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Permeation of a Spin-Label Phosphate into the Human Erythrocyte†

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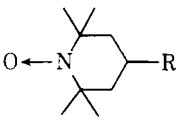
ABSTRACT: The reduction of spin-labels by human erythrocytes can be used to follow their penetration into these cells. The neutral spin-label alcohol Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl) diffuses through the membrane very quickly. The membrane is virtually impermeable to the positively charged spin-label Tempo-choline (*N,N*-dimethyl-*N*-(2′,2′,6′,6′-tetramethyl-4′-piperidinyl-1-oxyl)-2-hydroxyethylammonium chloride). The negatively charged spin-label Tempo phosphate (4-phospho-2,2,6,6-tetramethylpiperidinyl-1-oxyl) is reduced at 37°, with a half-time of about 1 hr. The reduction occurs internally following the rate-limiting transport of the label across the erythrocyte membrane. Reduction of this spin-label is greatly diminished by the specific inhibitor of anion transport, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). The rate of transport depends strongly on the transmembrane electrical potential.

There have been a large number of studies on the kinetics of chloride, sulfate, and phosphate ion transport across the erythrocyte membrane (Passow, 1968; Deuticke, 1970). Anion transport is a passive process (Chedru and Cartier, 1966; Schrier, 1970). For monovalent anions the exchange of one internal anion for one external anion is particularly rapid (Hunter, 1971). Anion transport can be saturated both by chloride ions (Gunn et al., 1973) and by phosphate ions (Ho and Guidotti, 1975). Br^-, I^-, Cl^-, SCN^-, SO_4^{2-}, and HPO_4^{2-} all have transport activation energies about 30–35 kcal/mol (Gunn et al., 1973).

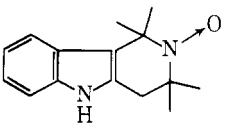
Facilitated anion transport is probably due to the 100,000 molecular weight membrane protein known as band III (Fairbanks et al., 1971). Both the diazo (Juliano and Rothstein, 1971) and the isothiocyanate (M. K. Ho and G. Guidotti, in press) derivatives of sulfanilic acid react strongly with band III, and 95% selectivity has been shown by SITS[1] derivatives for band III (Cabantchik and Rothstein, 1972, 1974). These compounds are potent anion transport inhibitors. Evidence that band III is one of the few proteins which completely spans the erythrocyte membrane makes its role as a transport protein quite plausible (Bretscher, 1971; Shin and Carraway, 1974). The present paper reports the effect of transmembrane potentials on the transport of the spin-label Tempo phosphate through the anion channel.



R = OH, Tempol
R = $(CH_3)_2N^+CH_2CH_2OH$, Tempo-choline
R = PO_4^{2-}, Tempo phosphate
R = $CH_2C_6H_5$, benzyltempo

Materials and Methods

Spin-Labels. Tempo-choline and benzyltempo (4-benzyl-2,2,6,6-tetramethylpiperidinyl-1-oxyl) were gifts of Dr. B. J. Gaffney, Tempol was a gift of Dr. P. F. Coleman, and the carboline label (2,2,4,4-tetramethyl-1,2,3,4-tetrahydro-γ-carbolin-3-oxyl) was a gift of Dr. E. J. Shimshick. Tempo phosphate was synthesized by the procedure of Weiner (1969).



carboline label

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[1] Abbreviations used are: RBC, red blood cell; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl; Tempo-choline, *N,N*-dimethyl-*N*-(2′,2′,6′,6′-tetramethyl-4′-piperidinyl-1-oxyl)-2-hydroxyethylammonium chloride; Tempo phosphate, 4-phospho-2,2,6,6-tetramethylpiperidinyl-1-oxyl; benzyltempo, 4-benzyl-2,2,6,6-tetramethylpiperidinyl-oxyl.

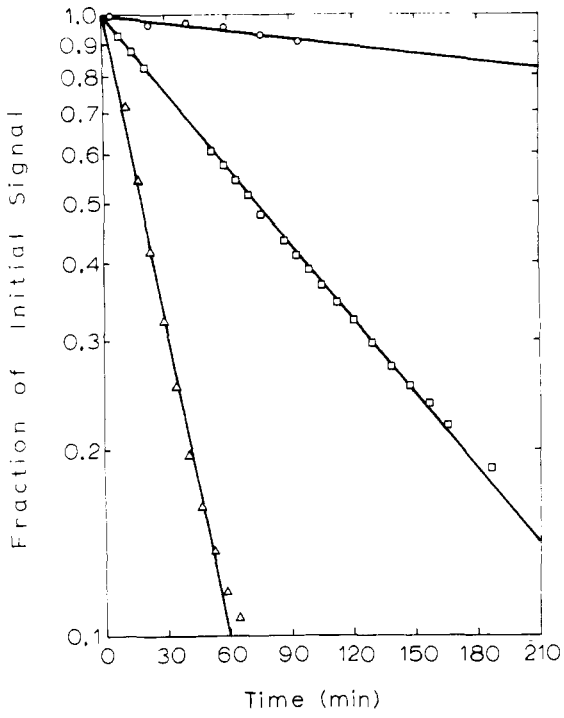


FIGURE 1: Reduction of spin-labels by carbon monoxygenated RBC (○) Tempo-choline, (□) Tempo phosphate, and (△) Tempol.

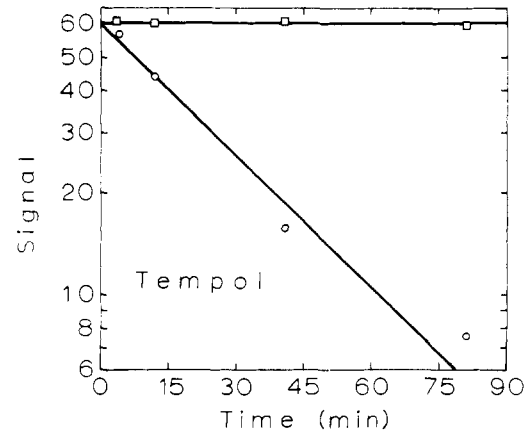


FIGURE 2: Supernatant ESR signal for Tempol before (○) and after (□) the addition of 0.533 m*M* $K_3Fe(CN)_6$ to the supernatant. The ESR signal amplitude before mixing (in arbitrary units) was 126. The time shown on the *X* axis is the time between mixing and centrifugation. The cells were first carbon monoxygenated.

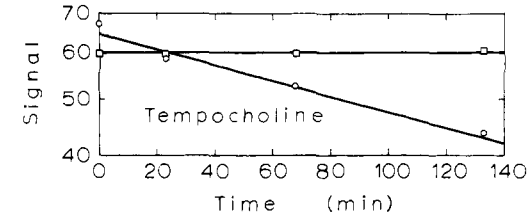


FIGURE 3: Supernatant ESR signal for Tempo-choline. The symbols have the same meaning as in Figure 2. The signal amplitudes before mixing were 72 without and 64 with ferricyanide. The difference between the amplitudes is due to spin-spin exchange broadening by ferricyanide. The cells were first carbon monoxygenated.

Preparation of Cells. Blood was drawn from healthy adults (Harbury et al., 1972). The buffer was 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 3.5 m*M* Na_2HPO_4, 1.5 m*M* NaH_2PO_4, and 10 m*M* dextrose adjusted to pH 7.0. The RBC were washed three times and incubated at 37° for 15 min prior to use. All experiments were done at a 50% hematocrit at 37° with a standard Varian E-4 or E-12 electron spin resonance (ESR) spectrometer with variable temperature accessory. Unless stated otherwise, the initial spin-label concentration was 100–150 μM. Lysates were made by mixing equal volumes of packed cells and distilled water. In some experiments RBCs were carbon monoxygenated by passing carbon monoxide over them for 30 min.

Measurement of Extracellular Nitroxide and Oxime Concentrations. Equal volumes of packed RBCs and spin-label (dissolved in buffer) were mixed and incubated at 37°. Then the cells were centrifuged and the ESR signal of the supernatant was measured with and without the addition to the supernatant of 0.533 m*M* $K_3Fe(CN)_6$. The extracellular nitroxide concentration is proportional to the signal without ferricyanide. The extracellular oxime concentration is proportional to the increase in signal following the addition of ferricyanide. In all of the above experiments ferricyanide was added only after the cells had been removed from the solution. Lysis was less than 2%.

Measurement of Intracellular Nitroxide Concentration. The intracellular nitroxide concentration was monitored by incubating RBCs with spin-label, and then adding the reducing agent sodium ascorbate and observing the protected signal (Kornberg et al., 1972). A 0.5-ml sample was incubated with spin-label as before and then placed on ice for 1 min before 0.2 ml of chilled 0.16 *M* sodium ascorbate (pH 7.0) was added (final concentration 45.7 m*M*) and the internal signal measured at 0–1°. All ascorbate and ferricyanide solutions were made up the day of the experiment.

Measurement of Transmembrane Potential. The transmembrane potential was measured following the method of Funder and Wieth (1966) using a Radiometer Model 26 pH meter and a Beckman combination microelectrode 39030 to determine the pH of the extracellular and intracellular fluids. Since the hydroxyl anion is in equilibrium across the membrane, the transmembrane potential is directly proportional to the difference in pH. Each point is the average of three measurements with an average deviation of about 1 mV. The potentials were time independent through the course of the experiment.

Results

Spin-Label Reduction. The reduction of nitroxide spin-labels is illustrated in Figure 1. The large difference in the observed reduction rates of spin-labels with different charges suggests that transport plays an important role in the reduction since it is known that small neutral molecules traverse the membrane faster than anions which in turn are faster than cations (Garrahan, 1970). The following experiments verify this hypothesis.

Location of Reduction. The reducing activity does not lie in the extracellular solution since spin-labels in isolated supernatant undergo no further reduction. Figures 2–4 show the results of experiments utilizing $K_3Fe(CN)_6$. Ferricyanide, an oxidizing agent (Kaplan et al., 1973), reverses the loss of signal caused by RBCs; therefore, the loss of signal is due to the reduction of the nitroxide, probably to the oxime.

The results in Figure 2 lead to the conclusion that the membrane is permeable to Tempol and to its oxime. The signal amplitude before mixing was 126, but the signal amplitude in the supernatant following mixing and centrifuga-

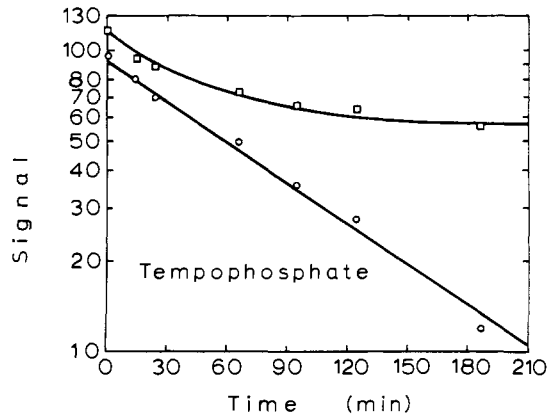


FIGURE 4: Supernatant ESR signal for Tempo phosphate. The symbols have the same meaning as in Figure 2. The signal amplitudes before mixing were 106 without ferricyanide and 129 with it. The difference is due to a significant Tempo phosphate oxime contaminant. The experiment shown used carbon monoxygenated cells, but we got essentially the same results with untreated cells.

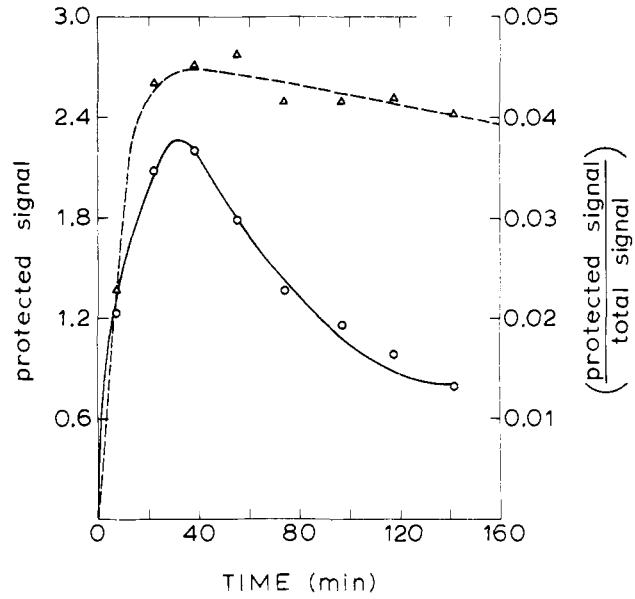


FIGURE 5: Time dependence of the ascorbate protected signal (○) and the fraction protected signal (△). The protected signal includes correction factors for dilution and the change in ESR spectrometer sensitivity due to the temperature difference. The time axis refers to the incubation period preceding the immersion of the sample in ice and the addition of sodium ascorbate.

tion was 60. Thus, the effect of mixing equal volumes of RBCs and spin-label solution is the same as that of diluting the spin-label solution with one volume of buffer. The signal amplitude following the addition of ferricyanide is independent of incubation time because the Tempol oxime also equilibrates across the membrane. Hence, the sum of Tempol nitroxide and oxime molecules in the supernatant is constant except for the initial equilibration which is too fast for our experiment to detect (less than 8 min). Since equilibrium is established across the membrane the rate-limiting step for the loss of Tempol signal is reduction not transport. Also there appear to be no biochemical reductions that ferricyanide cannot reverse.

Figure 3 shows that the erythrocyte is highly impermeable to Tempo-choline in agreement with Humphries and McConnell (1974). The supernatant signal amplitudes following mixing and centrifugation are about the same as that of the initial spin-label solution. There is no dilution of signal. The low rate of reduction is due to reducing sites on the exterior of the RBC membrane. The supernatant signal amplitude following the addition of ferricyanide is independent of incubation time since all oxime molecules stay in the supernatant.

The Tempo phosphate signal following oxidation by ferricyanide decreases with incubation time indicating that Tempo phosphate diffuses into the cell (Figure 4), and eventually levels off persumably because the Tempo phosphate oxime diffuses out of the cell.

Table I shows that lysis causes a large enhancement in Tempo phosphate reduction; hence, there is a potent Tempo phosphate reducing agent inside the cell. We do not see this enhancement with Tempol which supports our contention that the membrane is permeable to it.

Addition of the reducing agent, sodium ascorbate, to intact cells leaves a small protected signal inside the RBC. If the cell temperature is lowered to 0° before the addition of spin-label or the cells are lysed using 1:500 (w/v) melittin (Faynholc et al., 1972) or freeze-thawing, there is no protected signal. These experiments show that Tempo phosphate is capable of penetrating the membrane. On the other hand, there is no protected signal for the impermeable label Tempo-choline. In Figure 5, we show that the internal Tempo phosphate concentration quickly rises to a small steady-state fraction of the total signal. Each point shown

Table I: Lysis, Activation Energies, and Enzyme Inhibitors.

	($t_{1/2}$ Intact Cells/ $t_{1/2}$ Lysate)	Activation Energy (kcal/mol) Intact	Activation Energy (kcal/mol) Lysed	% Inhibition of Reduction by 1.23 m*M* Iodoacetic Acid[a]	% Inhibition of Reduction by 1.0 m*M* SITS
Tempol	~1	7–8	7–8	>90	0
Tempo phosphate	~3	24–29	9–11	0	65–70

[a] Similar results were obtained with lysate.

on Figure 5 is an extrapolation back to the time at which the sample was immersed in ice. This was necessary since the protected signal decays with a half-time of 15–25 min. This reduction is probably due to the RBC since halving the ascorbate concentration does not change the results.

If Tempo phosphate enters the cell and is reduced there, then the missing spin-label should be inside the cell in the form of oxime. Unfortunately, we cannot directly show this since it is impossible with ferricyanide to get a satisfactory recovery of signal in a concentrated lysate solution; however, there is a pool of oxime associated with the RBCs as demonstrated by the following procedure. RBCs are incubated 145 min with Tempo phosphate, washed, and resuspended in fresh buffer. The new suspension is incubated at 37°, aliquots are removed periodically and centrifuged, and the supernatant oxime and nitroxide concentrations were measured. There is a negligible amount of nitroxide present; however, there is a steady flow of oxime from the cells. The efflux has a half-time of 150–200 min and an amplitude of 10–15% of the supernatant signal immediately following the original mixing.

The apparent activation energies of reduction given in Table I are consistent with this scheme. The Tempo phos-

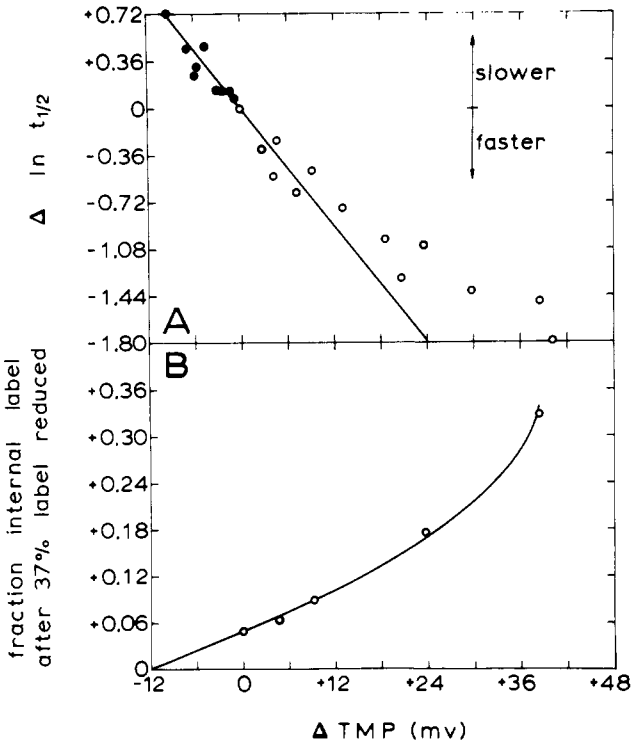


FIGURE 6: Tempo phosphate reduction and the transmembrane potential. (●) 62.5 μg/ml of valinomycin with varied $[Na^+]/[K^+]$ ratios; (○) sucrose partially substituted for NaCl and KCl. Valinomycin runs were incubated 1 hr before the addition of spin-label in order to allow the transmembrane potential to stabilize. Δ ln $t_{1/2}$ and ΔTMP are the differences in ln $t_{1/2}$ and transmembrane potential between the perturbed and unperturbed case. The protected signals were measured after two-thirds of a reducing half-time what is equivalent to the loss of 37% of the signal.

phate activation energy for intact cells is higher than that for lysed cells because Tempo phosphate has to pass through the membrane. Lysis has no effect on the Tempol activation energy since the membrane is already permeable to it.

Absence of Phosphatase Activity. Another property of $K_3Fe(CN)_6$ may be used to show that there is negligible Tempo phosphate phosphatase activity. $Fe(CN)_6^{3-}$ because of its spin paramagnetism broadens the nitroxide ESR signal (Hudson and Lockhurst, 1969). In our concentration ranges, the broadening is independent of spin-label concentration, but is a very strong function of spin-label charge. The concentrations of $K_3Fe(CN)_6$ necessary to halve the signal amplitude of Tempo phosphate, Tempol, and Tempocholine are 44.5, 14.3, and 2.6 m*M*. Another way to look at this charge dependence is to change the pH. In the presence of 50 m*M* $K_3Fe(CN)_6$ Tempo phosphate has twice as large a signal amplitude at pH 8 as at pH 4.[2] There is very little difference with Tempol. Our application of this effect is simple. At the end of five Tempo phosphate reducing half-times we detect no change in the line width for the supernatant signal in the presence of 22 m*M* $K_3Fe(CN)_6$ (Tempol and Tempo phosphate line widths are 2.44 and 1.98 G).[3] Hence, very few, if any, phosphate ester links have been hydrolyzed.

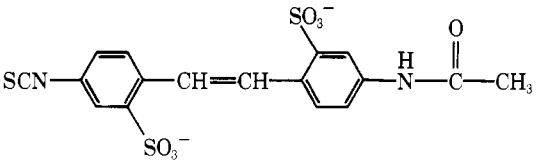
[2] A rough acid-base titration of Tempo phosphate gave a value of 6.2 ± 0.2 for the p*K* of the second proton.

[3] The line widths and peak heights are approximately linked in that the number of spins in the sample is proportional to the product of the derivative curve peak height and the square of the resonance line width (for a given line shape).

Table II: Effect of Valinomycin on Tempo Phosphate Reduction and Its Reversal by Substituting K^+ for Na^+.

	$t_{1/2}$ (min)
No valinomycin	
5 m*M* K^+	40.5
121 m*M* K^+	46.6
62.5 μg/ml of valinomycin	
5 m*M* K^+	92.0
77.5 m*M* K^+	59.0
121 m*M* K^+	41.6
146.4 m*M* K^+	42.0

Effect of SITS. SITS, the specific anion channel inhibitor, has a dramatic effect on Tempo phosphate reduction, but no effect on Tempol reduction or Tempo phosphate reduction by lysates (see Table I). Also, prior addition of SITS eliminates 85% of the ascorbate protected signal of Tempo phosphate. The SITS inhibition does not involve the transmembrane potential since valinomycin (94 μg/ml) treated cells using a 130 m*M* K^+ buffer give essentially the same results. The valinomycin could not have been inactivated by SITS since it has no free amino groups.



SITS

Transmembrane Potential Dependence. Figure 6A shows that the rate of Tempo phosphate reduction is a strong function of the transmembrane potential. The unperturbed transmembrane potential is about 15 mV. Valinomycin, a potassium ionophore, was used to make the transmembrane potential more negative. Substitution of sucrose for NaCl and KCl was used to make the transmembrane potential more positive. Table II shows that high potassium concentrations reverse the effect of valinomycin on Tempo phosphate reduction, which indicates that the valinomycin inhibition of Tempo phosphate reduction is a result of the transmembrane potential charge rather than a specific chemical effect. Valinomycin and sucrose have no effect on Tempol reduction by intact cells and Tempo phosphate reduction by lysate. When the transmembrane potential changes, there are small pH shifts. If we change the pH of the suspension a comparable amount by washing the cells in acid or basic buffer, there is no significant change in the transmembrane potential and Tempo phosphate reduction. The line in Figure 6A is drawn through the origin with slope $(2e/kT)$ where e is the fundamental unit of charge, and k is Boltzmann's constant. At high transmembrane potential there is deviation of the experimental points from this line and a larger ascorbate protected signal (see Figure 6B). These effects are probably due to saturation of the reducing capabilities of the RBCs.

Neutral Spin-Labels. The reduction of neutral spin-labels is quite different from charged ones. The signal vs. time curve for Tempol is roughly of the form $a + b(\exp(-kt))$, where a and b are constants (see Figure 7). Treatment of the cells with carbon monoxide eliminates the nonzero base line for Tempol reduction, but has no significant effect on Tempo phosphate reduction. If carbon monoxy cells are then treated with oxygen, there is only a small reversal of the effect. This phenomenon could be due to a carbon mon-

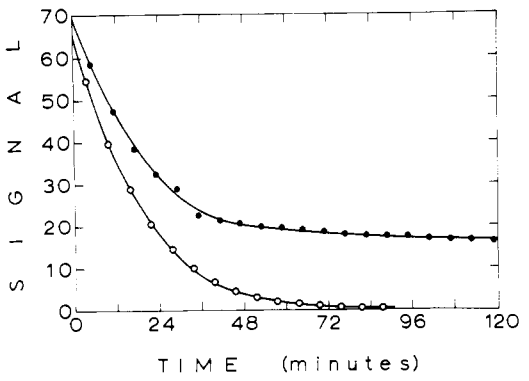


FIGURE 7: Reduction of Tempol with (O) and without (●) carbon monoxide.

oxide sensitive oxidizing enzyme located within the RBC membrane. Neither 1.2 m*M* NaN_3 nor 0.14 m*M* KCN eliminates the nonzero base line. We get the same results with benzyltempo and the carboline label except that the constant *a* for the former label is about twice as large as that for the other two.

Enzyme Inhibitors. Iodoacetic acid sharply inhibits Tempol reduction by both intact cells and lysate, but does not affect Tempo phosphate reduction (see Table I). Iodoacetic acid is a potent inhibitor of glycolysis; however, glycolysis is not involved with this effect since 10 m*M* NaF does not affect the reduction.

Saturation of Reduction. If we use higher spin-label concentrations, we can see saturation of the reducing systems. Figure 8A shows that Tempol reduction (using carbon monoxygenated RBC) saturates at lower spin-label concentrations than Tempo phosphate. Presumably, this is a result of Tempol's larger steady state internal fraction of nitroxide. Figure 8B shows that the saturation also leads to an elevation of the fraction of signal due to internal Tempo phosphate.

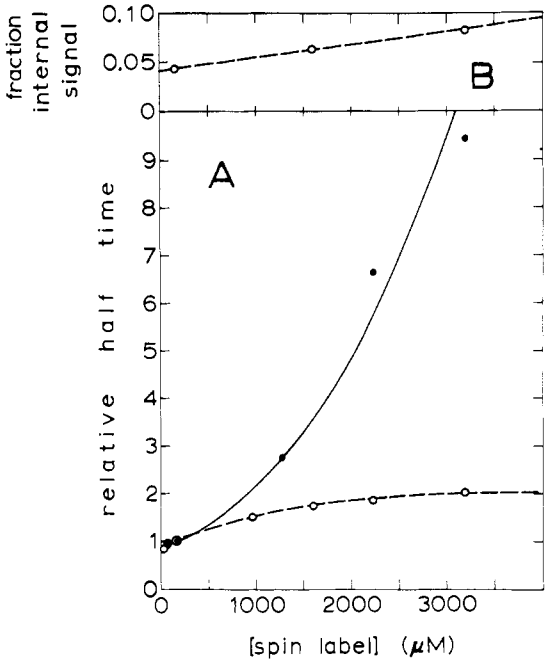


FIGURE 8: Saturation of the reducing capacity by Tempo phosphate (O) and Tempol (●). The relative half-time is the observed half-time divided by the half-time at 160 μ*M* initial spin-label concentration. The fraction internal signal is measured after two-thirds $t_{1/2}$.

Discussion

Circumstantial evidence for the involvement of an anion channel in Tempo phosphate penetration is fourfold. First, Tempo phosphate does not readily penetrate pure lipid vesicles (Kornberg et al., 1972). Second, the reduction of Tempo phosphate has a high activation energy, characteristic of anion transport (Gunn et al., 1973). Third, SITS strongly inhibits the reduction of Tempo phosphate and its ascorbate protected signal. Cabantchik and Rothstein (1972) observed an 80–85% inhibition of sulfate transport whereas we observed a 65–75% inhibition. The difference could be due to different conditions or a small amount of external reduction. Fourth, SITS inhibits the reduction of Tempo phosphate even in the presence of valinomycin, which is evidence against a nonspecific but transmembrane potential dependent penetration of the RBC membrane.

The transmembrane potential has a large effect on passive anion transport. Passow's fixed charge hypothesis (1968) has been used previously to explain sucrose acceleration of anion transport, but it cannot explain our valinomycin result. LaCelle and Rothstein (1966) have already found an ohmic resistance for cation transport in RBC. Our kinetic rates are consistent with an exponential dependence of the form $\exp(2e(\text{transmembrane potential})/kT)$ over a range of 15–20 mV. At high transmembrane potential, there is a deviation from the plot in Figure 6 caused by saturation of the reducing capacity of the cells. The "transition state" must require a net movement of two negative charges into the cell. These charges are evidently associated with Tempo phosphate.

Acknowledgments

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Regulation of Membrane Flexibility in Human Erythrocytes†

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ABSTRACT: We have used spin-labels to detect prostaglandin E induced changes in erythrocyte membranes. The observed changes in spin-label resonance spectra can be mimicked in erythrocyte ghosts by loading them with cAMP or cGMP. These changes can also be observed by adding either of these cyclic nucleotides to intact cells. This entry of cyclic nucleotides into intact cells is blocked by an inhibitor of the anion channel. We suggest that the observed changes in paramagnetic resonance spectra are due to changes in lipid "fluidity" that are brought about by changes in the biochemical state of membrane-associated proteins (such as spectrin) and in the direct or indirect biophysical interactions of these proteins with membrane lipids.

The morphology, chemical composition, and dynamical properties of the plasma membranes of most biological cells are doubtlessly subject to continuous regulation. The present paper is concerned with the membrane of the mature human erythrocyte. Recent studies by Allen and Rasmussen (1971) and by Kury et al. (1974) have shown that the rheological properties of erythrocytes at high hematocrit are affected by physiological concentrations of prostaglandins and epinephrine. These studies suggest but do not prove that the flexibility of an isolated erythrocyte can be controlled by these substances. Studies by Allen and Valeri (1974) indicate further that erythrocyte morphology is regulated by low concentrations of the prostaglandins PGE_1,[1] or PGE_2, which can decrease, or increase, respectively, the internal volume of the cell by a small amount (~3%). The paramagnetic resonance spectra of fatty acid spin-labels bound nonspecifically to these membranes show small but reproducible changes on the addition of low concentrations (10^{-10}–10^{-12} *M;* of the order of few molecules per cell) of the prostaglandins PGE_1 and PGE_2 (Kury et al., 1974), and also show reproducible changes on the addition of physiological concentrations of adrenaline and carbamyl choline (Huestis and McConnell, 1974). The spin-label concentration and resulting spectra suggest that the associated changes in membrane structure arise from small changes throughout much of the membrane, rather than large changes localized to small regions of the membrane. The resonance spectra are a measure of the flexibility of the spin-label fatty acid chains in the bilayer region of the membrane. It is interesting that substances that increase the apparent single cell flexibility (e.g., PGE_1) also increase this fatty acid chain flexibility, and vice versa (PGE_2 and epinephrine) (Kury et al., 1974). Recent studies of erythrocyte ghosts using circular dichroism also show changes in the presence of prostaglandins (Meyers and Swislocki, 1974).

The purpose of the present paper is to describe our paramagnetic resonance studies and to attempt to relate these biophysical changes to biochemical changes in the erythrocyte membrane that have been studied by other investigators.

Materials and Methods

Reagents. The 10,3-fatty acid spin-label is the *N*-oxyl-4′,4′-dimethyloxazoline derivative of 5-ketopalmitic acid.

Ghost Preparation. Human erythrocyte ghosts were prepared by a procedure adapted from Humphries and McConnell (1974). The cells were washed as described previously (Kury et al., 1974) and brought to 50% hematocrit in Allen's buffer (Allen and Rasmussen, 1971) (145 m*M* NaCl–5 m*M* KCl–1 m*M* $MgSO_4$–3.5 m*M* Na_2HPO_4–1.5 m*M* NaH_2PO_4–10 m*M* glucose–1 m*M* $CaCl_2$, final pH 7.0). A 1:4 dilution of Allen's buffer was prepared for the lysing solution. To 0.4 ml of 50% cells was added 2.0 ml of lysing solution. The cells were mixed and left on ice 10 min until translucent. They were centrifuged at g max 4300 for 10 min at 0°. Then 1.0 ml of the supernatant was carefully removed without disturbing the pellet. The membranes were resuspended in the remaining supernatant. Then 1.0 ml of resealing solution (252 m*M* KCl–10 m*M* NaCl–10 m*M* $MgCl_2$–3.9 m*M* Na_2HPO_4–1 m*M* Na_2ATP–2.4 m*M*

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; cGMP, guanosine 3′,5′-cyclic phosphate; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.