

Some Effects of Trinitrocresolate and Valinomycin on Na and K Transport Across Thin Lipid Bilayer Membranes: A Steady-State Analysis with Simultaneous Tracer and Electrical Measurements

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Received 3 January 1978; revised 10 April 1978

Summary. This paper describes the effect of trinitrocresolate anions (TNC^-) on the electrical conductance (G_m), and tracer-measured unidirectional Na and K fluxes (M_{Na} and M_{K}) across bilayers formed from sheep red cell lipids dissolved in decane. In the absence of TNC^- , typical low conductances were observed, while the cation fluxes were too low to measure by our techniques ($< 10^{-12}$ moles $\text{cm}^{-2} \text{sec}^{-1}$). In the presence of TNC^- (10^{-2} M), G_m increased and TNC^- was the main charge carrier in the system. The cationic fluxes were also much increased, but the membranes showed no significant selectivity between K and Na. Furthermore, the Na and K fluxes were at least two orders of magnitude larger than the ionic fluxes calculated from G_m . Thus, almost all of the K and Na transport across the membrane in the presence of TNC^- is electrically silent and is probably carried out as KTNC and NaTNC ion pairs.

In the presence of valinomycin (10^{-6} M) and no TNC^- , both the ion fluxes and G_m were 10^3 times larger in KCl than in NaCl, thus exhibiting the characteristic high selectivity of valinomycin for K over Na. In the presence of both valinomycin (10^{-6} M) and TNC^- (10^{-2} M), this selectivity disappeared in that both G_m and M_{Na} in the NaCl system were similar to the respective values in the KCl system. Even under these conditions, most of the Na is still transported by a process which does not carry charge.

Both G_m and M_x increased alike and monotonically with increasing temperature over the range 7 to 30 °C. In the absence of TNC^- the enthalpies of activation were invariably higher in KCl than in NaCl. Addition of TNC^- produced equal enthalpies of activation for both Na and K containing systems suggesting a common, temperature-dependent, rate-determining step in charge transfer and the electrically silent cation fluxes.

Ionophores are widely used in the study of biological systems for the enhancement of the translocation of ions otherwise impermeable across membranes. The ionophore-ion complex may be either charged and thus

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respond to the electrical gradient across the membrane, or uncharged, in which case the translocation of the complex is electrically silent. Most antibiotic molecules thus far studied produce complexes of the first type, while hydrophobic ions such as some uncouplers of oxidative phosphorylation behave like the second type. Sometimes both types of ionophores are used in conjunction on the presumption that each one behaves independently. However, recent studies on model lipid membranes indicate complex interactions as judged primarily from the measurement of electrical properties of these membranes in the presence of uncouplers, antibiotic and different metal alkali cations. Demin, Shkrob and Ovchinnikov (1972) showed that the lipophilic anion tetraphenylboron can block the potassium conductance induced by valinomycin (*val*). Kuo *et al.* (1976) found the same effect with the uncoupler TTFB (4,5,6,7-tetrachloro-*s*-trifluoromethylbenzimidazole) suggesting a competition between the two ionophores for absorption sites at the membrane interface. Tosteson (1971) reported that a lipid soluble anion (1-hydroxy,3-methyl,2,4,6-trinitrobenzene, (TNC^-)) increased the conductance of bilayers in the absence and presence of valinomycin. In the presence of *val* and TNC^- , the membrane conductance was the same when the cation was K or Na. The transference number for TNC^- was about 0.8 in both K and Na systems, while the transference numbers for K and Na were about equal. Thus, *val* apparently loses its selectivity for K over Na in the presence of TNC^- . He also reported, and Davis and Tosteson (1975) confirmed and extended spectroscopic data indicating a difference in the interaction between Na, *val*, and TNC^- as compared with K, *val*, and TNC^- in low polarity solvents. Recently Ginsburg and Stark (1976) have extended these studies and also concluded that in such systems the anion is mainly responsible for the charge transfer and the antibiotic facilitates the translocation of the anion across the membrane via the induction of lattice defects in the membrane rather than through specific carrier-substrate interactions.

This paper reports the results of experiments in which the transport of the alkali metal cations Na and K across lipid bilayers was measured directly through the use of tracers and compared with the steady-state electrical properties of the membranes. Such comparisons are important steps in the identifications of the charge carriers as well as the mechanisms of ion transport in membrane systems and have been made recently for monovalent anion transport across large spherical bilayers (Toyoshima & Thompson, 1975). In the experiments reported here, simultaneous measurements of Na and K fluxes and electrical properties were made in planar bilayers (area 1–2 mm²) which were modified by the presence of a lipid

soluble anion (1 hydroxy, 3 methyl, 2,4,6 trinitrobenzene or trinitrocresolate, TNC^-) or valinomycin or both simultaneously. Both of these agents have been shown previously to increase the cation permeability of red cell membranes (Gunn & Tosteson, 1971) and of lipid bilayers (Andreoli, Bangham & Tosteson, 1967; Tosteson, 1971).

It will be shown in accord with previous reports, that when present either alone or in the presence of valinomycin, TNC^- is the major charge carrier in the system. In the presence of TNC^- , transport of K and Na are about equal and occur by a mechanism that does not carry charge, probably as KTNC and NaTNC ion pairs. In the presence of TNC^- , the membrane selectivity induced by valinomycin for K as compared with Na, falls from a value of $>10^3$ to about 1 as estimated both from tracer and electrical methods. The enthalpies of activation for cation fluxes and membrane conductance are equal to one another in all systems studied.

Materials and Methods

Lipid Extraction and Bilayer Formation

Lipids were extracted from ghosts of sheep red blood cells either with *n*-butanol (Gutknecht & Tosteson, 1970) or with isopropanol-chloroform-methanol (Andreoli *et al.*, 1967*a*). The lipid extracts were kept in chloroform at -20°C and dissolved in *n*-decane (15–20 mg/ml) prior to bilayer formation. Lipid bilayer membranes were formed by brushing the lipid-decane mixture across a circular hole (1.5 mm^2) in a polyethylene partition (0.1 mm thick). This partition separated two open compartments which each contained 1.2 ml. Both compartments were stirred continuously with magnetic stirrers and could be perfused continuously (but not simultaneously) either by a perfusion pump or by gravity flow with vacuum aspiration. Membranes formed under these conditions could be maintained for up to 4 hr.

Solutions

All electrolyte solutions were prepared by dissolving reagent grade chemicals in double distilled (glass) water. All solutions contained 1 mM of phosphate buffer adjusted to pH 7.0 at room temperature. Trinitrocresolate (TNC^-) was obtained in the acid form from Eastman Kodak (Rochester, New York) and was dissolved in glass-distilled water by adding the hydroxide of the appropriate cation at equimolar amounts and finally adjusting to pH 7.0. Valinomycin (val) stock solution (10^{-3} M) was prepared by dissolving the antibiotic obtained from Calbiochem in ethanol and storing at -20°C .

Electrical Measurements

The electrical measurements were performed using four calomel electrodes. One pair was connected to a high input impedance differential amplifier to measure membrane potential (V_m). A third electrode was connected to the voltage source and the fourth was used to measure current flowing across the membrane (I_m). The experimental set up used for electrical and flux measurements is schematically shown in Fig. 1.

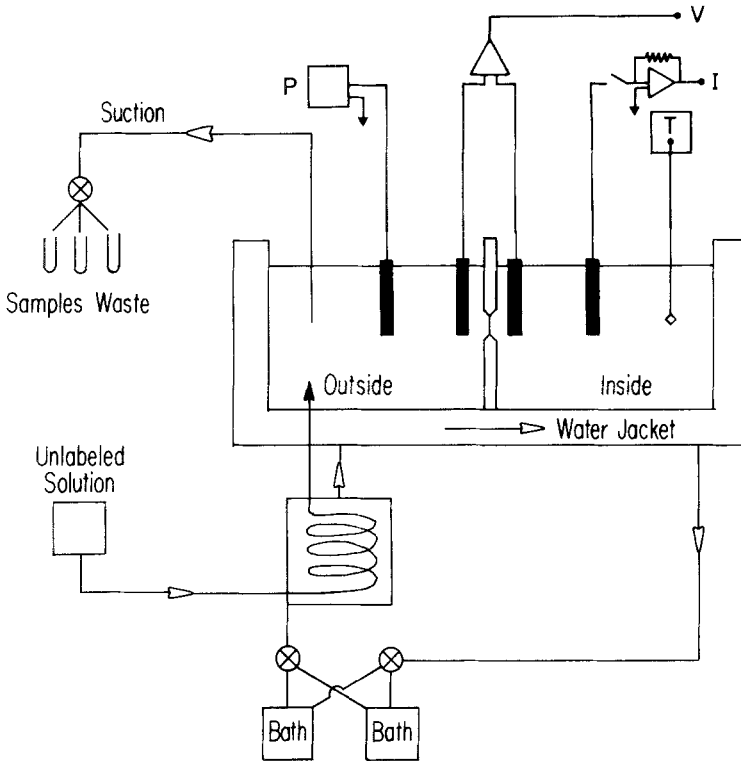


Fig. 1. Diagram of set-up used for flux experiments. *V*: high input impedance differential amplifier used to measure membrane potential. *I*: current amplifier. *T*: electronic thermometer. The nonradioactive solution was circulated through a system in which the temperature was controlled by a high capacity water bath. For further details see text

Flux Measurements

Unidirectional fluxes of K and Na were measured by means of ^{42}K and ^{24}Na , respectively, obtained as the chloride salts from Cambridge Nuclear, Cambridge, Mass., or from ICN, Irvine, Calif. First a membrane was formed in a nonradioactive medium. Then perfusion of the front chamber was started to test the stability of the membrane. Membranes were used only when they had conductances similar to those obtained previously in media of the same composition. Then the back ("inside") compartment was perfused with 2–3 ml of the radioactive solution to give a final specific activity of 0.1–1.0 Ci/mole, as measured from 5 μl samples withdrawn from this chamber. Perfusion of the front ("outside") chamber was at a rate of 2.5–3.5 ml/min. Samples were collected at 60-sec intervals in a vacuum trap (Fig. 1) and were subsequently dried on planchets in a drying oven and counted in a gas flow, low background detector (Beckman Wide Beta). Self-absorption was found to be negligible for both ^{42}K and ^{24}Na under these counting conditions.

The unidirectional fluxes were calculated by the following equation:

$$M_x = \frac{C_t + V(C_t - C_{t-1})}{60 \cdot S \cdot A}$$

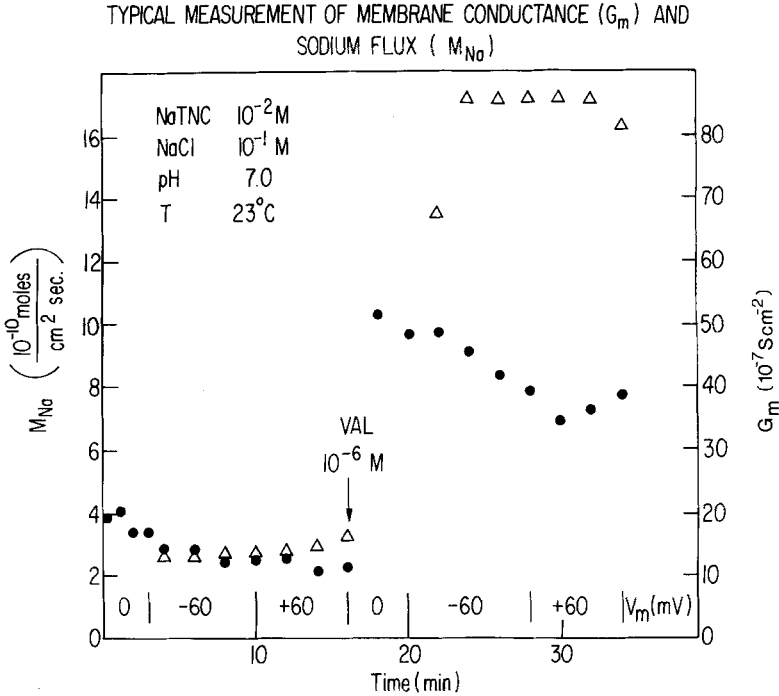


Fig. 2. Time course of sodium flux (M_{Na}) (●) and membrane conductance (G_m) (Δ). A small aliquot of a solution of valinomycin in ethanol was added at the arrow to give a final concentration in the chambers of 10^{-6} M. All points are from one membrane

where M_x is the unidirectional flux of cation x ; C_t is the total counts in the sample of collection interval t ; C_{t-1} is the total counts in the sample of the previous collection interval; $V = V_a/V_s$ where V_a is the volume of the front chamber; V_s is the volume of the sample; S is the specific activity (cpm/moles of x) in the back chamber; A (cm^2) is the surface area of the membrane; 60 sec is the duration of the collection interval. Backflow of radioactive isotope was negligible since the specific activity in the front chamber never exceeded 0.1% of the specific activity of the back chamber. Figure 2 shows the results of a typical flux experiment. With this technique we were able to measure fluxes $\geq 10^{-12}$ moles \cdot cm^{-2} sec^{-1} .

Temperature Experiments

In these experiments the flux chambers were modified to include specially designed water jackets for both front and back aqueous compartments. Water was pumped continuously through these water jackets, either in series or in parallel, from large capacity, temperature-controlled baths (see Fig. 1). The series connection was used for flux experiments in which the front compartment was perfused continuously with tracer-free media. In such experiments, the perfusion solution was maintained at a temperature slightly higher than the temperature of the water jackets in order to compensate for the heat loss in the dead space of the perfusion system. The series connection between the water-jackets of the front and back compartments prevented the development of a temperature gradient

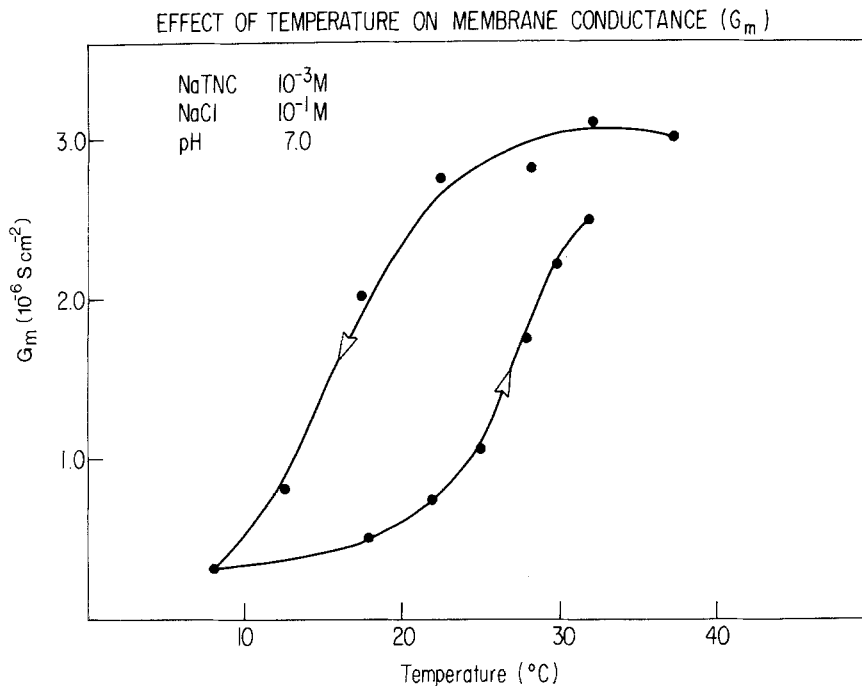


Fig. 3. Membrane conductance (G_m) was determined as the temperature was being changed continuously. The arrows indicate the direction of the temperature change. The points represent the values obtained using one membrane

between the two compartments. The parallel connection between the water-jackets was used in experiments in which the front compartment was not continuously perfused. Temperature in the back chamber was monitored continuously by means of an electronic thermometer. We first attempted to measure the electrical parameters continuously and simultaneously with temperature change. However, hysteresis (Fig. 3) indicated that equilibrium of the whole system had not been achieved. This could have been due to different heat capacitances for the different components of the system and/or adsorption and desorption of membrane constituents with changing temperature. Therefore, all experiments were performed in the steady state at different but constant temperatures. Usually, the shift from one temperature to the other was achieved within 10 min, but measurements were made only after 15 min as illustrated in Fig. 4. The values of the flux or conductance were computed as a mean of the last 3 sampling intervals at each temperature. Several attempts to measure electrical parameters at temperatures higher than 28–30°C failed since no steady state could be achieved, i.e., membrane conductance constantly increased for as long as 45 min.

Results

Effects of TNC^- on Conductance and Cation Fluxes

The effect of TNC^- concentration on the steady-state electrical conductance (G_m) of bilayers made from sheep red blood cell lipids is shown

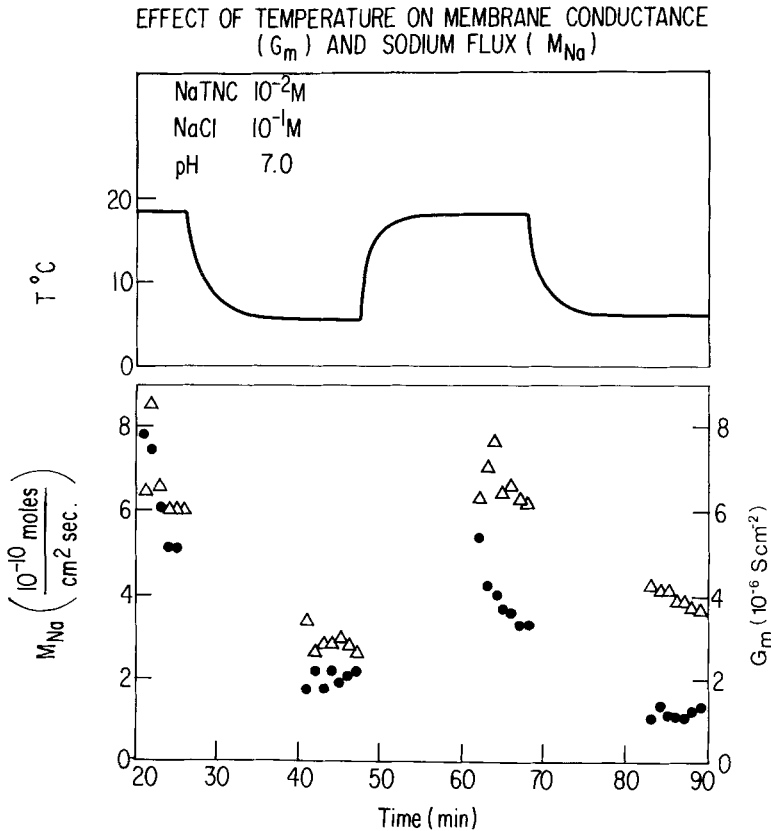


Fig. 4. Time course of sodium flux (M_{Na}) (\bullet) and membrane conductance (G_m) (Δ) at two different temperatures. The points represent values obtained in one membrane

in Fig. 5. Both in the presence of K and in the presence of Na, TNC^- caused a marked increase in membrane conductance, showing a tendency for saturation. Similar saturation of steady-state conductance as a function of lipid soluble anion concentration has also been reported in bilayers exposed to picrate (Ginsburg & Stark, 1976) and to tetraphenylboron (Andersen & Fuchs, 1975). This apparent saturation may be due to the adsorption of hydrophobic anions to the membrane interface, thus establishing a boundary potential and changing the electrostatic potential within the membrane (Andersen *et al.*, 1977). However, saturation in the present system occurs at higher concentrations of the hydrophobic anion as compared with the above mentioned systems. This may be due to the negative charge of some of the lipids of sheep red blood cells which would tend to reduce anion concentration at the surface of the membrane. Measurements of the zero-current electrical potential difference across

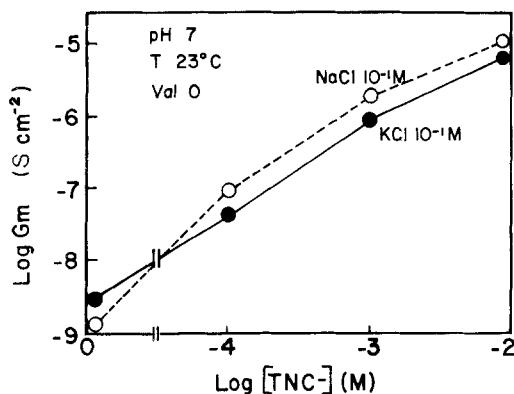
EFFECT OF TNC^- ON MEMBRANE CONDUCTANCE

Fig. 5. Double logarithmic plot of the steady-state membrane conductance (G_m) as a function of TNC^- concentration. The aqueous phases contained 10^{-1} M KCl (●) or 10^{-1} M NaCl (○). TNC^- was present on both sides of the membrane. Points represent mean values of at least three different membranes

membranes separating aqueous solutions of different activities of Na, K, Cl, and TNC, (not shown) indicate that TNC^- is the main charge carrier, its transference number being very close to unity.

Results of measurements of cation fluxes in the presence of TNC^- alone are shown in Table 1. In the presence of 10^{-2} M TNC^- , both ^{42}K and ^{24}Na fluxes (M_K and M_{Na} , respectively) were approximately 10^{-10} moles \cdot cm $^{-2}$ sec $^{-1}$ and were at least 100-fold larger than in control membranes in the absence of TNC^- . In the absence of TNC^- , K and Na fluxes were too low to be measured by our technique. Table 1 further shows that decreasing the TNC^- concentration from 10^{-2} to 10^{-3} M decreased the Na flux by approximately 10-fold.

From the values of G_m we can calculate the ionic flux associated with the translocation of charge by using the following relation $M_e = G_m RT/F^2$, where F is the Faraday constant, R is the gas constant, T the absolute temperature and M_e is the unidirectional flux of monovalent ions carrying the charge. From the ratios M_x/M_e depicted in Table 1, it is obvious that the measured fluxes of K and Na are at least two orders of magnitude higher than can be accounted for by the measured value of G_m . This discrepancy is even more striking if one takes into account the fact that the major charge carrier is not K or Na but TNC^- . Moreover, as shown in Table 1 and Fig. 2, these fluxes are not affected by an imposed electrical potential. The most reasonable explanation for these results is that the cations are moving across the membrane as KTNC or NaTNC ion pairs formed in the aqueous

Table 1. Effect of TNC⁻ on the fluxes of ⁴²K and ²⁴Na
a) $V_m = 0 \text{ mV}^a$

[TNC]	G_m	M_e	M_{Na}	M_{K}	M_x/M_e	T
M	(Scm^{-2})	(10 ⁻¹⁰ moles · cm ⁻² sec ⁻¹)				(°C)
0	(4.5 ± 1.2) 10 ⁻⁹	1.2 × 10 ⁻⁵		< 10 ⁻²	—	17
0	(1.9 ± 0.4) 10 ⁻⁹	4.9 × 10 ⁻⁶	< 10 ⁻²		—	17
10 ⁻²	(2.6 ± 1.1) 10 ⁻⁶	6.7 × 10 ⁻³	—	4.0 ± 1.5	6.0 × 10 ²	17
10 ⁻²	(4.9 ± 2.5) 10 ⁻⁶	1.3 × 10 ⁻²	2.2 ± 0.5	—	1.7 × 10 ²	17
10 ⁻³	(2.0 ± 0.3) 10 ⁻⁶	5.2 × 10 ⁻³	0.2 ± 0.05	—	0.4 × 10 ²	23
10 ⁻²	(9.0 ± 0.5) 10 ⁻⁶	2.3 × 10 ⁻²	3.0 ± 1.2	—	1.3 × 10 ²	23

b) $V_m = 60 \text{ mV}$, [TNC] = 10⁻² M^b

${}^iM_{\text{Na}}$	${}^oM_{\text{Na}}$	${}^iM_{\text{K}}$	${}^oM_{\text{K}}$	${}^iM_x/{}^oM_x$	T
(10 ⁻¹⁰ moles · cm ⁻² sec ⁻¹)					(°C)
		0.36	0.31	1.2	17
1.2	1.1	—	—	1.1	17
4.0 ± 1.1	3.6 ± 1.2	—	—	1.4 ± 0.1	23

^a Conductances (G_m) and unidirectional fluxes (M_x) were measured when $V_m = 0$. M_e was computed from the measured value of G_m , from the relation $M_e = \left(\frac{RT}{F^2}\right) G_m$. Data are presented as mean values followed by SEM. The ratios M_x/M_e are ratios of the mean values of M_x/M_e .

^b Unidirectional cation fluxes were measured in the same (iM_x , + → -) and opposite (oM_x - → +) direction to the electrical potential difference ($V_m = 60 \text{ mV}$) maintained across the membrane. All data, including the ratios ${}^iM_x/{}^oM_x$ are means of results from individual membranes followed by SEM. Aqueous solutions contained 10⁻¹ M XCl and 10⁻³ M phosphate buffer, pH 7.0 (X = Na or K, as indicated).

phases. Alternatively, these results could also be interpreted in terms of a carrier mechanism whereby the metal ions combine with TNC⁻ adsorbed at the membrane interface to produce an ion-carrier complex which is subsequently translocated across the membrane.

Effects of Valinomycin on Conductance and Cation Fluxes

Addition of valinomycin alone increased the conductance of bilayers when the bathing solutions contained KCl but not when they contained NaCl (Table 2), confirming observations previously reported from several laboratories (Mueller & Rudin, 1967; Lev & Buzhinsky, 1967; Andreoli *et*

Table 2. Effect of valinomycin on conductances and cation fluxes
a) $V_m = 0$ mV

VAL	G_m	M_e	M_{Na}	M_K	M_x/M_e
	(10^{-6} M) (Scm^{-2})	(10 ⁻¹⁰ moles · cm ⁻² sec ⁻¹)			
0	$(4.5 \pm 1.2) 10^{-9}$	1.2×10^{-5}		$< 10^{-2}$	—
0	$(1.9 \pm 0.4) 10^{-9}$	4.9×10^{-6}		$< 10^{-2}$	—
+	$(3.4 \pm 0.3) 10^{-4}$	0.89		6.4 ± 1.4	7.2
+	$(1.4 \pm 0.4) 10^{-8}$	3.6×10^{-5}	$< 10^{-2}$		—

b) $V_m = 60$ mV, [VAL] = 10^{-6} M

iM_K	oM_K	${}^iM_K/{}^oM_K$	I_m	I_K	I_K/I_m
(10 ⁻¹⁰ moles cm ⁻² sec ⁻¹)			(10 ⁻⁵ amp · cm ⁻²)		
9.7 ± 1.5	6.2 ± 0.5	2.5 ± 0.5	2.5 ± 0.3	3.3 ± 0.6	1.5 ± 0.3

The various quantities were derived as described in the text and in the legend to Table 1. The aqueous solutions contained 10^{-1} M XCl (X = Na, K, as indicated) buffered to pH 7.0 with 10^{-3} M phosphate; temperature 17°C. I_K , the current carried by K was calculated from $I_K = F({}^iM_K - {}^oM_K)$.

al., 1967*b*). The flux of Na in the presence of val (10^{-6} M) was too low to be measured ($< 10^{-12}$ moles · cm⁻² sec⁻¹). The value of M_e computed from the measurements of membrane conductance was 8.9×10^{-11} moles cm⁻² sec⁻¹, about 1/7 of the measured K flux. Furthermore, the ratio of the unidirectional K fluxes in the presence of an externally applied potential difference of 60 mV was only 2.5 rather than 10, the expected value for a system in which ions move independently. The measured net K current was not appreciably different from the total membrane current, indicating that K-val is the only charge carrier in the system. These facts are consistent with the idea that the val system is close to saturation under these experimental conditions, as shown by Stark and Benz (1971) using sheep red cell lipids.

Combined Effect of TNC and Valinomycin on Conductance and Cation Fluxes

The combined effect of TNC and valinomycin on conductance and fluxes is shown in Fig. 6 and Table 3. In the presence of val, the addition of TNC increased G_m by a factor of more than 10^4 in the Na system, while G_m was slightly reduced in the K system (compare with Table 2). The addition

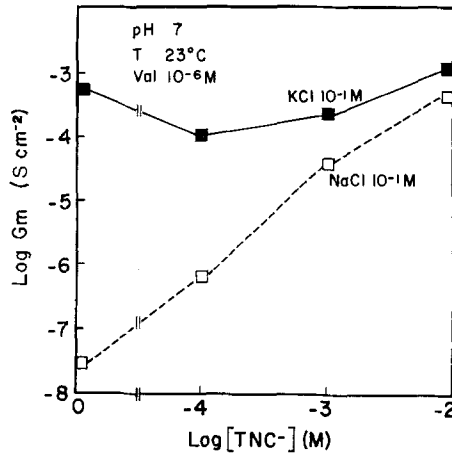
EFFECT OF TNC^- ON MEMBRANE CONDUCTANCE

Fig. 6. Double logarithmic plot of the steady-state membrane conductance (G_m) as a function of TNC^- concentration. The aqueous phases contained 10^{-1} M KCl (■) or 10^{-1} M NaCl (□) plus 10^{-6} M valinomycin. TNC^- was present on both sides of the membrane. The points represent mean values of at least three different membranes

Table 3. Effect of TNC^- and valinomycin on conductances and fluxes
 a) $V_m = 0$ mV

[TNC ⁻] M	G_m (S · cm ⁻²)	M_e (10^{-10} moles · cm ⁻² sec ⁻¹)	M_{Na}	M_{K}	M_x/M_e	T (°C)
10^{-2}	$(2.5 \pm 0.2) 10^{-4}$	0.64		1.4 ± 0.5	2.2	16
10^{-2}	$(3.5 \pm 1.0) 10^{-4}$	0.9	17.0 ± 4.0		18.9	16
10^{-3}	$(2.0 \pm 0.7) 10^{-4}$	0.52	6.0 ± 1.6		11.5	23

b) $V_m = 60$ mV, $[\text{TNC}^-] = 10^{-2}$ M

${}^iM_{\text{Na}}$	${}^oM_{\text{Na}}$	${}^iM_{\text{K}}$	${}^oM_{\text{K}}$	${}^iM_x/{}^oM_x$	T (°C)
		(10 ⁻¹⁰ moles · cm ⁻² sec ⁻¹)			
—	—	0.7 ± 0.1	0.5 ± 0.1	1.5 ± 0.1	17
30 ± 2.9	28 ± 2.4	—	—	1.1 ± 0.1	23

The various quantities were derived as described in the legend for Table 1. The aqueous solutions contained 10^{-1} M XCl and 10^{-3} M phosphate buffered to pH 7.0. Valinomycin concentration was 10^{-6} M.

of val when TNC is already present increased G_m by a factor of 70 in the Na system and 100-fold in the K system (compare with Table 1).

In the presence of TNC, addition of val substantially increased Na fluxes but left K fluxes relatively unchanged. As a result, the Na fluxes substantially exceeded K fluxes under these conditions. For both cations, M_x was greater than M_e but the ratios M_x/M_e were lower than when TNC is present alone. This implies that the effect of val is to promote preferentially the translocation of the TNC^- anion rather than the $XTNC$ ion pair. This effect is more pronounced in the K system and shows a marked dependence on TNC^- concentration in the Na system. The flux ratios for both K and Na when V_m was ± 60 mV were both about unity (Table 3) as would be expected in a system in which most of the cation transport occurs by a mechanism which does not carry charge.

Effects of Temperature on Conductances and Cation Fluxes

Table 4 summarizes the effects of temperature on Na and K fluxes and membrane conductances in the absence and/or presence of TNC^- and

Table 4. Effect of temperature on conductance and cation fluxes

TNC (10^{-2} M)	VAL (10^{-6} M)	XCl (10^{-1} M)	G (17°C) (S cm^{-2})	ΔH_g^\ddagger (kcal mol^{-1})	oM_x (17°C) (10^{-10} moles) $\text{cm}^{-2} \text{sec}^{-1}$)	ΔH_f^\ddagger (kcal/mol^{-1})
0	0	K	$4.5 \pm 1.2 \times 10^{-9}$	29 ± 8.6	$< 10^{-2}$	—
0	0	Na	$1.9 \pm 0.5 \times 10^{-9}$	11 ± 0.8	$< 10^{-2}$	—
+	0	K	$2.6 \pm 1.1 \times 10^{-6}$	18 ± 2.4	4.0 ± 1.5	14 ± 2.0
+	0	Na	$4.9 \pm 2.5 \times 10^{-6}$	12 ± 1.3	2.2 ± 0.5	8.5 ± 1.4
0	+	K	$3.4 \pm 0.3 \times 10^{-4}$	33 ± 5.9	6.4 ± 1.4	24 ± 5.0
0	+	Na	$1.1 \pm 0.4 \times 10^{-8}$	11 ± 0.8	$< 10^{-2}$	—
+	+	K	$2.5 \pm 0.2 \times 10^{-4}$	17 ± 2.2	1.4 ± 0.5	16 ± 2.7
+	+	Na	$3.5 \pm 1.0 \times 10^{-4}$	19 ± 4.3	17 ± 4	17 ± 3.0

The values for the enthalpy of activation of conductance (ΔH_g^\ddagger) and flux (ΔH_f^\ddagger) were calculated from measurement of conductance and flux made on the same membrane at two different temperatures, 17 and 7°C , from the relations $\Delta H_g^\ddagger = \frac{R \Delta \ln G_m}{\Delta(1/T)}$ and $\Delta H_f^\ddagger =$

$\frac{R \Delta \ln {}^iM_x}{\Delta(1/T)}$, respectively, where R is the gas constant in $\text{cal mol}^{-1} \text{ }^\circ\text{K}^{-1}$ and T is the temperature in degrees Kelvin. The values shown for ΔH^\ddagger are means of values so calculated for each membrane, followed by SEM. Values shown for G_m and iM_x are mean values followed by SEM for all experiments used in computing ΔH^\ddagger . In the cases where fluxes were too low to measure, G_m was calculated from the steady state I_m when $V_m = 60$ mV. In cases where fluxes were measured, G_m was estimated from measurements of I_m during 5-sec pulses of $V_m = 60$ mV.

valinomycin. Usually, membranes were formed at 17°C, and then the temperature was lowered to 7°C and raised again to 17°C to assure reproducibility. At least two such temperature cycles were performed on the same membrane. Fluxes and conductances were measured 10 min after temperature equilibration was reached. Enthalpies of activation were calculated for conductance (ΔH_g^\ddagger) and flux (ΔH_f^\ddagger) for each cycle and averaged.

When neither TNC^- nor val were present, ΔH_g^\ddagger was considerably higher in the presence of KCl than in the presence of NaCl. Addition of TNC^- in the presence of KCl reduced ΔH_g^\ddagger considerably, simultaneously with an increase in conductance. In the presence of NaCl, TNC^- had no effect on ΔH_g^\ddagger . Thus, the ratio of ΔH_g^\ddagger in the K to that in the Na system fell from a value of about 3 in the absence of TNC^- to about 1.5 in its presence. In the presence of TNC^- (10^{-2} M) alone, ΔH_g^\ddagger was slightly, but probably not significantly, higher than ΔH_f^\ddagger in both the K and the Na systems.

Adding valinomycin (without TNC^-) did not produce a significant change in ΔH_g^\ddagger in either the K or the Na systems, despite a marked increase in the conductance in the presence of K. In the K-val system, ΔH_g^\ddagger was higher than ΔH_f^\ddagger .

Addition of TNC^- to a membrane which had been modified by 10^{-6} M valinomycin had opposite effects in the presence of KCl and NaCl. ΔH_g^\ddagger and ΔH_f^\ddagger were reduced in KCl while ΔH_g^\ddagger was increased in NaCl. Furthermore, in a system already containing TNC^- , addition of valinomycin had no effect on ΔH_g^\ddagger and ΔH_f^\ddagger in the presence of KCl. ΔH_g^\ddagger and ΔH_f^\ddagger increased significantly in the presence of NaCl under similar conditions. Thus ΔH_g^\ddagger and ΔH_f^\ddagger were about the same in the presence of val (10^{-6} M) and TNC^- (10^{-2} M) in both K and Na systems, providing evidence that the temperature-dependent rate limiting step may be identical for TNC^- and for KTNC or NaTNC ion pairs.

Discussion

Na and K Transport in the Presence of TNC^- Alone

In the presence of TNC^- , the conductance of planar bilayer membranes formed from sheep red cell membranes in decane is almost exclusively anionic. The fact that the slope of the relation between membrane conductance and TNC^- concentration (Fig. 5) is never more than 1, suggests that the charge carrier is not some polymeric form of TNC^- as is the case with the analog 2, 4, 6 trinitrophenol (Ginsburg & Stark, 1976).

The effects of TNC^- on Na and K fluxes cannot be explained exclusively in terms of the actions of lipophilic anions on the electrical properties of bilayers. The data in Table 1 demonstrate clearly that the measured fluxes of K and Na exceed the fluxes predicted by the measured conductance by factors of from 40 to 600. Furthermore, changing the voltage across the membrane does not affect the fluxes, and the flux ratio in the presence of an electrical potential difference of 60 mV does not deviate appreciably from unity (Table 1*b*). These results are compatible with the idea that K and Na traverse the membrane as ion pairs with TNC^- or a mechanism in which TNC^- serves as a shuttling carrier which is saturated under the experimental conditions tested.

The hypothesis that Na and K can move across bilayers as NaTNC and KTNC ion pairs is further supported by measurements of the formation of ion pairs between alkali metal cations and picrate anions in water and in nitrobenzene, reported by Iwachido (1972). He found that the association constants of picrate for Na and K in water are $10^{1.38}$ and $10^{1.64}$, respectively, while the comparable values for nitrobenzene are $10^{5.80}$ and $10^{3.67}$, respectively. He also reported measurements of the partition coefficient (nitrobenzene/water) for Na picrate and K picrate ion pairs of $10^{-2.55}$ and $10^{-1.85}$, respectively. If we assume these values for the aqueous association constants and membrane/water partition coefficients of NaTNC and KTNC, we may estimate the diffusion coefficient of the ion pairs in the membrane. Since the partition coefficient nitrobenzene/water is probably an overestimate of the partition coefficient membrane/water, the resultant estimates for diffusion coefficients are *minimum* values. For the case of $[\text{TNC}] = 10^{-2}$ M, $[\text{NaCl}]$ or $[\text{KCl}] = 10^{-1}$ M, the aqueous concentration of NaTNC and KTNC ion pairs are 7.3×10^{-3} M and 8.3×10^{-4} and the *minimum* estimates of membrane diffusion coefficients are between 10^{-9} and 10^{-8} $\text{cm}^2 \text{sec}^{-1}$.

Ginsburg and Stark (1976) have proposed recently that macrocyclic compounds may introduce structural disorder in the lipid bilayer, thus creating free volumes moving across the membrane (*see also* Walz, 1976). Such mobile structural defects caused by the "mediator molecules" (i.e., valinomycin) could facilitate the diffusion of smaller "foreign molecules" (i.e., TNC^- and XTNC) across the membrane though no direct chemical interaction between val and TNC is assumed. This suggested mechanism is supported by the present results on the combined effect of val and TNC on cation fluxes (Table 3). It is also compatible with the observation that val enhances the translocation of TNC^- relatively more than that of XTNC ion pairs (compare M_x/M_e ratios of Tables 1 and 3). This latter point is based on the following arguments: first, we presume that

the interfacial concentration of TNC^- is larger than that of XTNC. Secondly, Ginsburg and Stark (1976) have approximated the adsorption density of picrate anions to be 1 for 7 phospholipid molecules (1 mM picrate in the aqueous phase), as compared with 1:200 for valinomycin (10^{-7} M valinomycin in the aqueous phase). This relatively greater density of packing of anions favors their occupation of free defect structures in the proximity of val molecules. Finally, due to charge repulsion of TNC^- anions adsorbed at the interface, the probability of occupation of free defect structures generated by val is markedly enhanced with increasing packing density of anions at the membrane surface. Altogether, this mechanistic interpretation offers a plausible explanation for the fact that val has a preferential effect on the translocation of TNC^- anions as compared with that of XTNC ion pairs, which may also be promoted by the induction of structural defects by val, but to a lesser degree.

The effects of temperature on membrane conductances and cation fluxes in the presence of TNC^- (Table 4) are of interest in several respects. First, the magnitudes of the enthalpies of activation of conductances (ΔH_g^\ddagger) and flux (ΔH_f^\ddagger), are about equal to one another, implying that the rate-limiting steps for charge transport by TNC^- and cation transport by NaTNC and KTNC ion pairs are energetically similar. Second, the values for both ΔH_g^\ddagger and ΔH_f^\ddagger are significantly greater in the K than in the Na system, indicating that the rate-limiting steps are energetically different in these two circumstances. This discrepancy between ΔH^\ddagger for K and Na is much less in the presence than in the absence of TNC^- , suggesting that the presence of the lipophilic anion opens possibilities for both charge transport (presumably by TNC^-) and cation fluxes (presumably as NaTNC and KTNC ion pairs) which are energetically similar irrespective of the cation. Third, ΔH_g^\ddagger and ΔH_f^\ddagger are *positive* and invariant in the temperature range 7 to 23°C. This result is in marked contrast to the behavior of red cells exposed to TNC^- (Gunn & Tosteson, 1971). In red cells ΔH_f^\ddagger for K and Na fluxes is *negative* in the temperature range 0–18°C, but becomes positive in the temperature range 18–37°C. Furthermore, the ΔH for partition of TNC^- between water and decane containing val is also negative in this temperature range from 0–18°C. The action of TNC^- on cation transport in red cell membranes must involve different processes than those related to the effect of TNC^- on cation transport in bilayers formed from red cell membrane lipids dissolved in decane. Since cation transport in bilayers in this system probably occurs as ion pairs with TNC^- , it follows that cation transport in red cells occurs by some other mechanism, possibly through a pathway opened by interactions between TNC^- and mem-

brane proteins. It is possible that TNC^- induced cation movements in red cells above 18°C do occur via ion pairs.

This work was supported by NIH grants HL 12157 and GM 24084.

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