.

All radioactive anions except $^{18}F^-$ were obtained and used in concentrations as described by Dalmark and Wieth (1972), where information about sampling and counting techniques is also stated. The radioactive fluoride ($^{18}F^-$) was a gift from the Niels Bohr Institute of Theoretical Physics, University of Copenhagen. The isotope has a half-life of 1.8 h, so the amount of radioactivity added to the cell suspensions was increased to 10 μCi per milliliter.

Electrolyte Media

Media containing monovalent anions were 165 mM solutions of the appropriate potassium salts, buffered with phosphate (0.5 mM), which was added as KH_2PO_4 and titrated to the desired pH with KOH. Minor modifications of these basic media are specified in the text. Control experiments showed that both natural and organotin-mediated exchange diffusion of chloride takes place at identical rates in KCl, NaCl, and LiCl media. The isotonic phosphate and sulphate media used in the heteroexchange experiments (Table IV) were 100 mM K_2SO_4 + 0.5 mM KH_2PO_4, and 130 mM KH_2PO_4. Both media were titrated to pH 6.8 (0°C) before being used for flux experiments.

Anion Exchange Experiments

The efflux of radioactive anions from labelled cells or ghosts was initiated by injecting a packed cell sample (usually 200 mg) into 40 ml of the well-stirred, thermostated, electrolyte medium. Serial samples of cell-free medium were obtained by the Millipore-Swinnex technique (Millipore Corp., Bedford, Mass.; Dalmark and Wieth, 1972) at

appropriate time intervals, thereby following the accumulation of radioactivity as a function of time. The rate coefficient for anion efflux (k, s^{-1}) was calculated from the slope of a graph of ln $(1 - a_t/a_\infty)$ vs. time (t), determined by linear regression analysis, a_t being the activity in the extracellular compartment at time (t), and a_∞ the activity at isotopic equilibrium. The unidirectional anion flux was determined from the relation:

$$J^{exch} = k(V/A) \cdot C_{A^-} \text{ mol}(\text{cm}^2 \cdot \text{s})^{-1}. \quad (1)$$

V is the solvent volume in red cells or ghosts determined from the mean cell volume with correction for the solids content assuming a density of 1.3 for hemoglobin; A is the mean surface area of erythrocytes and ghosts [$1.42 \cdot 10^{-6}$ cm^2 $(\text{cell})^{-1}$], cf. Funder and Wieth (1976); and C_A is the intracellular concentration of the anion mmol $(\text{cm}^3$ cell water$)^{-1}$. The term $k \cdot (V/A)$ cm $(\text{s})^{-1}$ equals the apparent permeability coefficient to anion exchange P_{Cl}^{exch}. In steady-state self-exchange experiments, we followed the isotope exchange to 80–90% completion. In the heteroexchange experiments, the initial efflux was calculated from the initial slope of isotope release.

Preparation of ^{42}K-Loaded Cells

10 ml of freshly drawn heparinized blood was titrated to pH 6.6 (38°C), washed in the 150 mM KCl medium, and resuspended to 15 ml. A small volume (10–15 μl) of a 150 mM KCl solution containing 15 μCi ^{42}K (AEK, RISØ, Denmark) was added, followed by 15 μl of a solution of valinomycin (grade A, Calbiochem Behring Corp., San Diego, Calif.) in ethanol (1 μmol/ml). When the cells were treated with DIDS, this compound was added dissolved in 150 mM KCl to give a resultant amount of 10^7 molecules per cell. The suspension was now incubated for 45 min at 38°C, and the cells were isolated for the subsequent flux experiments. Control analyses showed that the specific activity of ^{42}K after loading of the cells was between 90 and 100% of the specific activity in the extracellular phase.

^{42}K Efflux Experiments

Determination of the rate coefficients of ^{42}K efflux from the valinomycin-treated cells was performed by injecting ~200 mg of cells into 40 ml well-stirred thermostated medium containing valinomycin 10^{-6} M (ethanol 0.1%). The techniques of sampling and of calculating the rate coefficients were identical to those used in the $^{36}Cl^-$ efflux experiments. For each cell preparation, duplicate experiments were carried out at extracellular potassium concentrations of 0.15, 1.5, 15, 75, and 150 mM, in media containing 27 mM sucrose and (NaCl + KCl) equal to 150 mM. It was found that organotin-mediated self-exchange of chloride took place at the same rate in KCl and NaCl media in the presence and absence of valinomycin.

Membrane Permeability to Electrodiffusion of Chloride

It was essential to determine if tributyltin has any effect on the chloride conductance of the red cell membrane. Direct measurement of the conductance of human red cells is not feasible, but several quantitative estimates have been made by indirect methods, based on the qualitative observation of Chappel and Crofts (1966) that net transfer of anions becomes rate-limiting to the efflux of KCl from cells, if the normal low cation permeability of the membrane is raised a few orders of magnitude by ionophores that increase the potassium permeability. We have used the valinomycin method of Hunter (1971, 1977) to determine the effect of tributyltin on P_{Cl}^{net} of normal and of DIDS-treated human red cells. We determined the rate of $^{42}K^+$ efflux into the above-mentioned media at 38°C, pH 6.6. Stability of extracellular pH of the unbuffered media during the efflux

of $^{42}K^+$ showed that there was no significant transport of hydroxyl or hydrogen ions, suggesting that it is justifiable to consider chloride and potassium as the only permeating ion species, as it is implied in the constant field treatment employed by Hunter (1977).

The rate coefficient of $^{42}K^+$ efflux (k, s^{-1}) was determined by linear regression analysis of the data in experiments, where K_o was above 15 mM. At lower external potassium concentrations, where the cells shrink due to a net loss of KCl, we determined the rate coefficient from the initial rate of isotope release, i.e., from the samples taken before 20% of cellular KCl had been lost.

Under the present experimental conditions $K_i = Cl_i = Cl_o = 150$ mM at 38°C, pH 6.6. Therefore, the constant field equation takes the following simple form:

$$V_m = \psi_i - \psi_o = -RT/F \ln \frac{150 + P_K/P_{Cl}^{net} \times 150}{150 + P_K/P_{Cl}^{net} \times K_o} = -RT/F \ln B, \quad (2)$$

where K_o is the variable extracellular potassium concentration. The flux of $^{42}K^+$, which is determined experimentally, is affected by the membrane potential:

$$J_K = P_K K_i = \frac{\ln B}{B - 1}. \quad (3)$$

A sensitive way of determining P_{Cl} net is to plot $\ln B/(B - 1)$ vs. K_o as shown in Fig. 11. It should be noted that the value of $\ln B/(B - 1)$ is equal to the ratio k_K/k_{150}, where k_{150} is the rate coefficient of ^{42}K efflux in the 150 mM KCl medium, and k_K is the rate found at a lower KCl concentration. The potassium permeability (P_K) is equal to $k_{150}(V/A)$, V being the solvent volume and A the membrane area of the cells.

Partitioning of Organotin between Cells and Medium

As shown in Results, red cell membranes take up considerable amounts of the lipophilic organotin derivatives (T). The partitioning was analyzed by a biological method, previously used by Hunter (1974) to determine the uptake of valinomycin into red cell membranes.

The method is based on determinations of rates of transport in suspensions containing a fixed amount of organotin and a variable concentration of cells. Inasmuch as the organotin-mediated anion transport at a fixed hematocrit varied linearly with the amount of T in the medium, it is safe to assume that the adsorption of T to cell membranes follows Henry's law within wide limits (cf. Fig. 2):

$$T_t = T_m + T_c = T_m + \alpha h T_m, \quad (4)$$

where T_t is the total amount of organotin in the suspension, T_m is the amount of organotin in the aqueous medium, and T_c is the amount of organotin taken up by the cells. The linear adsorption coefficient, α, is the ratio: (mole T per liter cells/mole T per liter medium), and h is the fractional cell volume of the suspension. Anion self-exchange is a linear function of the amount of organotin in the cell membrane, so keeping T_t constant:

$$k_h = k_0[1/(1 + \alpha h)], \quad (5)$$

where k_0 is the rate coefficient of anion self-exchange as the hematocrit tends to zero, and k_h is the rate of self-exchange when the fractional cell volume of the suspension is h. The equation can be rearranged for a linear relation:

$$\tau_h = 1/k_h = 1/k_0 + \alpha h/k_0. \quad (5\ a)$$

Both k_0 and α can be found by linear regression analysis of a plot of $1/k_h$ vs. h. An

example of the determination of α is shown in Fig. 6. Experimental evidence showed that tributyltin is only adsorbed to the cell membranes, and not to any measureable degree to hemoglobin or to other cellular constituents. From the value of α, it is therefore possible to estimate the surface concentration of organotin molecules, assuming that they are located at the two membrane interfaces. The partition coefficient is redefined as the ratio between the organotin at the membrane surface (T_d mol cm^{-2}) and the concentration in the medium (T_m mol cm^{-3}),

$$\beta = \frac{T_d}{T_m} = \frac{\alpha}{A_{RBC}}, \tag{6}$$

where A_{RBC} (3.26×10^4 cm^2/cm^3 cells) is the total surface area (external + internal) of 1-ml red cells with a mean cell volume of 87×10^{-12} ml and a cell surface area of 1.42×10^{-6} cm^2. The number of organotin molecules per square centimeter (N) will now be given by:

$$N = \beta T_m N_A, \tag{6 a}$$

where N_A is Avogadro's number (6.023×10^{23} molecules/mole).

RESULTS

Chloride Self-Exchange

The effect of tributyltin on the rate of $^{36}Cl^-$ efflux from intact and from DIDS-treated human red cells at 0° is illustrated in Fig. 1. In the DIDS-treated cells, where the natural anion exchange mechanism is inhibited, addition of tributyltin (5.2 μmol/liter cell suspension) increased the exchange flux from 1.4 to 85 pmol

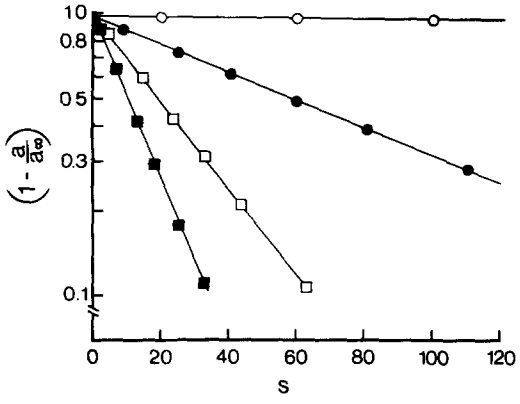

FIGURE 1. The effect of tributyltin (5.2 μmol/liter cell suspension) on steady-state chloride exchange in DIDS-treated and in normal red cells (0°C, pH 7.0, hematocrit 0.5%). The graph shows the fraction of $^{36}Cl^-$ remaining in the cells as a function of time; a is the activity in the medium at the time of sampling and a_∞ the activity after equilibrium has been attained. Control cells are indicated by open symbols: normal cells, □; DIDS-treated cells, ○. The self-exchange in the presence of organotin is depicted by the corresponding filled symbols. In both cases the increase of the chloride self-exchange flux was close to 100 pmol (cm^2 × s)$^{-1}$, which was the mean value found in a larger experimental series (cf. Table I).

$(cm^2 \times s)^{-1}$. A similar increase of the exchange flux was found in intact cells (Fig. 1), demonstrating that the artificial ionophore creates a transport pathway for chloride in parallel with the natural anion transport system.

Table I shows that the effect of tributyltin on chloride exchange was the same in red cells and in resealed ghosts, indicating that the presence of hemoglobin does not affect the tin-mediated transport of anions. This was further substantiated by the observation that the addition of a membrane-free red cell lysate to the flux medium did not change the rate of chloride exchange. Table I also demonstrates that phloretin, which is a potent inhibitor of the natural transport system, only has a moderate effect on the tributyltin-mediated chloride transport.

At a hematocrit of 0.5% the chloride self-exchange was found to be a linear function of the amount of tributyltin added to the suspension up to 10^{-5} mol/liter (Fig. 2). The flux leveled off, when more organotin was added, and an

TABLE I

CHLORIDE SELF-EXCHANGE IN DIDS-TREATED RED CELLS AND RESEALED GHOSTS MEDIATED BY TRIBUTYLTIN

	Chloride self-exchange flux	
Phloretin	DIDS-treated red cells	DIDS-treated ghosts
mM	$10^{12} \times (mol \times cm^{-2} \times s^{-1})$	
0	100.1 (8.4)	98.9 (11.9)
0.25	42.6 (1.7)	40.3 (6.5)

Tributyltin, 5.2 μmol/liter cell suspension; hematocrit 0.5%, pH 6.8, 0°C. Values are means (SD) from series of 10 experiments in each category.

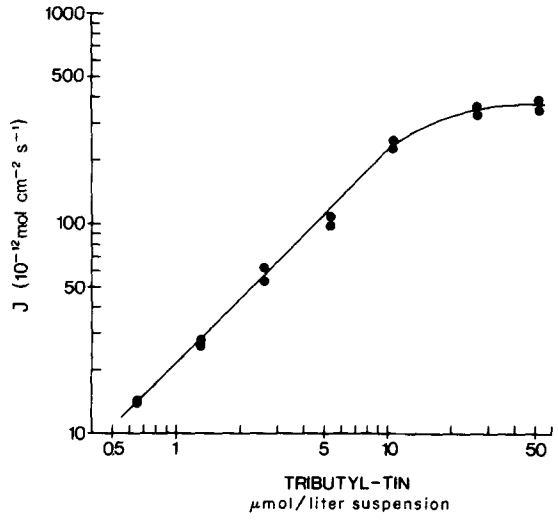

FIGURE 2. Chloride self-exchange flux (J) in DIDS-treated red blood cells as a function of the amount of tributyltin added per liter cell suspension (0°C, pH 6.8, hematocrit 0.5%).

increase from 30 to 50 μmol/liter suspension caused almost no increase of the self-exchange flux.

Organotin-induced chloride transport increased linearly with chloride concentration. Fig. 3 shows the result of an experiment, where chloride concentrations in cells and media were increased from 165 to 600 mM by the addition of ammonium chloride. Similar results were obtained by varying the concentration of KCl between 165 and 900 mM in resealed ghosts, demonstrating that the results shown in Fig. 3 were not caused by the presence of ammonium ions.

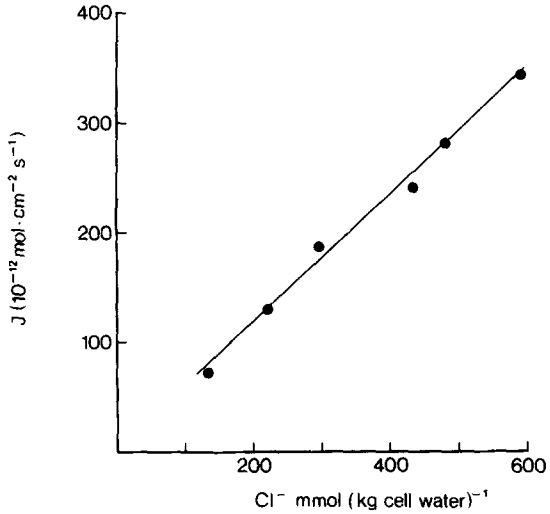

FIGURE 3. Chloride self-exchange flux as a function of chloride concentration in medium and cell water. The chloride concentrations were increased by the addition of NH_4Cl to the media used for preequilibration and experiments (0°C, pH 6.8, hematocrit 0.5%, tributyltin 5.2×10^{-6} mol/liter suspension).

The pH dependence of the tin-mediated chloride exchange was examined in ghosts to avoid difficulties imposed by the variable hydroxyl ion concentration difference across the erythrocyte membrane, when pH is varied. The results shown in Fig. 4 demonstrate that tributyltin-mediated chloride exchange decreased steeply with increasing pH. The inhibition of chloride exchange by OH^- was well described by assuming an apparent dissociation constant for R_3SnOH of 10^{-8} M, meaning that the flux was half-maximal at pH 6.9 at 0°C, where the ionization constant for water is $10^{-14.9}$. At pH 6.8 where most of the chloride self-exchange determinations were carried out, the flux would accordingly be 56% of the maximum tributyltin-mediated transport.

The red cell membrane has a very low permeability to hydrogen and hydroxyl ions, when the natural anion exchange is inhibited (Motais et al., 1977; Jennings, 1978). This was confirmed in the experiment shown in Fig. 5, which demonstrates that the DIDS-treated red cells were virtually impermeable to H^+ and OH^- at 0°C, and that an imposed pH gradient vanished rapidly when tributyltin was added. The rapid relaxation of extracellular pH after addition of tributyltin

confirms that this compound can perform a rapid Cl^-/OH^--exchange as first demonstrated by Selwyn et al. (1970).

In order to determine the partitioning of tributyltin between red cell membranes and medium, the rate of anion transport was determined in a series of experiments, where varied numbers of cells were exposed to a fixed amount of tributyltin. Fig. 6 shows that the time constant of anion exchange (τ) increased linearly with the cell concentration of the suspension. The mean value of the cellular partition coefficient (α) was 53.2 (SD 4.3, n = 5). Assuming that the tributyltin molecules were all located in the membrane-surfaces, we calculated a membrane partition coefficient (β) of 1.65 × 10^{-3} cm (cf. Methods). Similar

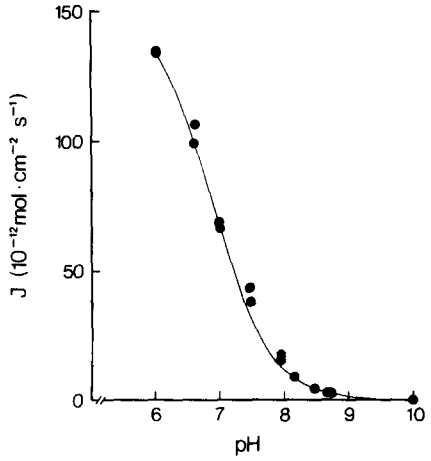

FIGURE 4. pH dependence of tributyltin-mediated chloride exchange in DIDS-treated resealed ghosts (0°C, cytocrit 0.5%, tributyltin 5.2 × 10^{-6} mol/liter suspension). The fully drawn line describes the relation:

$$J = \frac{150}{1 + \frac{10^{-(14.9-pH)}}{10^{-8}}} \text{pmol}(\text{cm}^2 \times \text{s})^{-1}.$$

$10^{-14.9}$ is the ionization constant for water at 0°C, $10^{-(14.9-pH)}$ is the hydroxyl ion concentration, and the apparent dissociation constant for membrane-bound tributyltin hydroxide is 10^{-8} M at a chloride concentration of 165 mM.

values were determined at 25°C, so it can be assumed that the adsorption of tributyltin depends little on temperature. This is important for the interpretation of the temperature dependence of tributyltin-mediated chloride exchange, which could be described by an apparent activation energy of 20 kcal/mol, as shown in the Arrhenius diagram of Fig. 7.

Self-Exchange of Other Inorganic Anions

Measurements of the rate of self-exchange of inorganic anions other than chloride showed that the relative transport rates were: SCN = I > Br > Cl >> F (Table II). The ability of tributyltin to transport fluoride was poor. The

exchange flux of iodide was about five times larger than the chloride exchange determined in media containing the same amounts of cells and of the organotin compound. In 165 mM KI medium it was found, in experiments similar to that shown in Fig. 6, that the partition coefficient of tributyltin was about four times larger than the value found in chloride media ($\alpha_{KI} = 200$). This result suggests that the differing tributyltin uptake induced by the anions determines the rates of chloride and iodide transport.

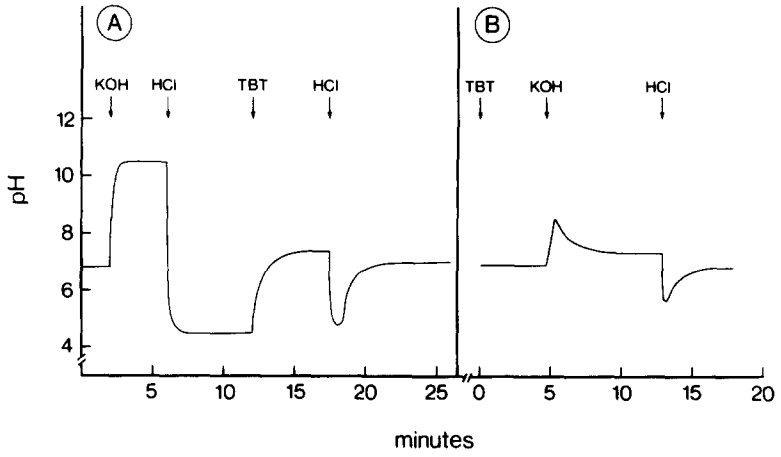

FIGURE 5. The low permeability of DIDS-treated cells to H^+ and OH^- at 0°C. (A) The pH response of a suspension of DIDS-treated cells to additions of acid and base (0°C, hematocrit 1%) before and after the addition of tributyltin. (B) The pH changes in tributyltin-treated cells. 40 ml of an unbuffered 165 mM KCl solution were used in both experiments. The additions of HCl and KOH were 100 μl of a 0.1 M solution (10×10^{-6} mol H^+ or OH^- corresponding to 2.5×10^{-4} mol/liter suspension. The amount of tributyltin (TBT) added in ethanolic solution was 1.5×10^{-7} mol (3.8×10^{-6} mol/liter cell suspension). Note the large pH changes in the cell suspension before the addition of organotin. The pH changes and the response time of the glass electrode were similar to those found in a cell-free medium indicating that the cells were practically impermeable to H^+ and OH^- at 0°C. When organotin had been added, the suspension responded to pulses of acid or base with much smaller pH excursions which relaxed to a new equilibrium value in the course of few minutes.

In the absence of tributyltin the rate of iodide self-exchange is extremely slow in DIDS-treated cells (the half-time of exchange being ~4 h at 0°C). The rate of exchange was doubled after the addition of 5×10^{-9} mol tributyltin per liter of cell suspension, and Table III shows that a further 1,000-fold increase of the tributyltin concentration to 5×10^{-6} mol/liter suspension caused a 1,000-fold increase of the self-exchange flux from 0.7 to 700 picomol $\times$ $cm^{-2} \times s^{-1}$. The Arrhenius activation energy of the tributyltin-mediated iodide transport between 0 and 38°C was 15.0 kcal/mol (SD 0.5).

Heteroexchange of Anions

So far we have only described tracer experiments in which there were no net movements of anions across the red cell membrane. The ability of tributyltin to exchange anions was also measured in heteroexchange experiments, where DIDS-treated chloride- or iodide-loaded cells were suspended in isotonic media of various potassium salts as shown in Tables IV and V. Because the cation permeability of the red cell is extremely low, intracellular anions can only leave

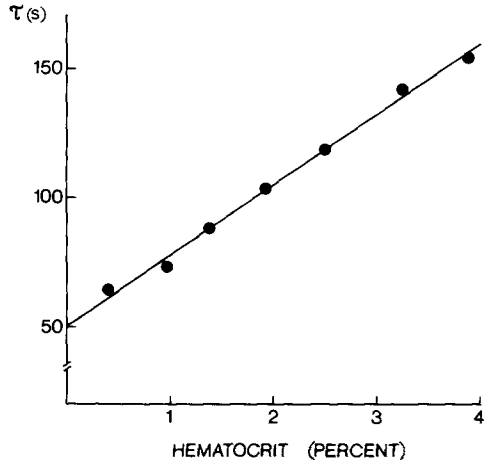

FIGURE 6. Time constant (τ) of chloride self-exchange as a function of the hematocrit in suspensions, which all contained 5.2 μmol tributyltin per liter. The time constant, which has the dimension of time (s), is equal to ($1/k$), the reciprocal rate coefficient of chloride exchange. The adsorption of tributyltin to the red cells was calculated from the linear relation between cell concentration (hematocrit) and the time constant as described in Methods. All experiments were performed at pH 6.8, 0°C.

the cells in exchange for the foreign anions of the extracellular phase. Therefore, one can measure the relative ease with which chloride or iodide can exchange with other anions. The results confirmed that bromide, and especially iodide and thiocyanate are transported more readily than chloride itself. In addition the results showed that the rates of fluoride, nitrate, sulphate, and phosphate transfer were extremely slow.

Fig. 8 shows that the slow rate of chloride efflux into a nitrate or fluoride medium was completely unaffected by the addition of 2 mM bicarbonate to the medium, in contrast to the dramatic effect of bicarbonate on chloride heteroexchange through the natural anion transport system (Fig. 5 in Wieth, 1972), supporting the conclusion of Motais et al. (1977) that bicarbonate is not transported by organotin derivatives. One might argue that heteroexchange of chloride with extracellular bicarbonate is impeded by the shift of pH from 6.8 to 7.1 after addition of bicarbonate. However, it may be noted that a similar pH change only reduced the rate of chloride self-exchange by 30% (cf. legend of Fig. 4).

Self-Exchange of Chloride or Iodide in the Presence of High Concentrations of Other Anions

The rates of chloride and iodide exchange were also determined in cells which had been preequilibrated in media containing 2 mM chloride or iodide plus 165 mM the anion in question. The results are shown in Tables VI and VII. It was not possible to demonstrate any kind of competition between chloride and the more rapidly transported anions such as bromide, iodide, or thiocyanate. On the contrary, the rate coefficients varied much in the same way as in the heteroexchange experiments, suggesting that ions favoring the partitioning of

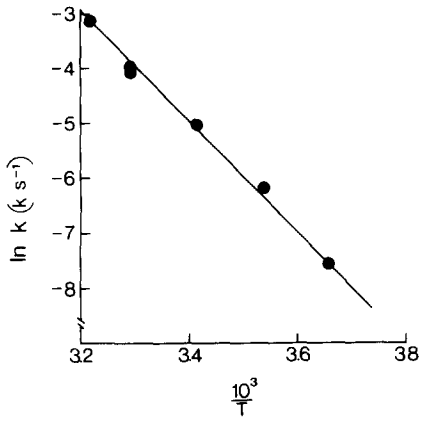

FIGURE 7. Arrhenius diagram of the relation between the reciprocal absolute temperature ($1/T$) and the natural logarithm of the rate coefficient of chloride self-exchange mediated by tributyltin in DIDS-treated red cells. All experiments were performed in a medium containing 5×10^{-7} mol tributyltin per liter suspension (hematocrit 0.5%, pH 6.8–6.6). It was necessary to keep the tributyltin concentration low in order to be able to cover the whole range from 0–38°C. The relation, determined by linear regression analysis, was:

$$\ln k = -9.9(10^3/T) + 28.8 \quad (r = 0.996).$$

tributyltin into the membrane increase the rate of $^{36}Cl^-$ efflux. The rates of chloride and of iodide self-exchange decreased by 50–70% in the nitrate medium. Nitrate has a very low affinity for organotin and the concentration of nitrate ion pairs in the membrane must accordingly be low. The decreased rates of halide exchange in the presence of nitrate fits with the concept that the total amount of ion pairs in the membrane plays an important role for the rate of halide transport, a point that is further dealt with in the Discussion. Halide exchange was strongly inhibited by fluoride, which is known to form water-soluble, long-chained complexes with organotin molecules, (Cotton and Wilkinson, 1966).

Anion Exchange by Other Organotin Compounds

Other trialkyl- and triaryltin derivatives than tributyltin can mediate exchange diffusion of anions in red cells as shown in Table VIII, which shows the chloride

exchange flux, induced by varying concentrations of trimethyl-, triethyl-, tripropyl-, and triphenyltin. The dose relationship for tributyltin-mediated chloride exchange was shown in Fig. 2. The relative effects of the tin compounds were compared to that of trimethyltin by dividing the organotin anion exchange by the concentration of organotin in the suspension. The normalized exchange transport induced by trimethyltin was thus: ($1.4 \times 10^{-12}/0.7 \times 10^{-3} = 2 \times 10^{-9}$ mol $\times$ $cm^{-2} \times s^{-1}$) at a hypothetical trimethyltin concentration of 1 mol per liter

TABLE II

SELF-EXCHANGE OF MONOVALENT INORGANIC ANIONS THROUGH DIDS-TREATED RED CELL MEMBRANES IN THE ABSENCE AND PRESENCE OF TRIBUTYLTIN

Radioactive anion	Tributyltin absent		Tributyltin	
	Rate coefficient	$T_{\frac{1}{2}}$	Rate coefficient	$T_{\frac{1}{2}}$
	min^{-1}	min	min^{-1}	min
$^{18}F^-$	1.4×10^{-2}	48.6	0.04	17.9
$^{36}Cl^-$	9.81×10^{-3}	70.7	0.84	0.83
$^{82}Br^-$	2.35×10^{-3}	294.5	1.77	0.39
$^{125}I^-$	2.92×10^{-3}	237.4	4.33	0.16
$[^{14}C]SCN^-$	3.55×10^{-2}	19.5	3.85	0.18

Tributyltin, 5.2×10^{-6} mol/liter cell suspension; hematocrit 0.5%, pH 6.8, 0°C.

TABLE III

IODIDE SELF-EXCHANGE IN DIDS-TREATED RED CELLS AS A FUNCTION OF TRIBUTYLTIN CONCENTRATION

Tributyltin	Iodide self-exchange flux
mol/liter cell suspension	$10^{12}\times(mol\times cm^{-2}\times s)$
0	0.006
5.2×10^{-9}	0.682
5.2×10^{-8}	6.48
1.04×10^{-7}	13.38
5.2×10^{-7}	97.3
1.04×10^{-6}	147.7
5.2×10^{-6}	711.5

Hematocrit 0.5%, pH 6.8, 0° C. The self-exchange flux was a linear function of the amount of tributyltin added to the suspension between concentrations of 5×10^{-9} and 5×10^{-6} mol/liter cell suspension.

cell suspension. The relative potencies with reference to trimethyltin were: methyl 1, ethyl 30, propyl = phenyl 1,000, and butyl 10,000.

It is likely that the lipophilicity of the organotin compound determines its ability to induce anion transport. We have attempted to determine the partitioning of triethyltin between medium and cells by the method used for determining partitioning of tributyltin (Fig. 6). The rate of chloride exchange was found to be independent of variations of the hematocrit between 0.5 and 5%, as would be expected if—as judged from the relative potencies—the uptake of triethyltin is a 100-fold smaller than the uptake of tributyltin. We have been able to determine the cellular partition coefficient for tripropyltin. The value ($\alpha = 5$) was ten times lower than that found for tributyltin, once more suggesting a simple relation

between the amount of organotin taken up by the membranes and the relative rates of transport.

Exchange Diffusion of Chloride without Increase of Chloride Conductance

In order to evaluate the mechanism by which organotin compounds induce anion transport in red cell membranes, it was essential to determine the effect of tributyltin not only on exchange movements of anions but also on the ability of chloride ions to carry electrical current through the membrane.

P_{Cl}^{net} was determined in the presence and absence of tributyltin by the method of Hunter (1977), according to which the rate of ^{42}K efflux from valinomycin-treated cells is measured as a function of extracellular potassium concentration.

TABLE IV

TRIBUTYLTIN-MEDIATED EFFLUX OF $^{36}Cl^-$ FROM DIDS-TREATED RED CELLS INTO ISOTONIC MEDIA CONTAINING THE POTASSIUM SALTS OF VARIOUS ANIONS

Extracellular anion	Rate coefficient of $^{36}Cl^-$ efflux	Initial chloride efflux
	$10^3 \times s^{-1}$	$10^{12} \times (mol \times cm^{-2} \times s^{-1})$
Chloride	15	112.5
Bromide	21	157.5
Iodide	36	270.0
Thiocyanate	35	262.5
Fluoride	0.9	6.8
Nitrate	0.9	7.0
Sulfate	1.1	8.2
Phosphate	0.6	4.6

Tributyltin, 5.2×10^{-6} mol/liter cell suspension; hematocrit 0.5%, pH 6.8, 0°C. The cells contained 172 mmol Cl^- per kilogram cell water, and the intracellular chloride was labelled with $^{36}Cl^-$. The initial rate of tracer efflux was determined, and the initial chloride efflux was calculated from the initial rate of isotope release. It is a self-exchange flux in the presence of extracellular chloride, and a net flux (heteroexchange) in experiments where chloride exchanges with other anions. All experiments were performed with the same set of cells. The chloride self-exchange flux was 1.1×10^{-12} mol$\times$cm$^{-2}\times$s^{-1} in the absence of tributyltin.

The rate of ^{42}K efflux should not be affected by the external potassium concentration, if the chloride permeability were orders of magnitude higher than the valinomycin-induced potassium permeability, as would be the case if the large chloride permeability measured by tracer exchange (P_{Cl}^{exch}) were a measure also of the permeability of the membrane to a net transfer of chloride (P_{Cl}^{net}). Fig. 9 shows that the rate of ^{42}K efflux was profoundly affected by changes in extracellular potassium concentration. The chloride permeabilities (P_{Cl}^{net}) calculated from these experiments are summarized in Table IX. It should be noted that P_{Cl}^{net} of intact red cells [3.0×10^{-8} cm(s)$^{-1}$] was 10^4 times lower than the exchange permeability determined in intact red cells at 38°C (Brahm, 1977). DIDS treatment of the red cells reduced P_{Cl}^{net} by 65%. We could not demonstrate any effect of tributyltin on P_{Cl}^{net} at a tributyltin concentration of 5 μmol per liter

cell suspension. The exchange permeability induced by this amount of tributyltin in DIDS-treated cells at 38°C is 5×10^{-5} cm(s)$^{-1}$ (cf. Fig. 7). The conclusion is, therefore, that the chloride permeability induced by organotin compounds in red cell membranes is largely electrosilent, although it is clear that a small increase of P_{Cl}^{net} cannot be detected with the indirect method employed.

TABLE V

TRIBUTYLTIN-MEDIATED EFFLUX OF $^{125}I^-$ FROM DIDS-TREATED RED CELLS INTO MEDIA CONTAINING 165 mM POTASSIUM SALTS OF VARIOUS ANIONS

Extracellular anion	Rate coefficient of $^{125}I^-$ efflux	Initial iodide efflux
	$10^3 \times s^{-1}$	$10^{12} \times (mol \times cm^{-2} \times s^{-1})$
Iodide	17	114.5
Chloride	2.8	19.3
Bromide	4.7	32.4
Thiocyanate	22.4	154.5
Fluoride	0.28	1.9
Nitrate	0.13	0.9

The experiments are analogous to the chloride heteroexchange experiments shown in Table IV, except that the tributyltin concentrations were 10 times lower (5.2×10^{-7} mol/liter cell suspension). The cells contained 180 mM I^- per kilogram cell water, and the intracellular iodide was labelled with $^{125}I^-$ (pH 6.8, 0°C, hematocrit 0.5%). All experiments were performed with the same set of cells. The iodide self-exchange flux was 6×10^{-15} mol$\times$cm$^{-2}\times$s^{-1} in the absence of tributyltin.

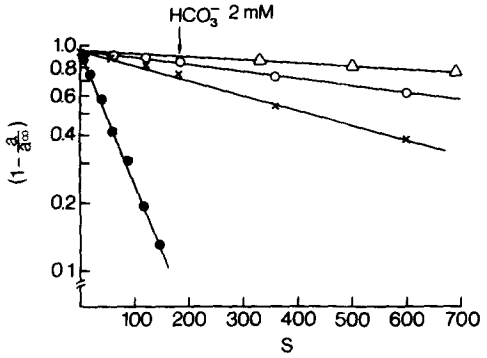

FIGURE 8. The rate of $^{36}Cl^-$ efflux from chloride-loaded, DIDS-treated cells into fluoride (○) and nitrate (x) media containing 5×10^{-6} mol tributyltin per liter cell suspension. The rate of exchange was not affected by the addition of 2 mM bicarbonate after 180 s. The rates of chloride self-exchange in the absence (△) and in the presence (●) of tributyltin are shown for comparison. The experiments were performed at 0°C, pH 6.8. The pH increased to 7.1 after the addition of bicarbonate to the fluoride and nitrate media.

DISCUSSION

The ability of organotin derivatives to exchange anions across biological membranes has previously been demonstrated by Selwyn et al. (1970) and by Motais et al. (1977). The present work represents a quantitative approach to the characterization of the exchange diffusion mechanism, and includes a study of

the failure of the organometallic carriers to mediate a net flow of anions through the membranes. The latter property is responsible for the tight 1:1 coupling of the anion exchange, making the transport process a perfect example of exchange diffusion, as defined by Ussing (1948).

The Mechanism of Organotin-Mediated Anion Exchange

The monovalent organic tin compounds dissociate in aqueous solution according to the scheme (Sillén, 1971):

$$R_3SnCl \rightleftharpoons R_3Sn^+ + Cl^-.$$

Our experiments showed that a constant rate of anion exchange was established within the first few seconds after injecting red cells into a medium containing organotin. The equilibration of organotin between medium and cells must, therefore, occur rapidly. We assume that the exchange diffusion of anions occurs through the lipid phase of the membrane by the mechanism illustrated

TABLE VI

CHLORIDE SELF-EXCHANGE IN DIDS-TREATED RED CELLS LOADED WITH $^{36}Cl^-$

Electrolyte medium	Chloride self-exchange	
	Rate coefficient	$T_{\frac{1}{2}}$
	min^{-1}	*min*
KBr	0.82	0.84
KI	1.11	0.62
KSCN	1.08	0.64
KF	0.07	9.96
KNO_3	0.38	1.84

Media contained 2 mM KCl in addition to 165 mM of the potassium salts indicated in the table. The cell suspension contained 5.2×10^{-6} mol tributyltin per liter, hematocrit 0.5%, pH 6.8, 0°C. The rate coefficient of chloride exchange of DIDS-treated control cells equilibrated in a 165 mM KCl medium was 0.77 min^{-1}. The results are mean values from duplicate experiments.

in Fig. 10. The electroneutral ion pair (R_3SnCl) can permeate the membrane much more easily than the cation (R_3Sn^+), which is largely excluded from the hydrophobic core of the membrane. The charges of the two ions in the ion pair are well shielded, because the ions display a perfect electron sharing in a low dielectric environment—comparable to that of a covalent bond (Clark and O'Brien, 1962). We have found no way of evaluating what fraction of the organotin in the surface membranes is present as ion pairs. In studies of artificial lipid membranes (Tosteson and Wieth, 1979) it was not possible to demonstrate any effect of tributyltin on surface potential, suggesting that a major fraction of the tributyltin in the membrane surface is electrically neutral, i.e., forms ion pairs. The ion pairs can shuttle between the two surface regions of the membrane by random walk and perform the exchange diffusion observed in self-exchange and in heteroexchange experiments. The linear increase of anion flux with both organotin concentration (Fig. 2 and Table III) and anion concentration (Fig. 3), shows that the organotin derivatives form 1:1 complexes with the transported anions.

The Role of the Organic Substituting Groups

The potency of a homologous series of organotin derivatives varied with the length (and therefore with the hydrophobicity) of the substituting organic group (Table VIII). The sequence of relative potencies of the homologous series was

TABLE VII

IODIDE SELF-EXCHANGE IN DIDS-TREATED RED CELLS LOADED WITH ^{125}I

	Iodide self-exchange	
Electrolyte medium	Rate coefficient	$T_{\frac{1}{2}}$
	min^{-1}	*min*
KCl	0.34	2.07
KBr	0.46	1.52
KSCN	1.32	0.53
KF	0.03	24.4
KNO_3	0.25	2.80

Media contained 2 mM KI in addition to 165 mM of the potassium salts indicated in the table. The cell suspension contained 5.2×10^{-7} mol tributyltin per liter, hematocrit 0.5%, pH 6.8, 0°C. The rate coefficient of DIDS-treated control cells equilibrated in a 165 mM KI medium was 0.93 min^{-1}. The results are mean values from duplicate experiments.

TABLE VIII

DOSE-RESPONSE DATA OF ORGANOTIN-MEDIATED CHLORIDE EXCHANGE IN DIDS-TREATED HUMAN RED CELLS

Organotin compound	Concentration of cell suspension	Organotin-induced chloride self-exchange
	mol/liter	$10^{12}\times(mol\times cm^{-2}\times s^{-1})$
Trimethyl	0.7×10^{-3}	1.4
–	1.4×10^{-3}	3.0
Triethyl	8.3×10^{-5}	4.0
–	1.7×10^{-4}	10.1
–	3.4×10^{-4}	20.8
Tripropyl	6.0×10^{-6}	17.7
–	1.2×10^{-5}	34.3
–	2.4×10^{-5}	71.1
–	5.8×10^{-5}	133.4
Triphenyl	6.0×10^{-6}	21.4
–	1.2×10^{-5}	39.7
–	2.4×10^{-5}	71.1
–	5.8×10^{-5}	128.0

pH 6.8, 0°C. The self-exchange flux in the absence of organotin [0.9×10^{-12} mol$\times$cm$^{-2}\times$s^{-1}] was subtracted from the flux found in the presence of organotin. The dose-response results for tributyltin are shown in Fig. 2.

the same as that reported by Selwyn et al. (1970) for the organotin-mediated Cl^-/OH^- exchange in red cells. The results of Fig. 2 and of Table VIII show that the self-exchange of chloride increased by a factor of 10^4, when methyl groups were replaced by butyl groups.

The Role of the Anion Partner

The exchange flux of anions also depends on the nature of the anion partner of the ion pair (Table II). The equilibrium between ion pairs in electrolyte medium and membrane is established rapidly, and it appears likely that the concentration of tributyltin ion pairs in the membrane is proportional to the concentration of ion pairs in the aqueous phase, which increases through the series $Cl^- < Br^- < I^-$. The association constants for ion pair formation between triphenyltin and chloride, bromide, or iodide are 10^3, 2×10^3, and 5×10^3 M^{-1}, respectively

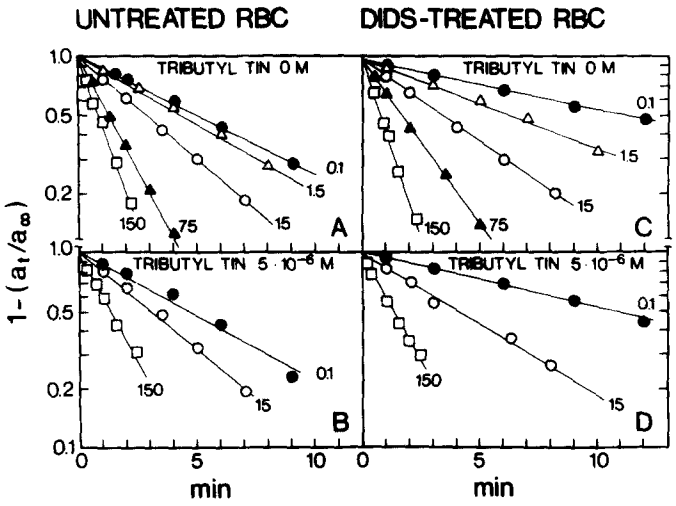

FIGURE 9. The rate of $^{42}K^+$ efflux into media with varying potassium concentrations (NaCl + KCl = 150 mM). The potassium concentrations are indicated on the efflux curves. Experiments were performed under four sets of conditions in untreated red cells (left hand panels) and in DIDS-treated cells (right hand panels), in the absence (upper panels) and in the presence (lower panels) of tributyltin (5×10^{-6} mol per liter cell suspension, hematocrit 0.5%, 38°C). The pH of the unbuffered media was 6.6. It changed maximally 0.05 during the experiments, showing that there was no significant flux of hydrogen or of hydroxyl ions. The membrane permeabilities to electrodiffusion of chloride ions under the four experimental conditions were calculated from these results as described in Methods. The values are shown in Table IX.

(Sillén, 1971). The relative affinities of the anions for ion pair formation correspond to the observed relative rates of halide transport by tributyltin (Table II). It is, therefore, possible that the magnitude of halide transport simply is determined by the concentration of ion pairs in the membrane phase which, in turn, in the cases of chloride, bromide, and iodide, bears a simple relation to the concentration of ion pairs in the aqueous phase. This view is supported by our finding that both organotin partitioning and anion transport were found to be four to five times larger in iodide than in chloride media. This simple relationship does not hold for fluoride. Fluoride ions have a high affinity for organotin (Sillén, 1971), but fluoride was not transported through the membrane by tributyltin (Table II), and fluoride did not act as an exchange

partner in the heteroexchange experiments (Tables IV and V). Nevertheless, fluoride was a strong inhibitor of chloride and iodide exchange (Tables VI and VII). The data on fluoride transport do not fit with the idea that a high concentration of fluoride ion pairs in the aqueous phase should result in a high concentration of organotin in the membrane. A possible explanation for the

TABLE IX

EFFECT OF TRIBUTYLTIN ON THE PERMEABILITY OF RED CELLS TO ELECTRODIFFUSION OF CHLORIDE

	Permeability to electrodiffusion of chloride	
Tributyltin	Untreated red cells	DIDS-treated red cells
mol/liter suspension	*cm (s)*$^{-1}$	
0	3.0×10^{-8}	1.1×10^{-8}
	(2.7–3.2×10^{-8})	(1.0–1.2×10^{-8})
5.2×10^{-6}	2.7×10^{-8}	1.1×10^{-8}
	(2.4–3.1×10^{-8})	(1.1–1.2×10^{-8})

Valinomycin 10^{-6}M; 38°C. Mean values and ranges of three experimental series. Ranges are indicated by parentheses.

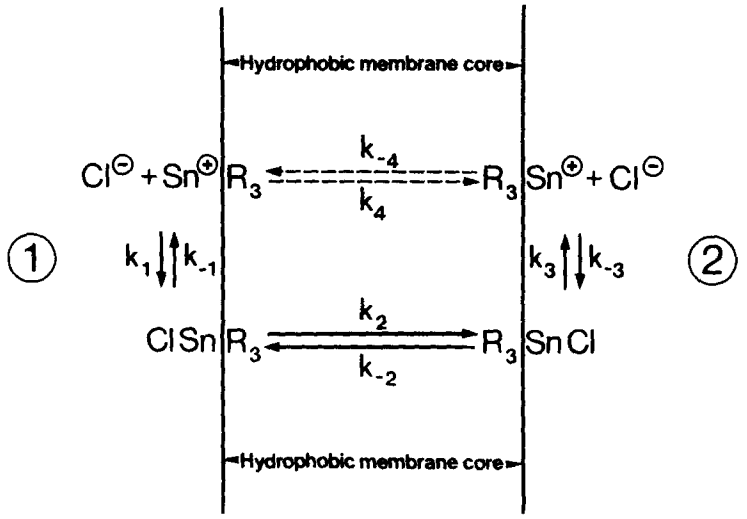

FIGURE 10. Schematic representation of the exchange diffusion of anions mediated by organotin compounds. The electroneutral ion pair (R_3SnCl) can permeate the hydrophobic core of the membrane, whereas the unpaired cation R_3Sn^+ permeates the membrane at a rate (k_4) which is orders of magnitude lower than that of the ion pair (k_2).

apparent discrepancy is provided by Cotton and Wilkinson (1966, p. 477), who reported that fluoride ions form water-soluble long-chained polyionic complexes with organotin derivatives. We, therefore, suggest that partitioning of organotin into the membrane is decreased, because the polyionic fluoride complexes cannot be adsorbed into the membrane.

Hydroxyl ions have an extremely high affinity for organotin. The association constant for triphenylhydroxide is $10^{9.2}$ M^{-1}, six orders of magnitude higher

than the affinity for chloride, bromide, and iodide (Sillén, 1971). It is, therefore, not surprising that hydroxyl ions exert a strong inhibitory effect on organotin-mediated anion exchange. The self-exchange of chloride was inhibited, when pH increased above 6 (Fig. 4), and the results of Fig. 4 fitted well with an apparent dissociation constant for tributyltin hydroxide of 10^{-8}M at a chloride concentration of 165 mM. In other words, chloride exchange was inhibited 50% at a hydroxyl ion concentration (10^{-8}M at pH 6.9), which is about 10^7 times smaller than the chloride concentration. Studies of the ability of organotin to equilibrate hydroxyl ions across the membrane have clearly demonstrated that OH^- exchanges readily with chloride (Selwyn et al., 1970; Aubert and Motais, 1975; Fig. 5 of this article). The slow response time of the glass electrode does not permit a determination of the initial rate of hydroxyl transport in the

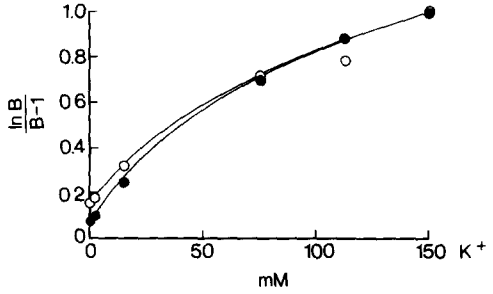

FIGURE 11. Determination of P_{Cl}^{net} from measurements of valinomycin-mediated $^{42}K^+$ efflux from red cells into media containing 150 mM (sodium + potassium chloride). Extracellular potassium concentration is shown on the abscissa, the ordinate ln $B/(B-1)$ is equal to k_K/k_{150}, where k_{150} is the rate coefficient of potassium efflux in the 150 mM KCl medium, and k_K is the rate found at a lower extracellular potassium concentration (cf. Methods). The ratio P_K/P_{Cl}^{net} was 19 in untreated cells (○), and 55 in DIDS-treated cells (●); the respective values of P_{Cl}^{net} were 3.0 and 1.1 × 10^{-8} cm $(s)^{-1}$. The experiments were performed at 38°C, pH 6.6.

experiment shown in Fig. 5 B. A very rough estimate of the apparent hydroxyl permeability induced by 3.8 × 10^{-6} mol tributyltin per liter cell suspension is a value of ~10^{-3} cm × s^{-1}, calculated by assuming that half of the total amount of hydroxyl ion transferred from medium to cells (1.8 × 10^{-9} mol/cm^2) is transferred during the 1st s after a sudden increase of the extracellular hydroxyl ion concentration by 2.5 × 10^{-4} M.

No evidence of saturation of transport was found, when the chloride concentration was increased to 600 mM in red cells (Fig. 3) or to 900 mM in ghosts. We cannot present a quantitative explanation for this low apparent affinity. Although the association constant of tributyltin chloride in aqueous solution is not known, it is likely to be somewhat larger than the previously cited value of 10^3 M^{-1} for triphenyltin chloride, because the tendency of organotin derivatives to form ion pairs with halides increase with the lipophilicity of the organic groups (Sillén, 1971). Therefore, it was surprising that chloride fluxes did not show any tendency to saturate (Fig. 3). It must be noted, however, that

several factors will tend to increase the concentration of tributyltin chloride in the membranes, when chloride concentration is increased. Increased formation of chloride ion pairs in the aqueous phase leads to an increased adsorption of ion pairs into the membranes. Moreover, the displacement of hydroxyl groups from tributyltin hydroxide at pH 6.8 will increase the amount of halide ion pairs by competition, contrasting the inhibitory effect of hydroxyl ions on chloride transport (Fig. 4). It is, furthermore, possible that the true association constant of tributyltin chloride of membrane-bound tributyltin differ from that found in the electrolyte solution, e.g., if the ion pair formation takes place in a microenvironment at the membrane surface that is characterized by a dielectric constant that is larger than that of the bulk solution. All these possible effects tend to lower the apparent affinity, as it can be evaluated by flux measurements.

We were not able to demonstrate clear evidence of competition between chloride and iodide for transport, when the self-exchange was studied at a concentration of 2 mM in the presence of 165 mM of the other anion (Tables VI and VII). The rate of iodide exchange decreased by a factor of three (from 0.9 to 0.3 min^{-1}), when most of the iodide was replaced with chloride (Table VII). This observation suggests that the decreased partitioning of tributyltin into the membrane in the presence of chloride is the cause of the decreased rate of iodide transfer. However, the simple relation between partitioning and transport did not hold for chloride: The rate of chloride exchange increased only by 40% (Table VI) in the presence of iodide, where the amount of tributyltin in the membrane is increased fourfold. In series of experiments where the effect of iodide substitution was studied over the whole concentration range between 0 and 165 mM, it was found that the small increase of the rate of chloride exchange shown in Table VI is significant. It is likely that the situation is complex, because the presence of iodide decreases the fractional amount of chloride ions forming ion pairs with organotin in the aqueous solution, while at the same time membrane transport of chloride is favored by the larger amount of tributyltin in the membrane, maybe because chloride transport through the membrane is facilitated by an exchange reaction of the type:

$$R_3SnI + Cl^- \rightleftharpoons R_3SnCl + I^-.$$

Adsorption of Tributyltin to the Membranes

We found no difference between the chloride self-exchange induced by tributyltin in DIDS-treated red cells or ghosts (Table I), suggesting that organotin was not bound to other cellular constituents than the membranes. This conclusion was further substantiated by the finding that the rate of self-exchange was not affected by addition of a membrane-free lysate to the medium. At a constant hematocrit, anion self-exchange in DIDS-treated cells was a linear function of the amount of organotin present in the medium, when the concentrations were below $\sim 5 \times 10^{-6}$ mol/liter suspension (Fig. 2, Table III), showing that the adsorption coefficient is constant within a wide concentration range. At a hematocrit of 0.5% the concentration of tributyltin in the medium (T_m of Eq. 4) is only 50–80% of the concentration in the suspension (T_t of Eq. 4) in iodide and chloride media, because a considerable fraction is taken up by the cells ($\alpha_I \sim$

200, $\alpha_{Cl} \sim 50$). For cells suspended in a chloride medium, the number of tributyltin molecules in the membrane surfaces at a concentration (T_m) of 4×10^{-6} M calculated by means of Eq. 6*a* was 4×10^{12} tributyltin molecules per cm^2. This membrane density is equal to 1.1×10^7 organotin molecules per cell, approximately one organotin molecule per 20 phospholipid molecules (Bar et al., 1966). If evenly distributed in the membrane, the mean spacing distance between the tin molecules is ~50Å. The membrane becomes apparently saturated with organotin if the number of molecules is doubled (cf. Fig. 2). A density of 10^{13} per cm^2 corresponds to a spacing of about 30Å.

The Turnover Number of Organotin-Mediated Anion Exchange

It is only possible to relate the anion transport to the total amount of tributyltin present in the membrane (T_d). At a concentration in the medium (T_m) of 4×10^{-6} M, the density of tin molecules in the membrane is $4 \times 10^{12} \cdot cm^{-2}$. The organotin-facilitated chloride exchange was 10^{-10} mol·$cm^{-2} \cdot s^{-1}$ = 6×10^{13} ions·$cm^{-2} \cdot s^{-1}$ at 0°C (Table I), and increased 22 and 90 times at 25 and 38°C, respectively (Fig. 6). The overall turnover numbers at 0, 25, and 38°C are, therefore, 15, 325, and 1350 s^{-1}. These rates are, of course, not measures of the transfer rates of ion pairs through the membrane, since the true turnover number cannot be determined as long as we do not know the degree of saturation of the membrane-bound carrier. According to the results of Fig. 4, chloride self-exchange was inhibited by ~45% at pH 6.8. Therefore, the true turnover number for chloride must be at least twice as big as the overall value, even if it is assumed that all tributyltin in the membrane is present as ion pairs: R_3SnCl and R_3SnOH.

Lack of Effect of Tributyltin on the P_{Cl}^{net} of Red Cells

The organotin-mediated chloride transport was found to be electrically silent. P_{Cl}^{net} was determined by the valinomycin method both in normal and in DIDS-treated red cells (Fig. 9). An example of our experimental fit is shown in Fig. 11. The P_{Cl}^{net} found by us in intact red cells (Table IX) agrees with values reported by Hunter (1971, 1977) and by Knauf et al. (1977). Also, our observation that DIDS treatment of red cells (which reduces natural chloride exchange by 99.6%) caused a reduction of P_{Cl}^{net} of 70%, is in accordance with the observation of Knauf et al. (1977).

There was no flux of hydroxyl or hydrogen ions under the four sets of experimental conditions employed for the determination of P_{Cl}^{net} (Fig. 9). This was clear, because the pH of the unbuffered media did not change by more than 0.03–0.05 units even in the low potassium media, where the change of membrane potential causes a considerable disequilibrium of hydrogen and of hydroxyl ions across the membrane. The stability of extracellular pH was also observed in the presence of tributyltin. This shows that a chloride-hydroxyl exchange cannot be induced by changing the membrane potential, underlining the electroneutral nature of the organotin-mediated anion transfer.

The results of Table IX show that tributyltin did not cause any detectable increase of P_{Cl}^{net} in normal or in DIDS-treated red cells. However, the determination of P_{Cl}^{net} by the valinomycin method is indirect, making it possible that a

small increase of P_{Cl}^{net} might not have been detected. It is obvious from inspection of Fig. 11 that, although the differences found in the rate of potassium fluxes at low potassium concentrations in normal and in DIDS-treated cells are very reproducible (cf. Table IX), the indirect method could fail to detect a minor effect of tributyltin on the membrane permeability to electrodiffusion of chloride ions. Therefore, we have also investigated the discrepancy between anion exchange and membrane conductance in artificial lipid membranes, where both conductance and tracer exchange can be determined directly with a high degree of precision. These studies, which confirmed that tributyltin mediates an electrically silent exchange diffusion of anions through thin lipid membranes, are reported in the following article (Tosteson and Wieth, 1979).

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REFERENCES

AUBERT, L., and R. MOTAIS. 1975. Molecular features of organic anion permeability in ox red blood cell. *J. Physiol. (Lond.).* **246:**159–179.

BAR, R. S., D. W. DEAMER, and D. G. CORNWELL. 1966. Surface area of human erythrocyte lipids: reinvestigation of experiments on plasma membrane. *Scence (Wash. D.C.).* **153:**1010–1012.

BRAHM, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. *J. Gen. Physiol.* **70:**283–306.

CABANTCHIK, Z. I., and A. ROTHSTEIN. 1974. Membrane protein related to anion permeability of human red blood cells. *J. Membr. Biol.* **15:**207–226.

CHAPPEL, J. B., and A. R. CROFTS. 1966. Ion transport and reversible volume changes of isolated mitochondria. *B.B.A. (Biochim. Biophys. Acta) Libr.* **7:**294–314.

CLARK, H. C., and R. J. O'Brien. 1963. Fluoro salts of the trimethyltin group. *Inorg. Chem.* **2:**1020–1022.

COTTON, F. A., and G. WILKINSON. 1966. Advanced Inorganic Chemistry. A Comprehensive Text. J. Wiley & Sons, Inc., Interscience Publishers, New York. 477.

DALMARK, M., and J. O. WIETH. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. *J. Physiol. (Lond.).* **224:** 583–610.

FUNDER, J., D. C. TOSTESON, and J. O. WIETH. 1978. Effects of bicarbonate on lithium transport in human red cells. *J. Gen. Physiol.* **71:**721–746.

FUNDER, J., and J. O. WIETH. 1976. Chloride transport in human erythrocytes and ghosts: A quantitative comparison. *J. Physiol. (Lond.).* **262:**679–698.

GUTKNECHT, J., J. S. GRAVES, and D. C. TOSTESON. 1978. Electrically silent anion transport through lipid bilayer membranes containing a long-chain secondary amine. *J. Gen. Physiol.* **71:**269–284.

HUNTER, M. J. 1971. A quantitative estimate of the non-exchange-restricted chloride permeability of the human red cell. *J. Physiol. (Lond.).* **218:**49–50P.

HUNTER, M. J. 1974. The use of lipid bilayers as cell membrane models: An experimental test using the ionophore, valinomycin. *In* Drugs and Transport Processes. B. A. Callingham, editor. Macmillan & Co. Ltd., London. 227–240.

HUNTER, M. J. 1977. Human erythrocyte anion permeabilities measured under conditions of net charge transfer. *J. Physiol. (Lond.).* **268**:35–49.

JENNINGS, M. L. 1978. Characteristics of CO_2-independent pH equilibration in human red blood cells. *J. Membr. Biol.* **40**:365–391.

KNAUF, P. A., G. F. FUHRMANN, S. Rothstein, and A. ROTHSTEIN. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**:363–386.

LEPKE, S., H. FASOLD, M. PRING, and H. PASSOW. 1976. A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS) and its dihydro derivative (H_2DIDS). *J. Membr. Biol.* **29**:147–177.

MOTAIS, R., J. L. COUSIN, and F. SOLA. 1977. The chloride transport induced by trialkyltin compound across erythrocyte membrane. *Biochim. Biophys. Acta.* **467**:357–363.

SELWYN, M. J., A. P. DAWSON, M. STOCKDALE, and N. GAINS. 1970. Chloride-hydroxide exchange across mitochondrial, erythrocyte and artificial lipid membranes mediated by trialkyl- and triphenyltin compounds. *Eur. J. Biochem.* **14**:120–126.

SHEAN, G. M., and K. SOLLNER. 1966. Carrier mechanisms in the movement of ions across porous and liquid ion exchanger membranes. *Ann. N.Y. Acad. Sci.* **137**:759–776.

SHIP, S., Y. SHAMI, W. BREUER, and A. ROTHSTEIN. 1977. Synthesis of tritiated 4,4-diisothiocyano-2,2-stilbene disulfonic acid (^{3}H-DIDS) and its covalent reaction with sites related to anion transport in human red blood cells. *J. Membr. Biol.* **33**:311–323.

SILLÉN, L. G. 1971. Stability constants of metal-ion complexes. *Chem. Soc. Spec. Pub.* No. 25, Suppl. No. 1.

TOSTESON, M. T., J. O. WIETH, and D. C. TOSTESON. 1977. Tri-butyl tin produces chloride exchange diffusion in bilayers. *Biophys. J.* **17**:211a.

TOSTESON, M. T., and J. O. WIETH. 1979. Tributyl-tin mediated exchange diffusion of halides in lipid bilayers. *J. Gen. Physiol.* **73**:789–800.

USSING, H. H. 1948. The use of tracers in the study of active ion transport across animal membranes. *Cold Spring Harb. Symp. Quant. Biol.* 193–200.

WIETH, J. O. 1972. The selective ionic permeability of the red cell membrane. *In* Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status. M. Rørth and P. Astrup, editors. Munksgaard, Copenhagen. 265–278.

WIETH, J. O., and M. T. TOSTESON. 1977. Tightly coupled exchange diffusion of anions in erythrocytes and in artificial lipid membranes-organotin compounds as anion exchange carriers. *Proc. Int. Union Physiol. Sci.* **13**:812. (Abstr.)