The Effect of 2, 4, 6-Trinitro-m-Cresol on Cation and Anion Transport in Sheep Red Blood Cells

ROBERT B. GUNN and DANIEL C. TOSTESON

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27706

ABSTRACT 2,4,6-Trinitro-3-methyl-phenol (trinitrocresol, H+TNC−) was found to inhibit anion and stimulate cation movements across the membranes of both high potassium (HK) and low potassium (LK) sheep red blood cells. The concentration of TNC− required to inhibit SO4− and Cl− efflux (10−5–10−3 M) was less than that required to increase Na+ and K+ leakage (10−3–10−2 M). Both the inhibition of anion and stimulation of cation permeation were reversed if TNC− was washed from the red cells. The cation leak caused by TNC− was much greater at 0° and 37°C than at room temperature (23°C). In sheep red cells, TNC− was found to be about 20 times more effective than salicylate and about 40 times more effective than thiocyanate in increasing cation leak. TNC− also inhibited the ouabain-sensitive potassium influx.

The red blood cell membrane is a million times more permeable to small anions such as chloride than to small cations such as Na+ and K+ (Tosteson, 1959). This permselectivity has been rationalized by the hypothesis that the membrane contains fixed positive charges (Mond, 1927). The analogy between the properties of the red cell membrane and those of an anion exchange membrane was explored by Wilbrandt (1942 a, b) using the theory developed by Teorell (1935) and Meyer and Sievers (1936), by Solomon (1960), and more recently by Passow (1964) who concluded that some characteristics of sulfate and phosphate permeability are consistent with a concentration of fixed positive charges of 2.5 to 3 moles/liter of red cell membrane water. From this viewpoint, the membrane permeability to ions is determined in part by the concentrations of permeant ions in the membrane which in turn are determined by the fixed charges. Positive fixed charges promote the accumulation of anions and the exclusion of cations within the membrane resulting in high anion and low cation permeability. The effects of some of the many agents which modify the permeability of the red cell membrane to ions are understandable in terms of interactions with the hypothetical fixed charges. For example, it has been suggested that the reduced anion permeability at a
more alkaline pH is due to titration of positive amino groups which have a 
P\text{K} near 9 (Passow and Schnell, 1969). The effects on red cells of treatment 
with amino acid reagents such as 1-fluoro-2,4-dinitrobenzene (Berg, Diamond, 
and Marfey, 1965) and 2-methoxy-3-nitropone (Passow and Schnell, 1969) 
reinforce this interpretation. These agents which both increase cation and 
reduce anion permeability react irreversibly with amino groups and in this 
manner may reduce the effective concentration of fixed positive charges in 
the membrane.

In our laboratory it was found that the anion of trinitrocresol (p\text{K} = 2.8 
in water [Moore and Peck, 1955]) was very effective as a counterion to K+ - 
valinomycin and other similar cationic complexes in hydrocarbon solvents. 
The concentration of K+ in a solution of valinomycin (10^{-4} M) in decane 
equilibrated with aqueous solutions of K+ salts was greatly increased by the 
presence of this lipid-soluble anion. Furthermore, the potassium conductance 
of lipid bilayers in the presence of valinomycin was greatly enhanced by 
addition of trinitrocresolate to the bathing solutions.

This paper reports the effects of 2,4,6-trinitro-3-methyl-phenol (trinitrocresolate [TNC−]) on the ionic permeability of sheep red blood cells. We have 
found that TNC− is a potent and reversible inhibitor of anion and stimulator 
of passive cation permeation. Inhibition of sulfate flux is nearly complete at 
10^{-3} M TNC− while the increased Na+ and K+ permeability is progressive 
above this concentration. Further, we have observed an augmentation of these 
effects at 0°C as compared with room temperature, which is qualitatively 
similar to that found by Wieth (1970a, b) for thiocyanate and salicylate in 
human red blood cells. However, TNC− is much more potent than thiocyanate 
or salicylate in producing these effects. A comparison of the effects of TNC− 
on red cells, lipid bilayers, and bulk phases of nonpolar solvents suggests that 
this aromatic anion interacts strongly with the groups which regulate passive 
ionic permeability in the red cell membrane, possibly amino groups, and that 
these interactions probably occur in a region with a low dielectric constant.

MATERIALS AND METHODS

Materials

Homozygous L (low potassium, LK) and M (high potassium, HK) crossbreeds of 
Suffolk, Rambouillet, and Hampshire sheep were bled by jugular venipuncture into 
a flask containing preservative-free heparin 0.1 mg/ml (Connaught Laboratories, 
Toronto, Canada). The flask was then placed on ice. Samples of whole blood were 
taken in quadruplicate for hematocrit and hemoglobin determinations. These meas-
urements were used to convert sample hemoglobin measurements into an equivalent 
volume of packed cells of the original whole blood. After centrifugation the plasma 
and white cells were removed by aspiration. The cells were then washed three times 
in a balanced salt medium which was constituted as follows: \text{NaCl} 111 \text{mM}, \text{KCl}
5.4 mM MgCl₂, 9.98 mM NaH₂PO₄, 9.6 mM NaHCO₃, 23.8 mM dextrose, 11.1 mM penicillin 0.107 g/liter, streptomycin 0.05 g/liter, pH 7.4 when equilibrated with 5% CO₂-95% air, osmolarity 0.282-0.287. Other solutions were obtained by modification of this medium. In K-free media, an additional 5.4 mM NaCl replaced the KCl. When trinitrotresol was added, equimolar amounts of NaCl were deleted and the solution titrated to pH 7.4 with NaOH. In the thiocyanate and salicylate media, 111 mM NaSCN or Na-salicylate replace the 111 mM NaCl, leaving 25.4 meq of chloride ions and the anions H₂PO₄⁻ and HCO₃⁻ unchanged.

**Experimental Procedures**

**Na⁺ and K⁺ influx experiments** Cells were washed once and then suspended (0.1 v/v) in flux media containing all components including TNC⁻. A 2 ml aliquot of this cell suspension was added to 10 ml Erlenmeyer flasks, gassed with 5% CO₂-95% air, stoppered, and placed in the incubator at the appropriate temperature for 15 min. As required, ouabain from a 10⁻² M stock solution in isotonic NaCl was added to give a final concentration of 10⁻⁴ M in the incubation flask. At time zero, 10 μCi of either ⁴²K⁺, ²⁴Na⁺, or 5 μCi of ²⁸Na⁺ was added to the cell suspension. Samples were taken at 5 min and 65 min or at shorter times when the flux was greatly increased.

Using a Schwartz Biopette, a 0.1 ml sample of cell suspension was transferred to a 12 ml polypropylene centrifuge tube containing 1.7 ml of dibutyl phthalate with 8 ml of isotonic MgCl₂ layered over it. In the absence of TNC⁻ the tubes were chilled; otherwise they were at room temperature. The tubes, remounted in a Sorvall S-24 centrifuge head, were immediately centrifuged at 12,000 g for 1 min (Sorvall Inc., Norwalk, Conn., model RC-2B). The cell button and the dibutyl phthalate above it were not disturbed while the MgCl₂ solution was aspirated and fresh isotonic MgCl₂ was layered onto the phthalate. This MgCl₂ wash solution was allowed to stand 3-4 min to promote mixing of any residual Na⁺, K⁺, or radioactivity from the side of the tube. The MgCl₂ solution and dibutyl phthalate were then completely removed; the cell button was lysed with 2.9 ml of hemolyzing fluid (1.25 ml concentrated NH₄OH, 0.0312 ml Non-Ion-Ox [A. S. Aloe Co., St. Louis, Mo.] and 0.004 M CsCl per liter of solution). At the end of the flux period the cell suspension was transferred to a centrifuge tube, spun, and a 0.100 ml supernatant sample was placed in 2.9 ml of hemolyzing fluid.

The samples were counted in a well-type crystal scintillation spectrometer, diluted with hemolyzing fluid, and then centrifuged to dispose any residual dibutyl phthalate. The hemoglobin concentration of the samples relative to that of the original whole blood was determined by measuring the optical density at 540 nm (Microsample spectrophotometer, 300 N, Gilford Instrument, Oberlin, Ohio). TNC⁻ does not absorb appreciably at this wave length. Sodium and potassium analysis was performed on an atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn., model 303).

**SO₄²⁻ efflux experiments** All solutions for sulfate studies contained 1 mM Na₂SO₄ in addition to the usual constituents. Cells were loaded with ³⁵SO₄⁻ by incubation for 4 hr at 37°C in which time specific activity equilibrium was achieved. Cells...
were then washed once with ice-cold media containing 1 mM Na2SO4 but no 35SO4 and added to the flasks at time zero. Samples were taken into small nylon tubes and centrifuged. An aliquot of supernatant was placed in scintillation fluid and counted in a Beckman liquid scintillation counter. When necessary the measurements were corrected for quenching from a standard curve made at the same time.

**CHLORIDE EFFLUX EXPERIMENTS** These were performed in the same way except that loading time with 36Cl was only 15 min and the cells were not washed before their addition to the flasks at zero time.

**CALCULATIONS** Cation flux, 4M, was calculated from the radioactivity per liter of original cells, counts per minute, at the two sample times A, B, and the external specific activity, 4a.

\[
4M = \frac{cpm (B) - cpm (A)}{(4a)\Delta t}
\]

The data are presented in terms of the rate coefficient, 4kXLE or 4kXPP, for the ion X with the i or o superscript denoting influx or outflux measurements and LE or P superscript denoting leak plus exchange diffusion (not inhibited by ouabain) or pump (ouabain-inhibited) flux of the ion. The rate coefficient is in reciprocal hours; however, for influxes it was obtained by dividing the observed flux, in mmoles/(liter of packed original cells X hour), by the observed external ion concentration at the end of the flux which is in mmoles/liter of supernatant. More precisely then, the rate coefficient for cation influx has the units of (liter of supernatant)/(liter of packed cells X hour).

Sulfate outflux rate coefficients in (liter cell water)/(liter of packed cells X hour) were calculated from: (1) the steady-state exchange coefficient \( b = \frac{-\ln(1 - \frac{4a(t)}{4a(\infty)})}{\Delta t} \) where \( 4a(t) \) and \( 4a(\infty) \) are the specific activity in the supernatant at time t and at equilibrium, (2) the cell sulfate concentration \( i(SO_4^-) \) in millimoles/liter cell water assuming a Donnan equilibrium and \( i(SO_4^-)/o(SO_4^-) = 0.45 \) and 69.5% of the cell volume is water (Tosteson and Hoffman, 1960), (3) the hematocrit, hct, using the relation

\[
4k_{SO_4}^{LE} = \frac{b(1 - hct)(0.695)}{(1 - hct) + hct(0.45)(0.695)}
\]

**PARTITION COEFFICIENTS** An aqueous phase containing 1.6 mM KTNC or KSCN and 1.0 mM 44KCl was equilibrated for 1 hr at the appropriate temperature with decane or chloroform containing 10-4 M valinomycin. The solution was mixed every 12 min during the hour, then centrifuged for 2 hr at 12,000 g. Aliquots of each phase were then counted for 44K+ and the K+ concentration of the aqueous phase was determined by flame photometry.

**RESULTS**

**Anion Permeability**

As shown in Fig. 1, the rate constant for sulfate transfer across the membrane of sheep red cells in a chloride medium at 37°C was found to be 0.6 hr⁻¹. This
value decreased progressively as the TNC\(^-\) concentration was increased. When the TNC\(^-\) concentration was 10\(^{-2}\) M the tracer sulfate efflux in 1 hr was not significantly different from zero. Chloride permeability was also inhibited by TNC\(^-\). At 0°C in a medium containing 10\(^{-2}\) M TNC\(^-\) the half-time for \(^{36}\)Cl\(^-\) exchange was nearly 1 hr (Fig. 2) (\(k_{cl}^{LH} = 0.72 \text{ hr}^{-1}\)) while in the absence of TNC\(^-\), isotopic equilibrium was reached within a minute.

**Cation Permeability**

The results of the experiments showing the effects of TNC\(^-\) on Na\(^+\) and K\(^+\) movement are presented in Figs. 3–5. All these experiments were performed.

---

**Figure 1.** Each point is the average, \(k_{SO_4}^{LH}\), from duplicate flasks. Each \(k_{SO_4}^{LH}\) was obtained from the least squares regression line for b. The percent inhibition by TNC\(^-\) is 8–13% at 10\(^{-5}\) M, 50–53% at 10\(^{-4}\) M, 90–93% at 10\(^{-3}\) M, >99% at 10\(^{-2}\) M.

**Figure 2.** All points for each curve were single samples from one flask. \(^{36}\)Cl\(^-\) outflux from LK sheep red cells, 0°C.

---
in the presence of $10^{-4}$ M ouabain in order to minimize active transport and demonstrate the effects of TNC$^-$ on the ouabain-insensitive (passive) transport of cations. In Fig. 3 the progressive increase in sodium permeability caused by TNC$^-$ is presented. While TNC$^-$ at $10^{-5}$ M was effective in reducing anion permeability and inhibition of $SO_4^-$ flux was virtually complete at concentrations above $10^{-3}$ M, the increased cation permeability due to TNC$^-$ occurred only at concentrations above $10^{-3}$ M. The rate coefficient, $\frac{dL_p}{d_{Na}}$, increased

![Figure 3](image-url)  
**Figure 3.** HK 177, LK 103, No. 9. Each point is the average of a single flux period from triplicate flasks.

![Figure 4](image-url)  
**Figure 4.** HK 177, 24-3, HK 105, 30-4, HK 105, No. 2. Each point is the average of the rates in duplicate flasks. Off the graph are the rate constants at $2 \times 10^{-2}$ M TNC$^-$ where $\frac{dL_p}{d_{Na}} = 1.17$ and $0.46 \text{ hr}^{-1}$ for experiments 30-4 and No. 2, respectively. The elevated control leak in 30-4 is due to a lower pH in the flask in that experiment.
from 0.025 hr⁻¹ in TNC⁻-free media to 0.11 hr⁻¹ in 10⁻² M TNC⁻. A fourfold increase in passive sodium movement due to 10⁻² M TNC⁻ occurred in both HK and LK sheep red cells. In the absence of TNC⁻, sheep red blood cells have a large component of Na⁺ exchange diffusion (Tosteson and Hoffman, 1960) which is included in the measured rate coefficient for Na⁺ influx. The extent to which TNC⁻ stimulates Na⁺ leakage as compared to exchange diffusion was not evaluated quantitatively but measurements of net movements of Na⁺ and K⁺ strongly suggest that the leakage process is primarily involved.

The passive potassium influx was similarly affected by TNC⁻ as shown in Figs. 4 and 5. In HK cells, the rate coefficient, \( k_{K}^{HK} \), increased from less than 0.01 hr⁻¹ to 0.14–0.16 hr⁻¹ in the presence of 10⁻² M TNC⁻. In 2 × 10⁻² M TNC⁻ (the solubility limit) influx rates of 0.5 and 1.2 hr⁻¹ have been obtained in HK cells at 37°C. In LK cells (Fig. 5), \( k_{K}^{LK} \) without TNC⁻ was 0.05 hr⁻¹ and increased to 0.24 hr⁻¹ in 10⁻² M TNC⁻ and 0.74 hr⁻¹ in 2 × 10⁻² M TNC⁻. In other experiments (LK 188, 30-4) potassium leaks as large as 2.0 hr⁻¹ have been observed in 20 mM TNC⁻. Thus the influx of K⁺ was increased by at least 17-fold in HK cells and 5-fold in the LK experiment shown. Other experiments with LK cells showed a 9-fold (LK 188, 30-4) and 12-fold (LK 188, No. 3) stimulation in 10⁻² M TNC⁻. At 37°C, the ratio of the rate coefficient for K⁺ influx to that for Na⁺ influx increased with TNC⁻ treatment, presumably because the induced increase in leakage of Na⁺ made the magnitude of Na⁺ influx by exchange diffusion negligible compared to total Na⁺ influx.

![Graph](image_url)

**Figure 5.** LK 103, No. 1. Each point is the average of the rates in duplicate flasks.
Temperature Dependence of the Trinitrocresolate Effect on Cation Permeability

Figs. 6–9 present the results of experiments designed to study the effect of temperature on cation permeability of sheep red cells in the absence and presence of TNC⁻. In the absence of trinitrocresolate the rate coefficient for cation permeation increased monotonically with temperature. By contrast, in the presence of trinitrocresolate, the rate coefficient for cation trans-

\[ Q_{10}(T) = \frac{\text{rate coefficient at } T + 10}{\text{rate coefficient at } T} \]

Because of incomplete data these probably represent upper and lower limits respectively for \( Q_{10} \) on the two branches of this curve.

\[ Q_{10}(0) = 0.65 \quad \text{and} \quad Q_{10}(25) = 2.3 \]

In the presence of TNC⁻, \( Q_{10}(0) = 0.69 \) (see Fig. 6). \( Q_{10}(25) = 1.8 \).

---

**Figure 6.** HK 105, No. 8. Each point is the average of rates in duplicate flasks. \( Q_{10}(T) \) is the rate coefficient at temperature \( T + 10 \) divided by the rate coefficient at \( T \). \( Q_{10}(0) = 0.65 \) and \( Q_{10}(25) = 2.3 \) in the presence of TNC⁻. Because of incomplete data these probably represent upper and lower limits respectively for \( Q_{10} \) on the two branches of this curve.

**Figure 7.** LK 104, No. 8. Each point is the average of rates in duplicate flasks. In the presence of TNC⁻, \( Q_{10}(0) = 0.69 \) (see Fig. 6). \( Q_{10}(25) = 1.8 \).
port was greater at 0°C than at room temperature. Cation transport in human red cells in the presence of high concentration of thiocyanate or salicylate has recently been reported to show a similar U-shaped dependence on temperature with the minimum occurring between 10° and 18°C (Wieth, 1970a). We have not determined precisely the temperature at which the minimum cation transport rate occurs in sheep red cells in the presence of TNC−, but it is certainly in the same range.

Figure 8. HK 177, No. 6. Each point is the average of rates in duplicate flasks. In the presence of TNC−, $O_{10}(0) = 0.70$ (see Fig. 6). $O_{10}(25) = 1.8$.

Figure 9. LK 103, No. 6. Each point is the average of rates in duplicate flasks. In the presence of TNC−, $O_{10}(0) = 0.75$ (see Fig. 6). $O_{10}(25) = 2.0$. 
Although at all temperatures studied in these experiments cation permeability increased with increasing TNC\(^-\) concentration, it is significant that at 0°C and 10\(^{-4}\) M TNC\(^-\) the rate coefficients for K\(^+\) and Na\(^+\) influx were the same (0.21 hr\(^{-1}\)) in both HK and LK sheep red cells. That is to say, under these conditions, sheep red cell membranes displayed no selectivity for K\(^+\) as compared with Na\(^+\).

**Reversibility**

Table I shows the results of experiments performed to test whether the TNC\(^-\) inhibition of anion and enhancement of cation permeation were reversible. A portion of the cells treated with TNC\(^-\), were washed 11 times using a total of 240 volumes of wash solution (23°C) which was sufficient to render the final wash solution free of the characteristic yellow color of TNC\(^-\) solutions. These washed cells and the remaining TNC\(^-\)-treated cells were used to measure the SO\(_4\)\(^-\) and K\(^+\) rate constants. The data indicate clearly that both effects of TNC\(^-\) on ionic permeability of sheep red cells were, in large part, reversible.

In order to test the possibility of using TNC\(^-\) as a means of altering the internal cation composition of red cells and studying the effect of these ions on the K\(^+\)-Na\(^+\) pump, the following experiment was performed. HK sheep red cells with initial composition of 83 mM K\(^+\)/liter of cells and 17 mM Na\(^+\)/liter of cells were washed eight times in K\(^+\)-free, high sodium medium containing TNC\(^-\) (10\(^{-4}\) M) at 0°C over a 7 hr period at the end of which their internal K\(^+\) and Na\(^+\) were 12 mM/liter of cells and 93 mM/liter of cells, respectively. The cells were then washed in the same medium without TNC\(^-\) 15 times at room temperature (23°C). The rate coefficients for K\(^+\) influx measured in a medium containing 5 mM K\(^+\) with and without 10\(^{-4}\) M ouabain were 0.02 and 0.43 hr\(^{-1}\), respectively giving a rate coefficient of 0.41 hr\(^{-1}\) for the K\(^+\) pump. These values are to be compared with those for HK sheep red cells with similar internal cation composition produced by exposure to
\( p \)-chloromercuribenzenesulfonate (PCMBS) followed by dithiothreitol where the rate coefficient for the leak was 0.014 hr\(^{-1} \) and for the pump 0.45 hr\(^{-1} \) (Hoffman, 1969; Hoffman and Tosteson, 1969). The slight increase in the leak as well as the slight reduction in the pump flux may be due to residual TNC\(^- \) in the red cell membranes. However, the important point is that the reduction of cell K\(^+ \) and elevation of cell Na\(^+ \) concentrations during the exposure to TNC\(^- \) produced the same fivefold stimulation of the Na\(^+\)-K\(^+ \) pump flux as was observed when PCMBS was used to alter the internal cation composition. Thus, TNC\(^- \) did not irreversibly alter the pump.

**The Relative Potency of TNC**

Anions other than TNC\(^- \) can promote cation permeation and inhibit anion flux in red cells. Wieth (1970a) has shown that sodium influx into human red blood cells at 0°C is increasingly stimulated by the following sequence: Cl\(^- \) = Br\(^- \) < NO\(_3^\) < I\(^- \) < SCN\(^- \) < salicylate. Further, the same sequence, except that I\(^- \) and NO\(_3^\) are reversed, described the relative potency of these anions as inhibitors of SO\(_4^\)\(^- \) permeation (Wieth, 1970b). In order to confirm the general relationship for sheep red cells, sodium influx was measured in media where 111 mM chloride was replaced by an equimolar concentration of thiocyanate or salicylate. Such measurements were made at 0°C, 23°C, and 37°C. The results for LK cells are shown in Fig. 10. At 0°C, compared to the values observed in Cl\(^- \) medium, Na\(^+ \) influx was increased 4-fold in thiocyanate and nearly 12-fold in salicylate. Although this stimulation is much less than the 20- and 70-fold stimulation found by Wieth (1970a) under the same conditions for human cells, the sequence Cl\(^- \) < SCN\(^- \) < salicylate is maintained. Furthermore, the convex temperature dependence of cation fluxes in sheep cells exposed to SCN\(^- \) or salicylate was also similar to that found in human cells. Fig. 11 shows that TNC\(^- \) at \( \lambda_1 \) of the concentration of salicylate increased the sodium influx by a factor of 2 in LK cells and by a factor of 3 in HK cells relative to the flux in salicylate media. Thus, the potency of TNC\(^- \) anion at 37°C is 22 times that of salicylate in LK cells and 33 times in HK cells when compared on an equivalent basis. We found that TNC\(^- \) like salicylate and thiocyanate had an appreciably greater effect on human red blood cells than on either LK or HK sheep red cells. Human red cells in 10\(^{-2} \) M TNC\(^- \) at 0°C have a rate coefficient for Na\(^+ \) influx, \( k_{Na}^{KNa} \) = 1.34 hr\(^{-1} \), 300 times the control value.

**The Effect of TNC\(^- \) on the Ouabain-Sensitive Pump**

In Table II are presented six experiments in which the inhibition of potassium influx by ouabain \( (10^{-4} \text{ M}) \) was measured in the absence (control) or presence of 5 \( \times \) 10\(^{-8} \) M TNC\(^- \). Five of the six show a decrease in the pump rate coefficient. At higher TNC\(^- \) concentrations the cation leak induced by TNC\(^- \)
EFFECT of TEMPERATURE on $\text{Na}^+$ INFLUX in LK SHEEP RED CELLS in MEDIA containing DIFFERENT ANIONS (111.0 mM)

Figure 10. LK 108, 10-3. Each point is the average of rates in duplicate flasks. Only 111.0 meq of the 136.4 meq/liter of chloride are replaced by salicylate or thiocyanate. The $Q_{10}(0)$ (see Fig. 6) for $\text{Cl}^-$, SCN$^-$, and salicylate$^-$ media are 1.5, 0.91, and 0.80, respectively.

RELATIVE POTENCY of the ANIONS, CHLORIDE, THIOCYANATE, SALICYLATE, and TRINITROCRESOLATE on $\text{Na}^+$ INFLUX into SHEEP RED BLOOD CELLS

Figure 11. Each bar is the average of rates in duplicate flasks. TNC$^-$ (10 mM) is twice as effective as salicylate (111 mM) in LK cells and three times as in HK cells. Experiment 11.

made the measurement of the small pump component difficult. At lower TNC$^-$ concentrations the pump inhibition was less pronounced.

Partition Coefficients

Table III presents the partition coefficient of $\text{K}^+$ between decane or chloroform and water at 37°, 23° and 0°C in the presence of valinomycin and TNC$^-$ or SCN$^-$. TNC$^-$ uptake into the organic phase was equimolar to $\text{K}^+$.
TABLE II
INHIBITION OF
THE OUABAIN-SENSITIVE K+ INFLUX
BY TNC− (5 × 10⁻³ M)

| Experiment             | Control  | TNC− |
|------------------------|----------|------|
| HK 105, 30-4           | 0.089    | 0.015|
| HK 105, No. 2          | 0.088    | 0.060|
| HK 4146, No. 7         | 0.102    | 0.090|
| LK 188, 30-4           | 0.019    | 0.000|
| LK 188, No. 3          | 0.032    | 0.053|
| LK 103, No. 1          | 0.012    | 0.001|

TABLE III
PARTITION OF K+
BETWEEN WATER AND NONAQUEOUS SOLVENTS
CONTAINING VALINOMYCIN AT THREE TEMPERATURES

| Solvent | Anion | 0°C     | 23°C    | 37°C    |
|---------|-------|---------|---------|---------|
| Decane  | TNC−  | 14 × 10⁻² | 3 × 10⁻² | 0.8 × 10⁻² |
| Decane  | SCN−  | 1 × 10⁻⁵  | 3 × 10⁻⁶ | 3 × 10⁻⁵  |
| CHCl₃   | TNC−  | 4 × 10⁻³  | 3 × 10⁻³ | 4 × 10⁻³  |
| CHCl₃   | SCN−  | 4 × 10⁻³  | 1 × 10⁻⁴ | 4 × 10⁻⁴  |

uptake which is consistent with the formation of a 1:1 salt (K+–valinomycin: TNC−). In the absence of either valinomycin or TNC− or both, no K+ was taken up into the decane phase.

DISCUSSION
Trinitroresololate (TNC−) reversibly inhibits anion and active cation transport but reversibly increases passive cation permeability of sheep red blood cells. At low concentrations of TNC− (10⁻⁸ m–10⁻³ m), sulfate permeability is progressively reduced. At 10⁻⁸ m TNC−, when sulfate flux inhibition is nearly complete, Na+ and K+ permeability is slightly elevated from control levels and increases monotonically with greater TNC− concentration. Accompanying this loss of permselectivity is an alteration in the selectivity among the cations. At 37°C, the addition of 10⁻² m TNC− decreases the ratio of Na+ to K+ rate coefficients, \( \frac{K_{Na}}{K_{K}} \), from ~3.5 to ~0.8 for HK red cells and from ~5.0 to ~0.5 for LK red cells. This ratio of tracer-measured rate coefficients includes both leakage and exchange and is therefore not identical to the parameter \( \alpha \) (Tosteson and Hoffman, 1960) which is the
ratio of rate coefficients for leakage without exchange. At 0°C and 10^{-2} M TNC⁻ the ratio is 1.0 with both Na⁺ and K⁺ rate coefficients equal to 0.21 hr⁻¹ in both HK and LK sheep red cells. These high exchange rates and the loss of Na⁺-K⁺ selectivity permit alteration of the internal cation composition by an equivalent exchange of Na⁺ for K⁺ when the ion gradients between the cell interior and the medium are maintained equal but in opposite directions.

One reasonable explanation for the effects of TNC⁻ on passive anion and cation movements is that a reaction between TNC⁻ anions and positively charged membrane ligands reduces their effectiveness at cation exclusion and other anion inclusion. According to this hypothesis, at low concentrations, TNC⁻ displaces other counterions such as chloride or sulfate, thereby reducing the intramembrane concentrations and thus the flux of these anions. At higher concentrations, TNC⁻ counterions have formed enough ion pairs with the fixed charges to permit the entry of cations such as Na⁺ and K⁺ into the membrane thereby increasing the flux of these cations. The reversibility of the TNC⁻ effects on membrane permeability to ions is consistent with the hypothesis that TNC⁻ forms ion pairs with fixed positive charges.

On the basis of evidence described in this paper, it is not possible to decide whether one or more classes of sites control passive ion permeation in sheep red cells. In particular, the contiguous but nonoverlapping concentration ranges for inhibition of anion and stimulation of cation permeation do not exclude a single class of positive sites controlling both processes. It is characteristic of ion exchange membranes that the extent of reduction of intramembrane fixed charge concentration required to produce inhibition of counterion transport is less than that required to produce a comparable increase in coion transport. This effect is even more prominent if steric factors are included in ion diffusion within the membrane (Passow, 1965).

The dependence of cation fluxes on temperature in the presence of TNC⁻ is strikingly different from the dependence in the absence of TNC⁻. The augmentation of cation flux at 0°C is similar to that reported by Wieth (1970 a) for human red blood cells in the presence of 120 mM thiocyanate or salicylate. The same explanation for this unusual temperature dependence suggested by Wieth may apply to our results with TNC⁻. According to this explanation, the reaction of TNC⁻ with the controlling sites is presumed to be exothermic. Thus, the amount of TNC⁻ bound to these sites increases with decreasing temperature. In the temperature range from 0° to 23°C this effect dominates and cation permeability increases with cooling at a given constant concentration of TNC⁻ in the aqueous bathing solution. In the temperature range from 23° to 37°C, despite a reduction in the amount of TNC⁻ bound to the sites, the positive activation heat for cation transport dominates and cation fluxes increase with increasing temperature at a con-
stant concentration of TNC$^-$ in the aqueous bathing solution. Thus, the U-shaped temperature dependence of Na$^+$ and K$^+$ passive transport in the presence of TNC$^-$ is presumed to be the result of two monotonic processes, exothermic adsorption of TNC$^-$ to membrane sites and endothermic diffusion. This hypothesis, which explains the unusual dependence of cation fluxes on temperature in the presence of TNC$^-$, is supported by the results of our experiments with TNC$^-$ in model systems.

The effects of TNC$^-$ on the red cell membrane are in many ways comparable to its interaction with the K$^+$ and Na$^+$ complexes of valinomycin in thin lipid bilayer membranes and bulk phases of decane and chloroform. In bilayers, valinomycin alone increases the K$^+$ conductance without an effect on Na$^+$ conductance (Andreoli, Tieffenberg, and Tosteson, 1967). The addition of TNC$^-$ in the presence or absence of valinomycin increases both K$^+$ and Na$^+$ conductance. TNC$^-$ seems to act as a lipid-soluble counterion which increases the amount of K$^+$-valinomycin in the membrane, and in some way alters the specificity of valinomycin so that it may more readily carry Na$^+$. In reciprocal fashion, valinomycin markedly increases the conductance of TNC$^-$, presumably by introducing the cationic valinomycin complexes as counterions, and, thereby promoting the accumulation of TNC$^-$ in the lipid membrane.

This ability of TNC$^-$ to act as a counterion to cationic complexes in nonpolar regions is demonstrated by the increased partition coefficient of K$^+$ between bulk phases of decane or chloroform containing valinomycin and water when TNC$^-$ is added to the system. Other anions such as SCN$^-$ and salicylate also have this effect though to a much smaller extent. The relative potency of an anion (SCN$^-$, salicylate, or TNC$^-$) to promote the uptake of K$^+$ from water into a nonaqueous solvent as a complex of valinomycin is apparently related to the dielectric constant, $\epsilon$, of the phase. In decane ($\epsilon = 1.99$), the relative potency of TNC$^-$ as compared to SCN$^-$ at 37°C was found to be 28, while in chloroform ($\epsilon = 4.8$) the relative potency of TNC$^-$ is 10. In the experiments presented here on sheep red blood cells, the potency of TNC$^-$ relative to that of SCN$^-$ is 26 (LK) and 40 (HK). If the dependency of the interaction between TNC$^-$ and the cationic complex on the polarizability of the surrounding region is the same in the red cell membrane (where the cationic species may be R-NH$_3^+$) as in a bulk phase (where it is K$^+$-valinomycin), then the region surrounding the fixed positive charges in the red cell membrane has a dielectric constant near 2.0. This value is in agreement with the gross properties of a 70 A thick membrane with a capacitance of 0.25 $\mu$F/cm$^2$; sheep red cells have a capacitance of 0.85 $\mu$F/cm$^2$ (Fricke, 1953).

The uptake of K$^+$ from water into decane, as a complex of valinomycin with TNC$^-$ as the counterion, has a striking negative temperature dependence.
(Table III) not unlike the temperature dependence (in the range from 0°
to 23°C) of cation permeation in red cells treated with TNC⁻. In contrast,
the partition of K⁺ from water into chloroform measured under identical
conditions showed much less dependence on temperature. Furthermore, the
relative temperature independence of cation rate coefficients in red cells
in the presence of thiocyanate (Fig. 10) compares with the negligible tem-
perature dependence of K⁺ partition between water and decane containing
valinomycin (Table III) in the presence of SCN⁻. Thus, the temperature
dependence of the effects of TNC⁻ and SCN⁻ on cation permeability in red
cells is similar to the temperature dependence of the interactions of these
anions with the cationic complexes of K⁺ with valinomycin in decane (ε =
1.99), but not in the more polar environment of chloroform (ε = 4.8).

Thus, on two accounts the model studies are consistent with the interpreta-
tion that positively charged centers in the red cell membrane are in a region
having characteristics more like decane than chloroform. First, the relative
potencies of SCN⁻ and TNC⁻ on the K⁺ permeability of red cells are more
comparable to the relative potencies of SCN⁻ and TNC⁻ on K⁺ uptake into
decane containing valinomycin than into chloroform. Second, the increasing
uptake of TNC⁻ and K⁺ into decane (but not chloroform) with decreasing
temperature, is consistent with the increasing K⁺ permeability of red cells
at lower temperatures in the presence of TNC⁻. We, therefore, tentatively
conclude that the positively charged sites which regulate passive cation
permeation are located in a region of the red cell membrane where the di-
electric constant is very low (circa 2.0). Experiments designed to test this
hypothesis are in progress in our laboratory.

We wish to thank Dr. J. O. Wieth for suggesting the temperature dependence studies and to thank
Mr. Paul Cook and Mrs. Carrie Parker for their technical assistance.
This work was supported in part by USPHS research grant HE-12157.

Received for publication 5 October 1970.

REFERENCES

Andresoli, T. E., M. Treffenberg, and D. C. Tosteson. 1967. The effect of valinomycin on
the ionic permeability of thin lipid membranes. J. Gen. Physiol. 50:2527.
Berg, H. C., J. M. Diamond, and P. S. Marfey. 1965. Erythrocyte membrane: chemical
modification. Science (Washington). 150:64.
Fricke, H. 1953. Relation of the permittivity of biological cell suspension to fractional cell
volume. Nature (London). 172:731.
Hoffman, P. G. 1969. Thesis. Kinetics of potassium transport in LK and HK sheep red cells.
Duke University, Durham.
Hoffman, P. G., and D. C. Tosteson. 1969. Kinetic differences between K-Na pumps in HK
and LK sheep red cells. Fed. Proc. 28:339.
Meyer, K. H., and J. F. Sievers. 1936. La perméabilité des membranes I. Théorie de la
perméabilité ionique. II. Essais avec des membranes sélectives artificielles. Helv. Chim. Acta.
19:649, 665.
Mond, R. 1927. Umkehr der Anionenpermeabilität der roten Blutkoerperchen in eine elektive Durchlaessigkeit fuer Kationen. Pfluegers Arch. Gesamte. Physiol. Mensch. Tiere. 217:618.

Moore, C. E., and R. Peck. 1955. Effects of steric hindrance on ultraviolet absorption spectra and ionization constants through mono- and di-alkyl substitution in 2, 4, 6 trinitrophenol. J. Org. Chem. 20:673.

Passow, H. 1964. In The Red Blood Cell. C. Bishop and D. M. Surgenor, editors. Academic Press, Inc., New York. 99.

Passow, H. 1965. Passive ion permeability and the concept of fixed charges. Proceedings of the 23rd International Congress of Physiological Sciences. Excerpta Med. Int. Congr. Ser. No. 87. 555.

Passow, H., and K. F. Schnell. 1969. Chemical modifiers of passive ion permeability of the erythrocyte membrane. Experientia (Basel). 25:460.

Solomon, A. K. 1960. Red cell membrane structure and ion transport. J. Gen. Physiol. 43 (No. 5, Pt. 2):1.

Theorell, T. 1935. An attempt to formulate a quantitative theory of membrane permeability. Proc. Soc. Exp. Biol. Med. 23:282.

Tosteson, D. C. 1959. Halide transport in red blood cells. Acta Physiol. Scand. 46:19.

Tosteson, D. C., and J. F. Hoffman. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. 44:169.

Wieth, J. O. 1970 a. Paradoxical temperature dependence of Na and K fluxes in human red cells. J. Physiol. (London). 207:563.

Wieth, J. O. 1970 b. Effect of some monovalent anions on chloride and sulphate permeability of human red cells. J. Physiol. (London). 207:581.

Wilbrandt, W. 1942 a. Die Kinetik des Ionenaustausches durch selektiv ionenpermeable Membranen. Pfluegers Arch. Gesamte. Physiol. Mensch. Tiere. 246:274.

Wilbrandt, W. 1942 b. Untersuchungen uber langsamen Anionenaustausch durch die Erythrocytenmembran. Pfluegers Arch. Gesamte Physiol. Mensch. Tiere. 246:291.