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Transport of Hydrophobic Ions in Erythrocyte Membrane: I. Zero Membrane Potential Properties

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Summary. The permeability and partition coefficients of tetraphenylarsonium (TPA) and several other organic cations were studied in the human erythrocyte using an ion-selective electrode. The permeability constant for the different cations could be explained quite well by differences in oil/water partition coefficients. No evidence for facilitated transport could be found. Binding of the organic ions occurred to both the cell membrane and to intracellular contents. Partitioning to the membrane remained relatively constant despite variation from ion intracellular binding with blood samples from different donors. TPA flux is stimulated by substoichiometric amounts of tetraphenylboron and other organic anions, suggesting an ion-pairing mechanism.

Key Words hydrophobic ions · red cell · permeability · tetraphenylarsonium · erythrocyte · ion binding · membrane transport

Introduction

Hydrophobic ions have been used extensively in the study of membrane structure and function. Research has focused on three interrelated problems: the elucidation of the mechanism of transport through membranes (Ketterer, Neumcke & Läuger, 1971), the functional dependence of transport parameters on membrane structure, and the use of passively distributed hydrophobic ions to reflect the membrane potential in a variety of systems.

In early experiments, Liberman and Topaly (1968) found that steady-state conductance of phospholipid bilayers increased in the presence of the hydrophobic cations such as dimethyldibenzyl ammonium, trimethylphenyl ammonium and the anion tetraphenylboron. Since the conductance was linearly related to the hydrophobic ion concentration and since a membrane potential difference of 58 mV could be attained by a 10-fold concentration difference across the bilayer, it was concluded that the membrane conductance resulted from the direct passage of these ions through the bilayer. From relaxation studies using the lipid-soluble ions dipicrylamine and tetraphenylboron, Ketterer et al. (1971) proposed that the transport of hydrophobic ions across the bilayer consists of three steps. Ions are adsorbed to the membrane solution interface, pass over a single energy barrier located in the membrane interior, and are desorbed into the opposite aqueous phase. Potential energy minima at the two membrane solution interfaces stem from the sum of chemical and electrical energy profiles in the membrane (Andersen & Fuchs, 1975). Application of a small transmembrane voltage resulted in transient currents which decayed with first-order kinetics. It was proposed that the currents were due to a redistribution of ions between the two potential energy wells and relevant rate constants were obtained from the data.

With respect to membrane structures, the dependence of tetraphenylboron and tetraphenylarsonium membrane transport on sterol content, lipid composition, and membrane thickness is, in general, in agreement with quantitative estimates based on theoretical calculations (Andersen, 1978). On this basis, Pickar and Benz (1978) concluded that these two ions are good probes of bilayer structure. Recently, Benz and Nonner (1981) have used dipicrylamine to explore the lipid bilayer component of nerve membranes based on information gained from artificial membrane studies.

Hydrophobic ions probably enter cells by simple diffusion through the membrane lipid phase without interaction with protein-mediated transport processes. Therefore measurements of their equilibrium distribution can be used to determine membrane potentials (Deutsch et al., 1979; Cheng et al., 1980). Ideally the probe should equilibrate rapidly, but not perturb the membrane potential. Further, the ion distributions must reflect free ions. The use of lipid-soluble ions in this context is complicated by binding of the ions to membranes or cytoplasmic constituents (Deutsch et al., 1979).

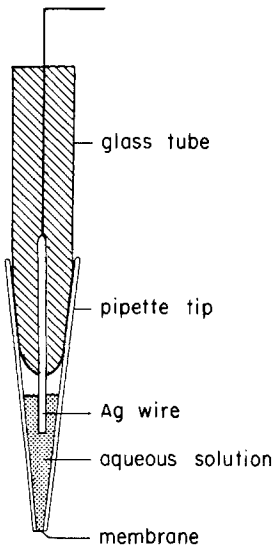

Fig. 1. Construction of TPA electrodes. The electrode holder consists of a tapered glass tube containing a Ag wire held in place with silicone rubber. The Ag wire is chloridized and dips into an aqueous solution containing Cl ions. The sensing membrane, which is described in the text is formed over the tip of a plastic pipette tip. This design makes it easy to rapidly replace the electrode in case of a malfunction

We have used the lipid-soluble ions tetraphenylarsonium, tetraphenylphosphonium, benzyltriphenylphosphonium and triphenylmethylphosphonium to characterize the permeability properties of the human red cell lipid bilayer. Ion fluxes were measured with organic ion-selective electrodes which achieve significantly better time resolution than isotope methods. Both kinetics and binding are considered in detail. Some of this work has been recently published in a preliminary report (Macey & Orme, 1980).

Materials and Methods

Drugs and Chemicals

Sources and abbreviations of chemicals are as follows: tetraphenylarsonium chloride (TPA) and tetraphenylphosphonium bromide (TPP) from K & K Chemical Co. (Plainview, N.J.); benzyltriphenylphosphonium chloride (BTPP) and sodium tetraphenylboron (TPB) from Aldrich (Milwaukee, Wis.); methyltriphenylphosphonium bromide (TPMP), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS) from Sigma (St. Louis, Mo.); valinomycin (VAL) from Cal-Biochem (San Diego, Calif.); sodium isethionate and 3,3′,4′,5-tetrachlorosalicylanilide (TCS) from Eastman Kodak (Rochester, N.Y.); 8-anilinonaphthalene (ANS) from J.T. Baker; 3,5-di-t-butyl-4-OH-benzylidene malonitrile (SK6847) was a gift from the Sumitomo Chemical Industry (Osaka, Japan); 5-Cl-3-t-butyl-2′-Cl-4′-nitro-salicylanilide (S-13) was a gift of P. Hann of Monsanto Chemical Co. (St. Louis, Mo).

Blood

Blood from human donors was washed three times in isotonic NaCl. For efflux experiments, cells were preloaded with organic cations at a 15% hematocrit (30°C) until equilibrium was reached. Unsealed RBC ghosts were made according to the procedure of Dodge, Mitchell and Hanahan (1963).

Drug Treatments

DIDS (1 mg/2 cc packed cells) was added to cells at 25% hematocrit and incubated at 0 to 5°C for 45 min in the dark. Cells were washed once to remove excess DIDS. Valinomycin and CCCP were added as concentrated solutions in ethanol. CCCP was mixed on the day of use.

Flux Experiments

Fluxes were measured at 30°C unless otherwise noted. For influx experiments, 1 cc of unloaded cells were injected by syringe into 5 cc of isotonic solution containing 3 μM lipid-soluble ion. The solution consisted of either 150 mM NaCl, mixtures of 150 mM NaCl and 150 mM KCl, or mixtures of 270 mM sucrose and 150 mM NaCl, depending on the experiment. Following a 2- to 4-min equilibration period, a small quantity of lipid-soluble ion was injected into the solution. For efflux experiments, 1 cc of loaded cells was injected into 5 cc of isotonic salt solution containing 3 μM lipid-soluble ion.

Electrodes

Cation fluxes were measured with ion-selective plastic membrane electrodes similar to those of Moody, Oke and Thomas (1970). To prepare the liquid ion exchanger, a precipitate of TPA/TPB was obtained by mixing solutions of these two ions, and 0.7 mg TPA/TPB was dissolved in 0.4 cc dimethylformamide in a capped test tube. Once dissolved, 0.5 cc dibutylphthalate and 2.0 cc 10% polyvinylchloride in tetrahydrofuran were added. The tube was shaken vigorously on a vortex mixer. The concentration of TPA/TPB in dibutylphthalate was 2 mM.

To prepare electrodes, the tips of 200-μl Eppendorf pipettes (West Coast Scientific Co., Berkeley, Calif.) were cut off to 2 to 3 mm diameter with a razor blade. A shallow groove was then made in the top half of the tip inside with a hot wire. This aided in dissipating positive pressure inside the tip which would have deformed the membrane. These tips were then dipped into the above electrode mixture and dried in a dust-free place for one to two weeks, prior to use. The tips were then filled with a solution of 150 mM NaCl containing 100 μM lipid-soluble ion, and used in conjunction with a standard Ag/AgCl electrode (Fig. 1).

The same electrode could be used for measuring all four of the organic cations tested. The selectivity order was: BTPP > TPA > TPP >> MTPP. The electrodes gave near-Nernstian responses to the first three cations down to 0.1 μM. Electrodes responded to a concentration pulse producing a 30-mV deflection in less than 2 sec. It is likely that this response time is limited by stirring and thus represents an upper limit.

Cell Water

Water was determined in packed cells by evaporation.

Chemical Determinations of Organic Cations and Anions

The organic cations and also TPB were determined by extraction into an organic phase, followed by measurement with a spectrophotometer. To aqueous solutions of the organic cations, KCNS was added to bring the concentration to 5 mM. The aqueous solutions were then extracted with an equal volume of chloroform (above 99% extraction was obtained). The organic phase was separated and the cations were read at 264 nm (TPA), 268 nm (TPMP) or 269 nm (TPP and BTPP).

TPB was extracted into an organic phase of 98% (by volume) octanol and 2% of the quaternary amine, Aliquat.[39] The organic extracts were diluted 1 : 1 with methanol and read at 257 nm.

Miscellaneous Chemical Procedures

Crude hemoglobin extracts were made by freeze-thawing red blood cells. Two volumes of the lysed cells were diluted with 1 volume 150 mM KCl and were then spun at 17,500 rpm for 30 min in a Sorvall SS-34 rotor to remove cell membranes and debris. Hemoglobin concentration was determined by the cyanomethemoglobin method (Beutler, 1975).

Lipid content of unsealed ghosts was determined by extracting with chloroform/methanol (Bligh & Dyer, 1959) and then evaporating the extract to dryness and weighing.

Membrane Potential

The criterion for zero membrane potential was the equality of pH on the two sides of the cell membrane. Although the membrane potential of the human red cell is set by the transmembrane Cl^- ratio, this procedure is valid because an extremely active HCO_3/Cl exchange system rapidly sets the H^+ ratio to the inverse of the Cl^- ratio. The low metabolic production of H^+, of the order of 2 to 4 mM/liter cells/hr (Grimes, 1980) and the high buffering capacity of hemoglobin (approx. 60 mM) in the red cell (Donlon & Rothstein, 1969), further prevent the H^+ ratio from departing significantly from the equilibrium value. Under experimental conditions similar to ours, the Cl^- and H^+ ratios of red cells have been shown to have a close inverse relationship (Waddell & Bates, 1969; Funder & Wieth, 1976). Internal pH was read with electrodes after sonication of the cells. The membrane potential for experiments reported here was 0 ± 2 mV.

Ion Binding

A blood suspension of known total volume, hematocrit, water weight and total amount of probe ion was incubated to equilibrium at 30°C in a shaker bath. Extracellular lipid-soluble ion concentration was either measured with electrodes or determined chemically. Intracellular concentrations were either measured directly in sonicated cell pellets, or calculated assuming the ion was in equilibrium. Packed cells were sonicated with a Sonifier model 350 cell disrupter. Bound ion was then calculated as the difference between total ion present and the sum of extracellular and intracellular free ion.

Calculations

The binding of organic cations to intracellular components of the red blood cell is a linear function of the free concentration of the cation over a large range of concentrations (Macey & Orme, 1980). Under these conditions, if C_i and V_i denote the internal concentration and internal volume, the moles M_b of bound organic cation is given by:

$$M_b = aC_iV_i \tag{1}$$

where a is a constant. If C_o and V_o denote external concentration and external volume, conservation of mass requires that the total number of moles M of the organic cation be given by:

$$M = M_b + C_iV_i + C_oV_o = C_iV_i(1 + a) + C_oV_o. \tag{2}$$

At zero membrane potential, the equation for ion flux (Macey & Orme, 1980) becomes:

$$AJ = V_o\frac{dC_o}{dt} = AP(C_i - C_o) \tag{3}$$

where A is the membrane surface area and P is the permeability constant. Eliminating C_i by substituting Eq. (2), and integrating gives:

$$C_o = \frac{M}{V_o + (1 + a)V_i} + \left[C_o(o) - \frac{M}{V_o + (1 + a)V_i}\right]e^{-t/\tau} \tag{4}$$

where $C_o(o)$ denotes the external concentration at time zero and τ is a time constant given by:

$$\tau^{-1} = \frac{A[V_o + (1 + a)V_i]}{(1 + a)V_oV_i}P. \tag{5}$$

The permeability can be calculated directly from the time constant. This is particularly advantageous because these time constants are independent of initial conditions and of slow drifts in ion electrodes.

In practice, electrode data was collected every second for 200 sec and fed into a PDP-11 computer. Time constants were calculated from the raw data by least-squares methods. Permeabilities were calculated from the time constants using Eq. (5). Literature values of the red cell area and volume, 138 μm^2 and 107 μm^3, were used in all calculations (Canham & Burton, 1968).

Partition coefficients have been defined in a number of arbitrary ways because even pure phospholipid membranes are heterogeneous so that the "concentration of solute in the membrane" has no precise definition. Some authors assume the measured membrane contents are uniformly distributed throughout the membrane volume while others (e.g., Cafiso & Hubbell, 1982) attempt to define a binding volume within the membrane, and still others (e.g., Ketterer et al., 1971) prefer to circumvent the issue by defining a surface partition coefficient where the measured membrane contents M_{memb} are simply normalized by the membrane surface area. To facilitate comparisons, we have adopted the latter approach; surface partition coefficients β are defined as

Table 1. Zero potential fluxes and permeabilities[a]

Ion	Initial Flux[b] (μmol/liter cells · sec)	P (cm/sec)	τ sec
TPA	$2.89 \pm 0.19 \times 10^{-1}$	7.2×10^{-7}	273
TPP	$2.90 \pm 0.19 \times 10^{-1}$	6.7×10^{-7}	294
BTPP	$1.51 \pm 0.08 \times 10^{-1}$	3.2×10^{-7}	547
TPMP	$1.04 \pm 0.05 \times 10^{-2}$	1.4×10^{-8}	6132

[a] Mean and SE for five samples from the same donor. Temperature = 30°C.
[b] Pre-equilibrated (45 μM internal free) red cells were injected into 150 mM NaCl containing 3 μM ion.

$$\beta = \frac{M_{\text{memb}}/2A}{C}. \tag{6}$$

In our case, M_{memb} is typically estimated from measurements of M_l, the total hydrophobic ion contents of membrane containing 1 g of lipid. Since the cell area = 1.37×10^{6} cm^2 and the mass of lipid per cell = 5×10^{-13} g/cell (Dodge et al., 1963), Eq. (6) becomes

$$\beta = \frac{1.82 \times 10^{7} M_l}{C}$$

where C is the aqueous concentration in moles/cm^3. To convert to a bulk concentration partition coefficient, simply divide by 1/2 the assumed thickness of the binding volume. This calculation makes no distinction between distributions in lipid in protein. If most of the ion is confined to the lipid phase, our computation could be an underestimate by a factor as large as 2.

Results

Zero Potential Fluxes and Permeabilities

Ion flux experiments were carried out in 150 mM NaCl at 30°C. It was consistently found that the membrane potential was close to zero under these conditions, as determined by the hydrogen distribution method. Results are summarized in Table 1. At zero membrane potential the efflux rates of TPA and TPP are almost equal. BTPP efflux is about 40% slower than TPA, and the TPMP efflux rate was slower by a factor of 27. Permeabilities ranged from 7.2×10^{-7} cm/sec for TPA to 1.4×10^{-8} cm/sec for TPMP. The data is from one blood donor and is intended to show the relative permeabilities of the four ions. Permeabilities were found to vary from one blood donor to the next. For seven other donors, the permeability of TPA ranged from 4.6×10^{-7} to 6.6×10^{-7} cm/sec at 30°C. TPA permeabilities for fresh blood were lower by a factor of 1.5 to 3 than those previously reported for blood bank blood (Macey & Orme, 1980). The permeability of TPMP in red cells (1.4×10^{-8} cm/sec) at 30°C is somewhat faster than the measured permeability of 3.8×10^{-9} cm/sec of a spin-labeled triphenylphosphonium analog in phosphatidylcholine liposomes (Cafiso & Hubbell, 1982). However, direct comparison of these results are compromised by differences in temperature and by differences in molecular structure.

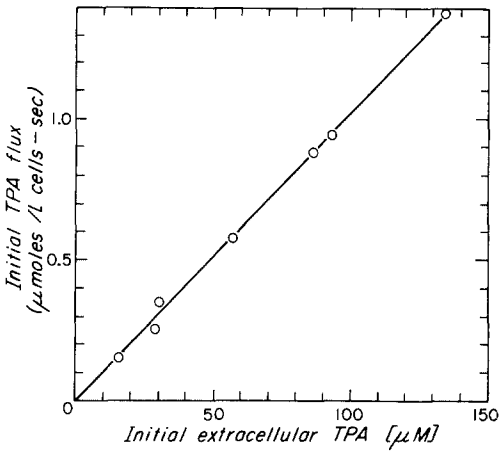

Fig. 2. Linearity of TPA influx as extracellular concentration rises. Blood at a hematocrit of 15% in isotonic saline, with an initial external TPA concentration of 3 μM, was pulsed with TPA to raise the external concentrations to the values indicated on the graph. Fluxes were measured with electrodes. The membrane potential was 0 and the temperature was 30°C

Concentration Dependence of Ion Fluxes

Figure 2 shows a linear dependence of initial TPA influx on extracellular concentration in the range 14 to 144 μM (which covers the range used in experiments described in this paper). This agrees with earlier experiments showing linearity to at least 1200 μM (Macey & Orme, 1980). Extracellular concentration was varied by administering pulses of increasing ion concentration. No evidence of saturation was found in this concentration range.

Temperature Dependence of Ion Fluxes

The activation energy for TPA flux, calculated from an Arrhenius plot in the temperature range 20 to 35°C, was found to be 22.5 kcal/mole (Fig. 3). In a control experiment, it was determined that the constant a of Eq. (1) varied insignificantly over the temperature range 20 to 35°C.

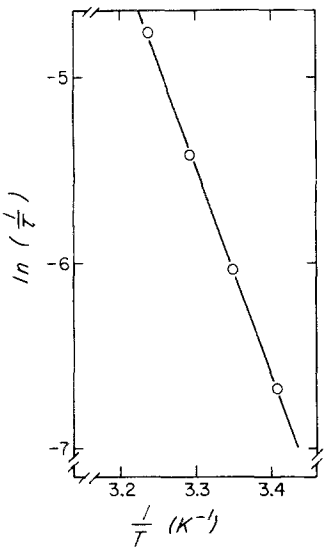

Fig. 3. Arrhenius plot of the temperature dependence of TPA efflux. Blood was preloaded with TPA and packed. The packed cells were then pipetted into 150 mM NaCl to give a hematocrit of 15% and the efflux was measured with an electrode. Temperature range was 20 to 35°C. The slope of the line corresponds to an activation energy of 22.5 kcal/mole

Efflux in the Presence of Organic Anions

A number of organic anions, including several "hydrogen-carriers," were found to increase the TPA efflux rate when present in 1 μM concentration. These include dipicrylamine, TPB, S-13, TCS, SK6847, and bromcresol green. The concentration dependence of the TPB stimulation is shown in Fig. 4. A maximum stimulation of about 30 times occurs at a few micromoles/liter. In a separate experiment the efflux of TPA increased by a factor of 22 when red cells were pretreated with 2 μM TPB. For TPMP the efflux rate increased by a factor of more than 400. For both of these ions, time constants were similar in the presence of 2 μM TPB.

Effect of Dipolar Molecules on TPA Efflux

Phloretin at a 0.25 mM concentration reduced the half-time for TPA efflux from 135 to 20.2 sec, a 6.7-fold stimulation.

Effect of Ouabain on TPA Efflux

Ouabain at 0.2 μM had no effect on TPA efflux. Apparently there is no significant efflux through the Na/K transport system.

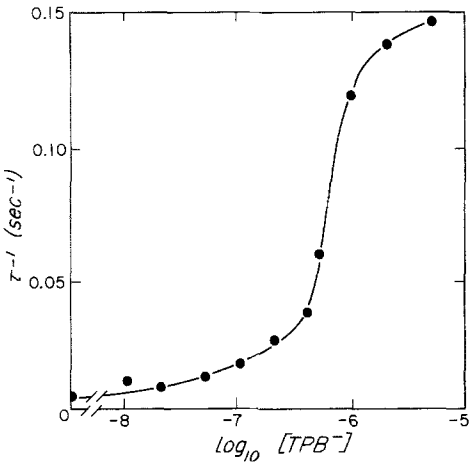

Fig. 4. Stimulation of TPA efflux by TPB. Packed cells previously loaded with TPA were added to 150 mM NaCl plus TPB and the efflux was measured with an electrode. The hematocrit was 13% and the temperature was 30°C

Organic Cation Binding

When red cell suspensions were incubated with the lipid-soluble ions TPA, TPP, BTPP and TPMP, there was a consistent discrepancy between total amount of ion and the sum of extracellular and intracellular free amounts of ion. Table 2 shows binding data obtained from the same blood donor, at zero membrane potential. While TPA and TPP binding are comparable, TPMP is bound to a much lesser extent.

Ion binding varies from one blood sample to another. The average value of a for TPA for seven donors was found to be 3.72 ± 0.37.

A linear relation exists between the amount of ion bound and the total amount of ion, as shown in Fig. 5. Further, the amount of ion bound is proportional to the internal amount of free ion.

Localization of the Binding Sites

If TPA is added to leaky erythrocyte ghosts, equilibration is rapid (within 3 sec, *see* Fig. 6); the slow equilibration (several minutes) when TPA is added to intact cells can be ascribed entirely to the permeation process. If binding to the external cell membrane surface was significant, then the initial external TPA concentration after a TPA pulse should be considerably lower than that calculated from dilution. This was not the case—the measured and calculated values agreed within a few percent (1.8 ± 0.4% SD). We conclude that TPA binding is almost entirely within the membrane and cell.

Table 2. Ion binding to human red cells[a]

Ion	M_o (moles)	M_{if} (moles)	M_b (moles)
TPA	$1.414 \pm 0.014 \times 10^{-7}$	$1.463 \pm 1.174 \times 10^{-8}$	$9.829 \pm 0.159 \times 10^{-8}$
TPP	$1.411 \pm 0.023 \times 10^{-7}$	$1.534 \pm 0.025 \times 10^{-8}$	$9.861 \pm 0.257 \times 10^{-8}$
BTPP	$1.558 \pm 0.014 \times 10^{-7}$	$1.694 \pm 0.015 \times 10^{-8}$	$8.222 \pm 0.155 \times 10^{-8}$
TPMP	$2.076 \pm 0.029 \times 10^{-7}$	$2.257 \pm 0.033 \times 10^{-8}$	$2.481 \pm 0.331 \times 10^{-8}$

Ion	$a = M_b/M_{if}$	M_b/M	% in free
TPA	6.397 ± 0.168	0.385 ± 0.006	13.5 ± 0.3
TPP	6.433 ± 0.278	0.387 ± 0.010	13.5 ± 0.5
BTPP	4.853 ± 0.134	0.322 ± 0.006	17.1 ± 0.4
TPMP	1.101 ± 0.160	0.097 ± 0.013	47.8 ± 3.9

[a] Mean and SE for five samples from the same donor. Temperature 30°C; pH 6.9; $H = 0.148$; membrane potential zero. Total blood volume 6.17 cc. M_{if} = internal free amount; M_o = extracellular free amount; $M = 2.55 \times 10^{-7}$ moles; M_b = amount bound.

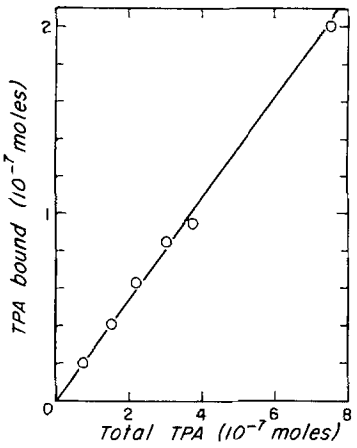

Fig. 5. Linear relationship between TPA binding and the amount of TPA added to a red blood cell suspension. Cells were incubated at 30°C with different amounts of TPA and the external TPA at equilibrium was measured with an electrode. Binding was calculated from the amount disappearing from the external solution. The membrane potential, determined from the pH gradient, was zero

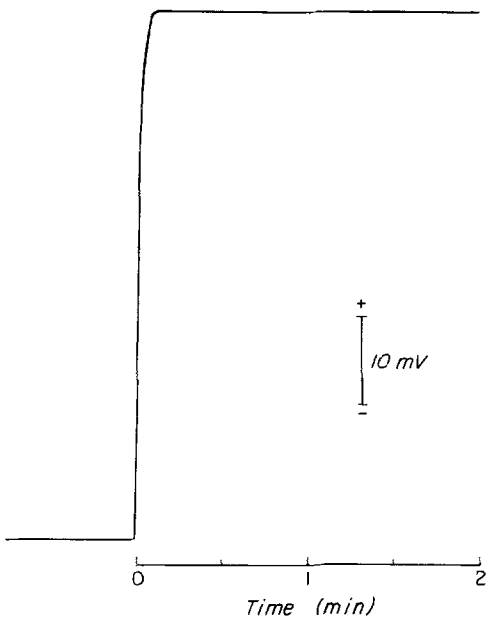

Fig. 6. Strip chart recording of electrode voltage showing rapid equilibration when TPA is added to Dodge (leaky) ghosts. The experiment shows that binding to the cell membrane is a relatively rapid process. Ghost volume fraction was 19% and the temperature was 30°C

The localization has been carried one step further by measuring the amounts of TPA bound to the cell membrane (Dodge ghosts) and to a crude hemoglobin extract at pH 7.1 (Table 3). There is 9.7 μM TPA bound to ghost membrane per gram of membrane lipid at a free TPA concentration of 100 μM. Presumably most of this TPA resides within the lipid fraction. Binding to the hemoglobin fraction, when normalized to the amount of hemoglobin present, was quite variable and may reflect binding to components other than hemoglobin itself (organic acids are known to fluctuate considerably in the red blood cell, for example). In terms of hemoglobin, the average binding at 100 μM free TPA was 469 nM/g Hb.

The calculated and measured % free TPA agreed well with one another. In our observations, the % free TPA varied widely (10 to 40%) from subject to subject, probably reflecting different pH's and concentrations of cytoplasmic binding components. Notice that despite these variations, the amount bound to the membrane, i.e., the sur-

Table 3. TPA binding to different biochemical components of the red blood cell at 30°C[a]

Sample	Binding to membrane (μM/g lipid)	Binding to "Hb", pH 7.1 (μM/g Hb)	Calculated TPA distribution			Measured % free	Surface partition coefficient (cm × 10^5)
			% bound to "Hb"	% bound to membrane	% free		
A	9.22	567	62.4	14.9	22.7	18.5	1.68
B	9.52	502	59.2	16.5	24.3	19.0	1.74
C	10.9	373	50.5	21.7	27.9	28.6	1.99
D	9.10	434	56.1	17.3	26.6	27.6	1.66
Average	9.68	469	57.4	17.4	25.2	23.4	1.77

[a] The measured cellular percentages are based upon TPA electrode measurements in sonicated cells. All figures assume an internal free TPA of 0.1 μM/cm^3 water and that there are 5 g lipid and 340 g Hb per liter of packed cells. Measurements were obtained from samples from four different blood donors.

face partition coefficient remains relatively constant.

TPB Binding

The binding and distribution of TPB was studied because of its stimulatory effect on organic cation flux. When intact cells are equilibrated with TPB about 98% of the TPB becomes bound to cellular components.

When cells were treated with 2 μM TPB at a 15% hematocrit, the equilibrium free concentration was 0.38 μM. The cellular TPB was distributed as follows:

free	2.4%
bound to membrane	53.6%
bound to hemoglobin fraction	44.0%

The membrane fraction was at a concentration of 857 μM (assuming that the membrane is 1% of the cellular volume (Dodge et al., 1963). At 100 μM free TPB, the binding to membrane lipid and hemoglobin was 257 and 4.59 μM/g, respectively. These values are much greater than those for TPA.

Discussion

Evidence for Lipid Bilayer Transport

In a previous paper (Macey & Orme, 1980), we showed that it was unlikely that there was any significant flux of TPA through the anion exchanger, or the glucose, glycerol or urea transport systems of the RBC. On the basis of experiments with ouabain, we can now add the Na/K transport system to the list of membrane transport proteins which apparently do not interact with TPA. While we have not eliminated all membrane proteins from consideration, the hypothesis that TPA flux is mainly through the lipid portion of the membrane is very attractive, especially in light of the observed transport through artificial lipid bilayer membranes (Andersen et al., 1976). The linearity of the TPA influx over a wide range of concentration also argues against a protein carrier system and the large size of the molecule (radius = 4.2 Å) argues against any significant cation channel transport.

Permeability: Comparison with Artificial Bilayers

The TPA permeability of 5.9×10^{-7} cm/sec is more than an order of magnitude greater than that found in lecithin: cholesterol bilayers (Andersen et al., 1976). The negative asymmetric surface potential of the red cell bilayer could enhance the TPA^+ permeability, but by hardly more than a factor of 3. This follows because the inner membrane surface has a potential on the order of −60 mV at physiological ionic strengths (Heinrich, Gaestel & Glaser, 1982). This would increase the local univalent cation concentration near the inner membrane interface by a factor of 10, but it would also depress the transmembrane potential by 60 mV. These factors are antagonistic; using both of them, the calculated flux (based on constant field analysis) is only increased 2.6 times by the presence of red cell surface charge (using a single-barrier model, the flux is increased 3.2 times). It is likely that surface potential does play some role but it cannot be the major cause for the high apparent TPA permeability of red cells.

If the red cell membrane dielectric constant is higher, this would also contribute to the elevated permeability. However, an additional intriguing possibility is suggested by our observation that the phloretin stimulation of TPA transport in the RBC

Table 4. Relative values of organic cation permeability compared with relative values of partition coefficients for alcohols having the same groups

Compound	4th Group	Organic cations relative permeability	Alcohols octanol/water partition coefficient
TPA	phenyl	100	100
TPP	phenyl	93.0	100
BTPP	benzyl	44.4	42.0
MTPP	methyl	1.9	0.6

(about 7×) is much less than stimulation in artificial bilayers (about 1000×; *see* Andersen et al., 1976). Phloretin is believed to act by lowering the natural membrane dipole potential, increasing cation and decreasing anion fluxes. The small stimulatory effect, together with the low membrane permeability, in the RBC would be consistent with a low natural dipole potential in these cells (Macey & Orme, 1980). Artificial bilayers with low dipole potentials are relatively insensitive to phloretin (Andersen et al., 1976).

Using the binding data for TPB, we can estimate a surface partition coefficient of 4.7×10^{-4} which is small in comparison to the value 3×10^{-2} estimated by Ketterer et al. (1971) in lipid bilayers. Superficially, this suggests that TPB permeability in red cells is much lower than the corresponding permeability in artificial bilayers so that the ratio of TPB/TPA permeabilities would also appear to be lower in red cells and support the suggestion that red cell dipole potentials are small. This argument suffers because partition coefficients are not necessarily related to permeability, particularly in cases like TPB^- where most solute partitions near the aqueous interfaces, far from the main energy barrier for permeation in the center of the membrane. However, in these cases the binding is affected by dipole potentials and anion binding would be expected to increase with increased dipole potential. Thus the lowered TPB^- binding remains a viable support of the conjecture that red cell dipole potentials are comparatively smaller. Clearly, a direct measurement of TPB^- permeability would be desirable.

Relative Fluxes and Partitioning

The four organic cations studied can be regarded as central positive charges surrounded by a hydrophobic shell which facilitates their partition into the membrane. Because of their large size, to a first approximation the outer shell may be regarded as controlling ionic selectivity. All four cations are identical in having three phenyl groups, but the fourth shell group differs. For TPA and TPP the fourth group is a phenyl, for BTPP it is a benzyl and for MTPP it is a methyl. If the differences in permeability can be ascribed to this fourth group, the effect on permeability follows the order: phenyl > benzyl ≫ methyl.

One way in which the fourth shell group could influence permeability is by changing the membrane/water partitioning of the cations. To test this idea we examined the partition coefficients of three alcohols: phenol, which has a phenyl group, benzyl alcohol with a benzyl group and methyl alcohol with a methyl group. The octanol/water partition coefficients for these compounds are 30.0, 12.6, and 0.18, respectively (Leo, Hansch & Elkins, 1971), in the same order as the cation permeabilities. Even more striking is the close *quantitative* agreement between the magnitudes of the partition coefficients of the alcohols and the permeability constants for cations containing the same groups (Table 4). We conclude that the relative permeabilities of the organic cations can be explained in terms of differences in partition into the membrane.

Temperature Effects

The activation energy for TPA efflux was 22.5 kcal/mole, considerably higher than values usually found for bulk phase diffusion in liquids (5 kcal/mole), except those of extremely high viscosity, such as glasses (Doremus, 1969). Similar values have been found for the organic anions, TPB and dipicrylamine, for the K^+-valinomycin complex and for the spin-labeled analog of TPMP in artificial phosphatidyl choline (Ketterer et al., 1971; Stark et al., 1972; Bruner, 1975; Kolb & Läuger, 1977; Cafiso & Hubbell, 1982). According to Ketterer et al. (1971), image force calculations for the TPB anion (4.2 Å radius; Grunwald, Baughman & Kohnstan, 1960) would predict an activation energy of approximately 17 kcal/mole and other factors such as membrane dipoles would be expected to raise the value for organic cations.

Synergistic Effects of Organic Anions

Stimulation of TPA efflux by organic anions can be explained by a carrier model and Stark (1980) has found evidence of such a mechanism in artificial phosphatidylcholine membranes for TPA/TPB interactions. The formation constant K_f for a TPA/TPB complex is given by:

$$TPA + TPB = TPA \cdot TPB$$
$$K_f = (TPA \cdot TPB)/(TPA)(TPB).$$

The magnitude of K may be estimated from Bjerrum's equation for ion-pair formation (Robinson & Stokes, 1959):

$$K_f = \frac{4Ne^b d^3}{3000}$$

where N is Avogadro's number, d is the distance of closest approach, and b is given by

$$b = \frac{e^2}{DkTd}$$

where e is the electronic charge, D is the dielectric constant, k is Boltzmann's constant and T is the absolute temperature. It is not clear where the complex forms in the membrane; however, if we assume a value of 3 for the membrane dielectric constant and 8.4 Å as the distance of closest approach (Grunwald et al., 1960), then for 30°C:

$$K_f = 5.9 \times 10^8 \text{ liters/mole}.$$

In a typical experiment, with a 15% hematocrit, the initial TPA and TPB concentrations are 20 and 2 μM, respectively. Using these conditions together with the calculated value of K_f, it can be shown that virtually all (>99%) of the TPB is confined to the membrane either free or in complex with TPA. Even with a dielectric constant of 5, the same conclusion holds. The flux of TPA in the red cell can thus be treated as a type of facilitated diffusion with TPB acting as a carrier confined to the membrane phase.

An interesting finding was that the same concentration of TPB (2 μM, nominal) stimulated TPA and TPMP fluxes by 22 and 400, respectively, reducing the time constant for both to the same value. This suggests that the rate-limiting step for transmembrane transport in the presence of TPB is the backflux of the charged TPB^- ion. A model incorporating this idea will be developed in a future paper.

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