The Effect of Valinomycin on the Ionic Permeability of Thin Lipid Membranes

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ABSTRACT Optically black membranes prepared from sheep red cell lipids have a high electrical resistance (1–3 × 10^8 ohm-cm^2). The ionic transference numbers (T_i) for cations (Na^+ or K^+) are equal to each other but at least four to five times greater than for Cl^-. The cyclic depsipeptide valinomycin produces a striking decrease in the membrane resistance when K^+, but not when Na^+ is in the solutions bathing the membrane. The ratio T_{Na}/T_K, estimated from membrane voltages in the presence of ionic concentration gradients, approaches zero. The order of membrane monovalent cation selectivity, in the presence of valinomycin, is $H^+ > Rb^+ > K^+ > Cs^+ > Na^+$. Addition of the antibiotic to one side of a membrane which separates identical solutions of NaCl produces a substantial (up to 80 mV) membrane voltage (side opposite valinomycin negative). These data are consistent with the hypothesis that valinomycin can interact with appropriately sized cations (hydrated diameter $\lesssim$6 A) to increase their membrane permeability, perhaps by forming hydrogen bonds between the solvation shell of the cations and carbonyl oxygens in the valinomycin molecule which are directed toward the aperture of the ring.

INTRODUCTION

Previous reports from this laboratory (1, 2) described the properties of optically black, thin lipid membranes separating two aqueous phases. The membranes were formed from lipids extracted from high potassium (HK) and low potassium (LK) (3) sheep red cell lipids dissolved in decane (4), and were similar in nature to thin lipid membranes formed from other lipids (5, 6). Ionic transference numbers (T_i), estimated from steady-state membrane voltages in the presence of ionic concentration gradients, indicated that the sheep erythrocyte lipid membranes were approximately four to five times more permeable to the alkali metal cations, sodium and potassium, than to chloride, and were slightly more selective for potassium than for sodium

(2). Sheep erythrocyte lipids contain substantial amounts of the negatively charged phospholipids, phosphatidyl ethanolamine and phosphatidyl serine (7), and the cation selectivity of the artificial membranes was rationalized in terms of fixed negative membrane charges attributable to the phospholipids (2).

Bangham et al. observed that valinomycin, a cyclic dodecadepsipeptide (mol wt = 1111), produced a greater increase in the potassium than in the sodium permeability of concentric phospholipid lamellae with bimolecular dimensions (8, 9). Lev first demonstrated (10) that valinomycin also produced striking selectivity for K^+ as compared with Na^+, in thin lecithin membranes separating two aqueous phases. Independently, Mueller and Rudin (11) have made similar observations on the effect of valinomycin on the ionic permeability of thin lipid membranes prepared from a variety of lipids, and also have suggested the possibility of hydrogen bonding between appropriately sized cations and the valinomycin molecule. A preliminary report of the observations described in this paper (12), presented evidence that naturally occurring valinomycin also increased the potassium, but not sodium permeability, of intact HK and LK sheep erythrocytes and of thin lipid membranes prepared from lipids extracted from these cells. On the basis of consideration of the probable conformation of the valinomycin molecule in these membranes, we suggested the possibility that carbonyl oxygens in the valinomycin ring might form hydrogen bonds with water molecules in the hydration shells of appropriately sized cations.

This paper reports in detail the effects of valinomycin on the electrical properties and ionic permeability of thin membranes prepared from lipids extracted from HK and LK sheep red cells. In a companion manuscript (13), similar results are presented for the effects of valinomycin on the ionic permeability of intact sheep erythrocytes. The striking similarity between the effect of valinomycin on the passive ionic permeability of intact sheep erythrocytes and on thin membranes prepared from lipids extracted from these cells further illustrates the usefulness of the artificial membrane technique (1, 2, 4–6) as an approach to the study of biological membranes.

METHODS

A detailed discussion of the experimental techniques and their justification has been presented previously (2). With minor modifications, the same methods were employed in these studies.

Unless otherwise indicated, the lipid solutions from which the membranes were formed contained approximately 40 mg HK sheep erythrocyte lipids/ml decane. The lipids were applied by a brush technique (5) to an aperture (1–2 mm diameter) in a polyethylene partition separating a front and rear chamber.

When the apparatus was assembled, the rear chamber was sealed and filled with

fluid (2); there was electrical and hydrodynamic continuity with the fluid phase in the front chamber only through the aperture on which the membranes were formed. A three-way stopcock was also connected to the rear chamber. By rinsing through the rear chamber to the front chamber with relatively large amounts (100–150 ml) of a given solution, in the absence of an intact membrane, it was possible to form a series of membranes in varying media without dismantling the apparatus. A rapid flow system (5–10 ml/min) allowed changes in the composition of the solution in the front chamber bathing an intact membrane (2).

All experiments were carried out at room temperature (22–24°C). Except where indicated, the aqueous solutions were unbuffered (pH $\sim$ 5.6).

The DC electrical circuit was arranged so that the rear chamber was positive or negative and the front chamber was grounded. The input resistance (R_i) of the circuit varied between 10^6–10^{10} ohms, depending on the membrane resistance (R_m), and was adjusted so that approximately half of the potential of the applied DC pulse (E_i) used to compute membrane resistance was dissipated across the input resistor.

Preliminary experiments indicated that the activity of valinomycin,[1] when assayed by the increase in potassium permeability of thin lipid membranes, deteriorates greatly when the antibiotic is in aqueous solution at room temperature for 24 hr. Such deterioration did not occur when valinomycin was dissolved in ethanol. Consequently, a stock solution of valinomycin (1–2 mg/ml) in 95% ethanol was stored at 4°C. In the experiments, aliquots of the stock ethanolic solution were added to aqueous solutions less than an hour prior to use. Appropriate control solutions, at the same or greater concentrations of ethanol (<0.1%), but without valinomycin, did not affect the zero frequency capacitance or DC resistance of the membranes.

In order to minimize the possibility that the observed effects of valinomycin might be attributable to low resistance electrical "leakage" pathways in the apparatus for any given experiment, the following procedure was employed. A thin lipid membrane ("control" membrane) was formed in valinomycin-free solutions and DC resistances and membrane voltages in the presence of different salt solutions were recorded during a period of approximately 20–60 min. Subsequent experiments to evaluate the effects of valinomycin were carried out only when the resistance and ionic selectivity of this control membrane were the same as previously described (2). When one surface of the membrane was exposed to valinomycin, an aliquot of the stock valinomycin solution was added to an aqueous solution identical with the one bathing the membrane. The test solution containing valinomycin was then flowed into the front chamber, while the control membrane was intact. When both surfaces of a membrane were exposed to valinomycin, the control membrane was ruptured, but the apparatus was not dismantled. Both chambers were then refilled with identical solutions to which valinomycin had been added, and a new membrane was formed in the presence of the antibiotic. All subsequent solutions to which the formed membrane was exposed

[1] Naturally occurring valinomycin (mol wt = 1111), was kindly furnished by Dr. W. S. Lynn, Duke University, and Dr. J. C. MacDonald, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada.

also contained the same valinomycin concentration as the initial medium. Under these conditions, the observed changes could reasonably be attributed to the effects of valinomycin.

RESULTS

Membrane Electrical Resistance

In the absence of valinomycin, the DC resistance of thin lipid membranes prepared from sheep erythrocyte lipids was in the range $1.0–3.0 \times 10^8$ ohm-cm^2, and was usually stable for the duration of the membrane (2). Fig. 1 illustrates the effect of valinomycin on the electrical resistance of these membranes when the aqueous medium bathing the membrane was 0.1 M KCl or 0.1 M NaCl. If the same membrane was exposed to multiple concentrations of valinomycin (Fig. 1, experiment VI-34), the lowest concentration of the antibiotic was added first. Prior to adding a higher concentration of valinomycin, the initial medium without valinomycin was flowed into the front chamber until the electrical resistance had risen at least ten-, and ordinarily a hundredfold. When the flow rate was 6 ml/min, this ordinarily required a rinsing period of 15–20 min. Subsequently, the next higher concentration of valinomycin was introduced into the front chamber, and the new steady-state electrical resistance recorded.

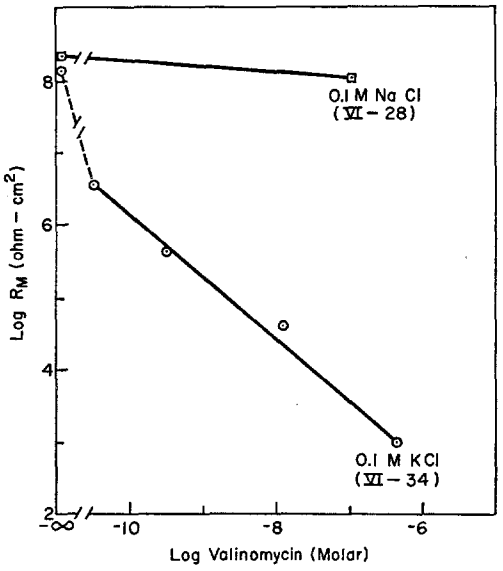

FIGURE 1. The effect of valinomycin on the electrical resistance (R_m) of thin lipid membranes. The aqueous solutions bathing the membrane were 0.1 M KCl (⊙) or 0.1 M NaCl (⊡) both at pH ~ 5.6. In the KCl experiments, valinomycin was added to the front chamber only. In the NaCl experiments, since the membrane was formed with valinomycin in the initial medium, it was present in both chambers. The numbers in parentheses indicate the experiment. For experimental details, see Methods and the text.

As shown in Fig. 1, the relationship between the logarithm of membrane resistance and the logarithm of valinomycin concentration, in the range $5 \times 10^{-11} - 10^{-6}$ M, had a slope of approximately minus one when potassium

was present in the aqueous media bathing the membrane. At 4.6×10^{-7} M valinomycin in 0.1 M KCl, membrane resistance was 10^3 ohm-cm^2. In contrast, when 0.1 M NaCl was the only solute in the media, membrane resistance fell only slightly in 10^{-7} M valinomycin. Furthermore, as indicated in Table I, the relatively small decrease in membrane resistance produced by valinomycin was independent of the NaCl concentration over a hundredfold range.

The relationship of the fall in membrane resistance produced by valinomycin to the concentration of potassium in the aqueous solutions bathing the membrane is shown in Fig. 2. When valinomycin was absent, membrane resistance was independent of the KCl concentration over the range 5×10^{-6}-0.25 M, but fell when the KCl concentration was 0.5 M. When the membranes were formed in the presence of valinomycin and KCl, there was a sigmoid re-

TABLE I

THE EFFECT OF VALINOMYCIN ON MEMBRANE RESISTANCE IN VARYING CONCENTRATIONS OF NaCl

The DC resistance (R_m) of thin lipid membranes was measured in the presence and absence of valinomycin. When present, the antibiotic was in the initial medium in which the membranes were formed. The pH of the solutions was approximately 5.6. Experimental details are described under Methods.

Experiment	Medium	Valinomycin	R_m
	M	M	ohm-cm^2
VI-31-a	0.001 NaCl	0	1.9×10^8
VI-31-c	0.001 NaCl	3.1×10^{-7}	5.7×10^7
VII-20	0.1 NaCl	0	2.1×10^8
VII-32	0.1 NaCl	1.03×10^{-7}	9.2×10^7

lationship between the logarithm of membrane resistance and the logarithm of KCl concentration, with nearly a thousandfold fall in membrane resistance occurring in the concentration range $10^{-3} - 10^{-2}$ M. An increase in the KCl concentration beyond 0.1 M had comparatively negligible effects. The fall in membrane resistance produced by valinomycin when the KCl concentration was 0.1 M was approximately the same when the antibiotic was present in one (Fig. 1) or both (Fig. 2) of the solutions bathing the membrane. These data indicate clearly that valinomycin produces a striking increase in the conductance of these thin lipid membranes to potassium, but little, if any, increase in sodium conductance.

Current-Voltage Properties

Figs. 3 and 4 illustrate the current-voltage properties of the lipid membranes when the bathing medium was 0.01 M KCl. In the absence of valinomycin (Fig. 3), the membrane resistance was constant for applied potentials over

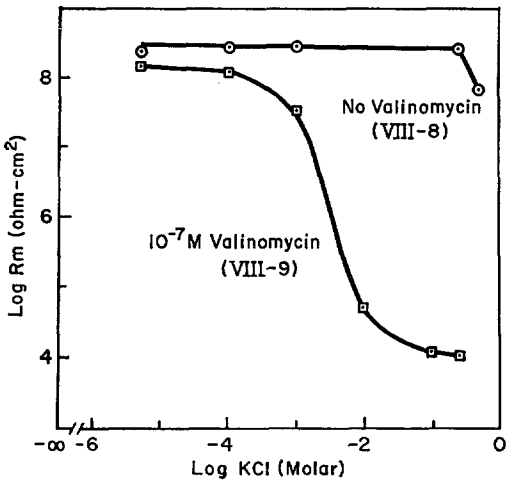

FIGURE 2. The effect of the KCl concentration in the solutions bathing the membrane on membrane electrical resistance (R_m) in the absence (⊙) and presence of 10^{-7} M valinomycin (⊡). In the latter case, since the membranes were formed in a solution containing the antibiotic, it was present in both front and rear chambers. Experimental details are described under Methods. The numbers in parentheses indicate the experiment.

the range ±120 mv (4-6, 14). When valinomycin was present (Fig. 4), membrane resistance fell, but the current-voltage relationship remained linear over at least the range ±30 mv, at the highest concentration (6.2 ×

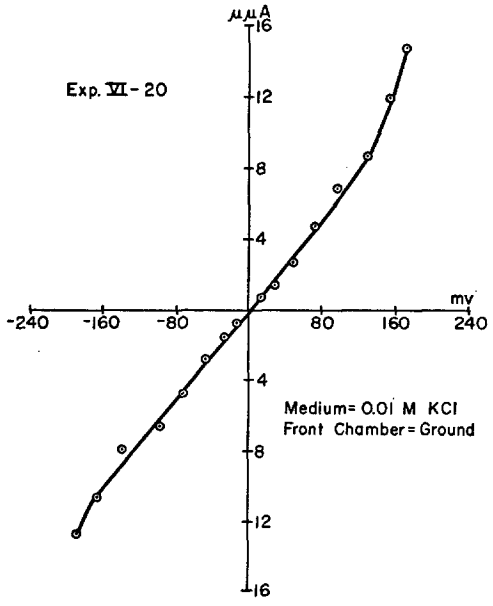

FIGURE 3. Current-voltage characteristics of thin lipid membranes. The membrane was formed in 0.01 M KCl. Experimental details are given under Methods.

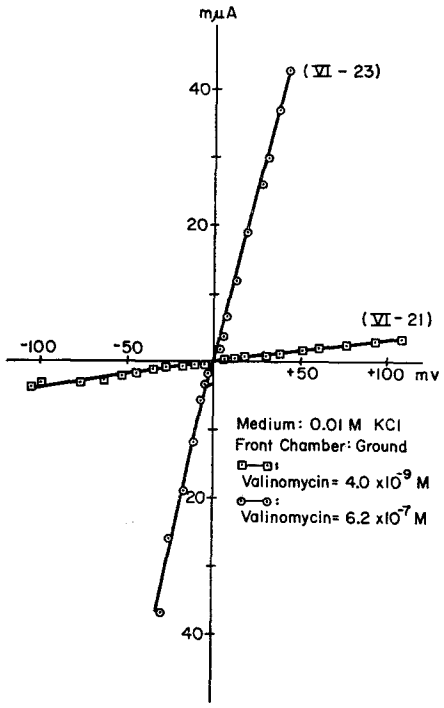

FIGURE 4. Current-voltage characteristics of thin lipid membranes in the presence of valinomycin. The membranes were formed in 0.01 M KCl and the indicated concentrations of valinomycin (⊡ = 4.0×10^{-9} M; ⊙ = 6.2×10^{-7} M) in 0.01 M KCl were flowed into the front chamber. Experimental details are described under Methods. The numbers in parentheses indicate the experiment.

10^{-7} M) of valinomycin tested (11). Membrane resistance values, computed from the slopes of the lines in Figs. 3 and 1, are presented in Table II. The results indicate that the fall in membrane resistance produced by valinomycin is dependent on the concentration of KCl in the medium over at least a hundredfold range of the antibiotic.

Ionic Selectivity

Further evidence that valinomycin produces a selective increase in the potassium permeability of these membranes is presented in Table III. When valino-

TABLE II

THE EFFECT OF KCl CONCENTRATION ON THE FALL IN MEMBRANE RESISTANCE PRODUCED BY VALINOMYCIN

The DC membrane resistances(R_m) in 0.01 M KCl were computed from the slopes of the lines in Fig. 4, and in 0.1 M KCl from the data shown in Fig. 1.

Experiment	Medium	Valinomycin	R_m
	M	M	ohm-cm²
VI-23	0.01 KCl	4×10^{-9}	5×10^{5}
VI-34	0.1 KCl	4×10^{-9}	5.6×10^{4}
VI-21	0.01 KCl	6.2×10^{-7}	8×10^{3}
VI-34	0.1 KCl	6.2×10^{-7}	6.3×10^{2}

mycin was absent, the cation transference numbers (T_{Na} or T_K) were approximately 0.85, but the membranes did not distinguish between sodium and potassium (1, 2). When valinomycin was added, the single salt potential for KCl was approximately equal to the equilibrium potential for potassium, and

TABLE III

THE EFFECT OF VALINOMYCIN ON SODIUM AND POTASSIUM PERMEABILITY OF THIN LIPID MEMBRANES

Ionic transference numbers for sodium and potassium were computed from the steady-state membrane potential (V_m) in the presence of single salt or biionic concentration gradients as previously described (2). The lipid preparations from which the membranes were formed were extracted from high potassium (HK) or low potassium (LK) sheep erythrocytes. Preparation HK-ac was treated with acetone (2) to remove cholesterol. When NaCl was the only solute in the media bathing the membrane, the indicated concentration of valinomycin was in the initial medium. Otherwise, valinomycin was added to the front chamber only. All solutions were unbuffered (pH ~ 5.6). Potential differences are expressed as those of the back chamber with respect to the front chamber at ground. Experimental details are described under Methods.

Experiment	Lipid preparation	Chamber medium: Rear	Chamber medium: Front	Valinomycin	V_m	T_{Na}	T_K
			Single salt potentials				
		M	M	M	mv		
VI-21	HK	0.01 KCl	0.1 KCl	0	+36	—	0.83
VI-21	HK	0.01 KCl	0.1 KCl	4×10^{-9}	+55	—	1.0
II-47-a	LK	0.1 KCl	0.01 KCl	0	−38	—	0.84
VII-48	LK	0.1 KCl	0.01 KCl	2.0×10^{-7}	−56	—	1.0
VI-27	HK	0.01 NaCl	0.1 NaCl	0	+39	0.85	—
VII-2	HK	0.01 NaCl	0.1 NaCl	2.1×10^{-7}	+39	0.84	—
			Biionic potentials				
VI-6	HK	0.001 KCl 0.1 NaCl	0.1 KCl 0.001 NaCl	0	+5	0.37	0.43
VI-6	HK	0.001 KCl 0.1 NaCl	0.1 KCl 0.001 NaCl	3.6×10^{-9}	+113	0	1.0
VI-2	HK-ac	0.001 KCl 0.1 NaCl	0.1 KCl 0.001 NaCl	4×10^{-8}	+107	0.02	0.98

T_K was one. In contrast, T_{Na}, computed from the single salt potential, was unaffected by the presence of valinomycin. A direct demonstration of the selective increase in the potassium, but not sodium, permeability of thin lipid membranes attributable to valinomycin is provided by the biionic potentials in Table III, where the ratio T_{Na}/T_K approaches zero. The increase in mem-

brane potassium permeability produced by valinomycin occurred when the membranes were formed from HK or LK sheep erythrocyte lipids, and was independent of the cholesterol content of the lipid extract (Table III). In computing the transference numbers in Table III, the contribution of protons or hydroxyl anions to the total membrane conductance was neglected (2). When valinomycin was present, T_K was one and this assumption was strictly valid.

In this context, the effect of a reduction in pH on the ionic selectivity of the membranes is of particular interest (Table IV). When the contributions of protons or hydroxyl anions to the total membrane conductance are specifi-

TABLE IV
THE EFFECT OF pH ON MEMBRANE POTENTIAL
Experimental details are given under Methods and in the text.

Experiment	Front chamber		Rear chamber		V_m	T_+	T_-
	Salt	pH	Salt				
	M		*M*	*ph*	*mv*		
VII-6	0.01 KCl	5.6	0.10 KCl	5.6	−43	0.97	0.03
VII-6	0.10 KCl	4.6	0.10 KCl	5.6	9		
VII-20	0.01 NaCl	5.7	0.1 NaCl	5.7	−44	0.96	0.04
VII-20	0.1 NaCl	4.7	0.1 NaCl	5.7	7		
VII-16	0.01 KCl	3.6	0.1 KCl	3.6	−14	0.72	0.28
VII-16	0.1 KCl	4.6	0.1 KCl	3.6	−11		
VII-24	0.01 NaCl	3.6	0.1 NaCl	3.6	−15	0.68	0.32
VII-24	0.01 NaCl	4.6	0.1 NaCl	3.6	−6		

cally considered, the expression (2) for membrane voltage (V_m) becomes:

$$V_M = T_H E_H + T_X E_X + T_{OH} E_{OH} + T_{Cl} E_{Cl} \qquad (1)$$

where T is the ionic transference number and E the equilibrium potential for a particular ion (in the experiments listed in Table IV, T_X and E_X refer to sodium or potassium). The pH of the solutions was adjusted to the indicated value in Table IV with HCl. When the pH of the solutions in both chambers was identical, equation (1) reduces to:

$$\frac{V_M}{E_X} = (T_X - T_{Cl}). \qquad (2)$$

Note that $E_X \cong E_{Cl}$, since the amount of HCl added to adjust pH was negligible with respect to total chloride content. When the salt concentration was

the same in both chambers, equation (1) reduces to:

$$\frac{V_M}{E_H} = (T_H - T_{OH}). \tag{3}$$

Furthermore,

$$1 = \sum_i T_i \tag{4}$$

Assuming that the transference numbers are independent of concentration, simultaneous solution of equations (1)–(3) yields

$$T_+ = (T_X + T_H) = \frac{1 + \frac{V_M}{E_X} = \frac{V_M}{E_H}}{2} \tag{5a}$$

and

$$T_- = (T_{OH} + T_{Cl}) = (1 - T_+). \tag{5b}$$

As illustrated in Table IV, the membranes were nearly anion-impermeable at low hydrogen ion concentration (pH $\cong$ 5.5). When the pH was reduced below 4.0, the contributions of anions to the total membrane conductance became substantial.

In order to compare directly the effects of valinomycin on membrane permeability to several cations, including protons (Table V), the experimental conditions were modified as follows. The pH of the initial medium was adjusted to 4.0, and the concentration of the salts was in the range 10^{-2} – 10^{-4} M. The expression (2) for membrane voltage (V_M) then becomes:

$$V_M = T_H E_H + T_X E_X + T_K E_K + T_{OH} E_{OH} + T_{Cl} E_{Cl} \tag{6}$$

where T is the ionic transference number and E the equilibrium potential for a particular ion in the experiments listed in Table V (T_X and E_X refer to the test cation which is compared with K^+ in a given experiment). In the concentration range 10^{-2} – 10^{-4} M, the activity coefficients for the salts used in Table V, at any particular concentration, are very nearly the same (15). Consequently, at the same concentration ratios for different salts, $E_K \cong E_X$.

When valinomycin was absent, the data in Table V were insufficient to permit calculation of the individual ionic transference numbers. However, the experiments provide at least a qualitative estimate of the relative ionic membrane permeability. As indicated by the biionic potentials (Table V), the membranes were more permeable to protons than to any of the alkali-metal cations, but distinguished only slightly among Rb^+, K^+, Cs^+, and Na^+. Com-

TABLE V

THE EFFECT OF VALINOMYCIN ON CATION PERMEABILITY OF THIN LIPID MEMBRANES

The pH of all solutions was adjusted to 4.0 with HCl. Valinomycin, in the indicated concentrations, was present in the initial medium in which the membrane was formed and in all subsequent replacement solutions. Ionic transference numbers (T_i) were computed from the steady-state membrane potential as described in the text. Experimental details are given under Methods.

Ionic concentration potentials

Rear chamber	KCl, 10^{-3} M ; KCl, 10^{-2} M XCl, 10^{-4} M ; XCl, 10^{-3} M pH = 4.0			Front chamber
Experiment	Test ion (X)	Valinomycin	V_m	T_X and T_K
		M	mv	
VII-55a	H	0	+24	—
VII-55b	H	10^{-7}	+50	—
VII-39	Rb	0	+30	—
VII-40	Rb	10^{-7}	+61	1
VII-41a	Cs	0	+11	—
VII-41b	Cs	10^{-7}	+56	1
VII-45a	Na	0	+10	—
VII-45b	Na	10^{-7}	+57	1

Biionic potentials

Rear chamber	KCl, 10^{-3} M ; KCl, 10^{-4} M XCl, 10^{-4} M ; XCl, 10^{-3} M pH = 4.0				Front chamber
Experiment	Test ion (X)	Valinomycin	V_m	T_K	T_X
		M	mv		
VII-55a	H	0	+20	—	—
VII-55b	H	10^{-7}	+25	—	—
VII-39	Rb	0	+6	—	—
VII-40	Rb	10^{-7}	+17	0.36	0.64
VII-41a	Cs	0	−2	—	—
VII-41b	Cs	10^{-7}	−8	0.57	0.43
VII-45a	Na	0	0	—	—
VII-45b	Na	10^{-7}	−49	0.92	0.08

parison of Tables III and V shows that in the absence of valinomycin, the membrane voltage produced by a tenfold KCl concentration ratio in the two solutions bathing the membrane, was less at pH 4.0 than at pH 5.6. It is unlikely that the fall in membrane voltage is attributable to the lower KCl concentrations used in the experiments described in Table V, since membrane voltage is linearly related to the logarithm of the ionic activity ratio on either side of the membrane in the concentration range 0.001–0.1

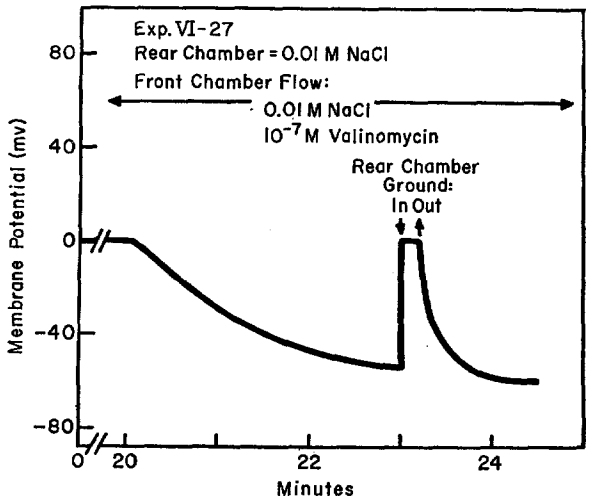

FIGURE 5. Development of membrane potential in the absence of ionic concentration gradients. The membrane was formed in 0.01 M NaCl. The identical solution containing 10^{-7} M valinomycin was flowed into the front chamber (flow rate = 6.0 ml min^{-1}). At the indicated points, the rear chamber was grounded, and the ground lead subsequently removed. Experimental details are described under Methods.

M (2). The effect was not referable to a competitive interaction between Na^+ and K^+, since a similar reduction in membrane voltage at low pH was observed when the medium bathing the membrane contained only a single salt (Table IV). Rather, the effect seemed to be due primarily to a pH-dependent reduction in cation permselectivity, as illustrated in Table IV.

TABLE VI
VALINOMYCIN-DEPENDENT MEMBRANE POTENTIALS IN THE ABSENCE OF SALT CONCENTRATION GRADIENTS

The membrane potential (V_m) and DC resistance (R_m) were measured as previously described. Valinomycin was added to one (front chamber) or both the solutions bathing the membrane, as described under Methods. The pH of the solution was approximately 5.6.

Experiment	Chamber medium		Valinomycin		V_m	R_m
	Rear chamber	Front chamber	Rear chamber	Front chamber		
	M	M	M	M	mv	ohm-cm²
VI-27	0.01 NaCl	0.01 NaCl	0	4.0×10^{-9}	−80	1.2×10^7
VI-30	0.1 NaCl	0.1 NaCl	0	8.0×10^{-9}	−15	—
VI-3	0.1 NaCl	0.1 NaCl	4×10^{-8}	4×10^{-8}	0	5×10^7
VI-23	0.01 KCl	0.01 KCl	0	6.2×10^{-7}	0	1.8×10^4
VI-26	0.1 KCl	0.1 KCl	0	6.2×10^{-7}	0	6.0×10^3
VIII-9e	0.01 KCl	0.01 KCl	10^{-7}	10^{-7}	0	5.3×10^4
VIII-9f	0.1 KCl	0.1 KCl	10^{-7}	10^{-7}	0	1.1×10^4

When valinomycin was present, the ionic concentration potentials (i.e., the potential in the presence of a tenfold concentration ratio for both test alkali metal chlorides) were approximately equal to E_K or E_X except when the test ion was H^+ (Table V). Hence, equation (6) for the ionic concentration potentials in the presence of valinomycin could be reduced to:

$$\frac{V_M}{E_K} = 1 = T_X + T_K \,. \tag{7}$$

Stated another way, T_{Cl} and T_{OH} were negligible, except when the test ion was H^+. When the test ion was Rb^+, Cs^+, or Na^+, the cation transference numbers could be computed from the biionic potentials. In agreement with the observations of others (10, 11), the order of cation selectivity for the thin lipid membranes in the presence of valinomycin was $H^+ > Rb^+ > K^+ > Cs^+ > Na^+$.

Membrane Potentials without Salt Gradients

An unexpected finding was the development of stable membrane potentials of considerable magnitude when the membranes were exposed to identical concentrations of NaCl, and valinomycin was added to one of the solutions bathing the membrane. A typical record of this phenomenon is shown in Fig. 5. The membrane was formed in 0.01 M NaCl. At 20 min the identical solution, but containing 10^{-7} M valinomycin, was introduced into the front chamber, and a membrane potential of approximately -60 mv developed. If the circuit was opened after grounding the rear chamber, the potential was reestablished, as would be expected if it represented a diffusion potential. In the absence of valinomycin, no membrane potentials are observed when the solution in the front chamber is changed unless the salt concentrations in the front and rear chambers are different (2). From Table VI, it is evident that the requisites for the establishment of membrane potentials in the absence of salt concentration gradients were an asymmetrical distribution (front chamber only) of valinomycin in the solutions bathing the membrane, and the presence of the relatively impermeable sodium, rather than the highly permeable potassium ion in the aqueous phase. Under these conditions, a stable and consistently negative membrane potential (front chamber = ground) could be demonstrated. However, the magnitude of the potential was variable (Table VI).

DISCUSSION

The data presented in this paper indicate that valinomycin radically alters the ionic selectivity properties of thin membranes prepared from sheep eryth-

rocyte lipids. Consequently, it seems appropriate to consider individually the possible mechanisms regulating ion penetration in these membranes in the presence and absence of valinomycin.

From energetic considerations, Haydon and Taylor (16) have suggested that the most favored structure for thin lipid membranes separating two aqueous phases is a flat, tightly packed, bimolecular leaflet. Experimentally, these same workers have shown that the capacitance and conductance of lecithin-in-decane membranes are almost entirely referable to the hydrocarbon region, and that the possibility of segregated fixed charges in this core is negligible (17). The electron microscopic appearance of thin lipid membranes is also compatible with a bilayer structure (18). Hence, it seems likely that the high electrical resistance of thin lipid membranes (1, 2, 4–6, 14) is largely attributable to the relatively restricted penetration of the polar ends of phospholipid molecules into the dielectric "core" of the membrane.

In this connection, it is interesting to note that Schwan[2] using an AC method, has recently found the specific resistivity of the sheep red cell lipid solutions in decane, which have been used to form thin artificial membranes in this laboratory, to be about 2×10^8 ohm-cm. A layer of such a solution which is 100 A thick would have a "membrane" resistance of 200 ohm-cm^2. The actual measured resistance of membranes made from these solutions is about 2×10^8 ohm-cm^2. Presumably, the polar groups of the phospholipids are free to carry current in a decane solution, but are restricted to the aqueous phases on the two sides of the membrane. Thus, the apparent specific resistivity of the lipid bilayer ($\sim 10^{14}$ ohm-cm) is closer to that of pure hydrocarbon (e.g. decane or the aliphatic chains of hydrocarbon) than to that of phospholipids dissolved in decane.

The cation-selective properties of thin sheep erythrocyte phospholipid membranes appear to be determined primarily by fixed negative charges on the surface of the membrane (2). When the data are calculated as in Table IV, it is evident that, at low hydrogen ion concentration, the membrane is nearly anion-impermeable. At the lower pH, the net negative charge on phosphatidyl ethanolamine and phosphatidyl serine is presumable reduced and, concomitantly, the cation selectivity of the membrane is diminished. These observations further support the conclusion (2) that the negatively charged membrane phospholipids regulate a "gating" (25) mechanism facilitating cation permeability. Hence, when valinomycin is absent, the experimental results (Tables I, III–V, Fig. 3, references 1 and 2) are quite consistent with a site-free model of membrane structure (19–21). In this mechanism the membrane is pictured operationally as being composed of an homogeneous, nonpolar core, the outer surfaces of which are lined with the polar ends of phos-

[2]H. P. Schwan. Personal communication.

pholipid molecules. The latter behave as ion exchangers or "gates" (25), which regulate the penetration of ions into the membrane.

The effects of valinomycin on the electrical resistance of these thin lipid membranes (Figs. 1 and 2; Table I) and on membrane potentials in the presence of ionic concentration gradients (Tables II, III, and V) are attributable to a striking increase in the permeability of these membranes to potassium, but not sodium. To a first approximation, the enhanced potassium permeability is independent of net fixed membrane charges, since the antibiotic has a similar effect on thin lipid membranes formed from uncharged lipids (10). There is a marked decrease in membrane resistance, directly related to the valinomycin concentration, when potassium, but not sodium, is present in the aqueous media (Fig. 1). Although valinomycin produced a slight decline in membrane resistance when the membrane was exposed to a sodium-containing solution, the effect was independent of the sodium concentration over at least a hundredfold range (Table I). In contrast, the fall in membrane resistance produced by a given concentration of valinomycin is directly dependent on KCl concentration (Fig. 2) over at least a hundredfold concentration range of the antibiotic (Table II). The sigmoid relation between potassium concentration and membrane resistance (Fig. 2) suggests the possibility that membrane resistance, in the presence of sodium and valinomycin, might be reduced to a greater degree if the sodium concentration were considerably elevated. Alternatively, it is possible that the slight decrease in membrane resistance in a sodium-valinomycin medium is referable to increased proton permeability (Table V).

It is remotely possible that the potassium-, rather than the sodium-dependent fall in the electrical resistance of the system produced by valinomycin is referable to an increase in the potassium conductance of the bulk phase torus surrounding the lipid membrane. In this case, the measured system resistance might not be that of the membrane, but that of the shunt pathway. However, this seems unlikely since valinomycin also increases the potassium permeability of other membrane systems (9, 13) where no bulk phase torus is present. Hopefully, studies on the effect of valinomycin on the bulk conductance of lipid systems, currently in progress in our laboratory, will provide a more definitive answer to this problem.

A variety of experimental procedures can lower the electrical resistance of thin lipid membranes separating two aqueous phases. These include variations in surface area (2, 22); addition of chloroform-methanol to the solution from which the lipids are formed (6); exposure of the membranes to unidentified, water-soluble molecules, thought to be proteinaceous in nature (5, 23, 24), anionic and cationic detergents (25), or egg albumin (26); and, the occurrence of enzyme-substrate (27) or specific antigen-antibody (27, 28) reactions in the solutions bathing the membrane. Exposure of thin lipid mem-

branes to iodide-containing solutions will also lower their electrical resistance, possibly by facilitating an electronic, rather than an ionic mode of conduction (29). However, in contrast to the observed effects with valinomycin (10–12), none of these modifications allows this type of membrane to distinguish between sodium and potassium.

Corey-Pauling models of valinomycin (10, 12) indicate that the diameter of the central hole in the compound is approximately 6–7 A when it is in its most open conformation. When the molecule is in this shape, most of the functional groups (carbonyl oxygens, ester oxygens, and peptide nitrogens) are directed toward the aperture of the ring. Hence, it is reasonable to suppose that the interactions between cations and valinomycin might involve hydrogen bonding between carbonyl oxygens in the ring and the hydration shell of appropriately sized cations (10, 12). The results of the experiments shown in Table IV, in agreement with the observations of others (10, 11), indicate that the order of cation selectivity of thin lipid membranes exposed to valinomycin is $H^+ > Rb^+ > K^+ > Cs^+ > Na^+$. The hydrated diameters of the permeable cations rubidium, cesium, and potassium, estimated by a method described by Robinson and Stokes (30), are 5 A or less, while that of the impermeable sodium ion is 6.5 A (13), suggesting that the critical ionic diameter limiting the interaction between cations and valinomycin is in the range 5–6 A.

It is possible that valinomycin might form hydrogen-bonding pores in thin lipid membranes, which mediate the penetration of protons, rubidium, potassium, and cesium, but not sodium. In this case, the charge-carrying element crossing the membrane would be the cation itself. According to this hypothesis, any pores formed by valinomycin would be sufficiently small, probably less than 8 A in diameter, to exclude hydrated sodium ions. Mullins has suggested that the cation selectivity of membranes may be determined by "small" pores (less than 20 A diameter) which facilitate permeation of those ions which fit the pore closely, and consequently can replace water from their hydration shell with solvation from the pore (31). Stein and Danielli postulated the presence of hydrogen-bonding polar pores extending through the thickness of the membrane as a possible mechanism for the diffusion of sugars into red blood cells (32). However, it is also possible that the charged species crossing the membrane is a complex between valinomycin and a permeable cation (11). The experimental data presented here and by others (10, 11) are insufficient to distinguish between a narrow pore (in the sense described above) or a carrier mechanism (33), or a combination of the two, for the effect of valinomycin on the ionic permeability of these membranes and sheep red blood cells (12, 13).

The development of stable, large membrane voltages (Table VI, Fig. 5) when valinomycin was distributed asymmetrically in the solution bathing

the membrane, and the concentration of the impermeable sodium ion was the same on either side of the membrane, was a surprising finding. With experimental conditions used thus far in this laboratory, ((2); Methods), it is the only situation in which significant membrane voltages have been observed in the presence of identical salt solutions on either side of the membrane. The side of the membrane opposite to the solution containing valinomycin invariably becomes negative. If this membrane voltage is a diffusion potential, it is necessary to postulate the presence of an anion concentration gradient directed away from the phase containing valinomycin or a cation concentration gradient directed toward the solution containing the antibiotic. Further work is obviously necessary before this interesting phenomenon can be adequately explained.

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REFERENCES

1. ANDREOLI, T. E. 1966. Formation and properties of thin lipid membranes from sheep red cell lipids. *Science.* **154**:417. (Abstr).
2. ANDREOLI, T. E., J. A. BANGHAM, and D. C. TOSTESON. 1967. The formation and properties of thin lipid membranes from HK and LK sheep red cell lipids. *J. Gen. Physiol.* **50**:1729.
3. EVANS, J. V. 1954. Electrolyte concentrations in red blood cells in British breeds of sheep. *Nature.* **174**:931.
4. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1964. An investigation by electrical methods of lecithin-in-hydrocarbon films in aqueous solutions. *Proc. Roy. Soc. (London), Ser. A,* **281**:377.
5. MUELLER, P., D. O. RUDIN, H, TITIEN, and W. C. WESTCOTT. 1962. Reconstitution of excitable cell membrane structure in vitro. *Circulation.* **26**:1167.
6. HUANG, C., L. WHEELDON, and T. E. THOMPSON. 1964. The properties of lipid bi-layer membranes separating two aqueous phases: Formation of a membrane of simple composition. *J. Mol. Biol.* **8**:148.
7. DE GIER, J., and L. L. M. VAN DEENEN. 1961. Some lipid characteristics of red cell membranes of various animal species. *Biochim. Biophys. Acta.* **49**:286.
8. BANGHAM, A. D., M. M. STANDISH, and J. C. WATKINS. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13**:238.
9. CHAPPELL, J. B., and A. R. CROFTS. 1966. Ion transport and reversible volume changes of isolated mitochondria. *In* Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater. editors. *Biochim. Biophys. Acta Library.* Elsevier Publishing Co., Amsterdam. **7**:293.
10. LEV, A. A., and E. P. BUZHINSKY. 1967. Cation specificity of the model bimolecular phospholipid membranes with incorporated valinomycin. *Cytology* (U.S.S.R.). **9**:102.
11. MUELLER, P., and D. O. RUDIN. 1967. Development of $K^+ - Na^+$ discrimination

in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398.

12. ANDREOLI, T. E., P. COOK, and D. C. TOSTESON. 1967. Valinomycin—a molecular sieve for cations? Abstracts of the Biophysical Society 11th Annual Meeting. Houston, Texas. 9.
13. TOSTESON, D. C., P. COOK, T. E. ANDREOLI, and M. TIEFFENBERG. 1967. The effect of valinomycin on potassium and sodium permeability of HK and LK sheep red cells. *J. Gen. Physiol.* **50**:2513.
14. VAN DEN BERG, H. J. 1965. A new technique for obtaining thin lipid films separating two aqueous media. *J. Mol. Biol.* **12**:290.
15. LATIMER, W. M. 1952. The Oxidation States of the Elements and Their Potentials in Aqueous Solutions. Englewood Cliffs, New Jersey, Prentice-Hall, Inc. 2nd edition. 354.
16. HAYDON, D. A., and J. TAYLOR. 1963. Stability and properties of bimolecular lipid leaflets in aqueous solutions. *J. Theoret. Biol.* **4**:281.
17. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1965. Polar group orientation and the electrical properties of lecithin bimolecular leaflets. *J. Theoret. Biol.* **9**:278.
18. HENN, F. A., G. L. DECKER, J. W. GREENAWALT, and T. E. THOMPSON. 1967. Electron microscopic study of lipid bilayer membranes. Abstracts of 11th Annual Meeting. Houston, Texas. 63.
19. TEORELL, T. 1953. Transport processes and electrical phenomena in ionic membranes. *Progr. Biophys. Biophys. Chem.* **3**:305.
20. GOLDMAN, D. E. 1964. A molecular structural basis for the excitation properties of axons. *Biophys. J.* **4**:167.
21. EISENMAN, G., J. B. SANDBLOM, and J. L. WALKER, JR. 1967. Membrane structure and ion permeation. *Science.* **155**:965.
22. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1965. The variation of capacitance and conductance of bimolecular lipid membranes with area. *J. Theoret. Biol.* **9**:433.
23. MUELLER, P., and D. O. RUDIN. 1963. Induced excitability in reconstituted cell membrane structure. *J. Theoret. Biol.* **4**:268.
24. MUELLER, P., and D. O. RUDIN. 1967. Action potential phenomena in experimental bimolecular lipid membranes. *Nature.* **213**: 603.
25. SEUFERT, W. D. 1965. Induced permeability changes in reconstituted cell membrane structure. *Nature.* **207**:174.
26. TSOFINA, L. M., E. A. LIBERMAN, and A. V. BABAKOV. 1966. Production of bimolecular protein-lipid membranes in aqueous solution. *Nature.* **212**:681.
27. DEL CASTILLO, J., A. RODRIGUEZ, C. A. ROMERO, and V. SANCHEZ. 1966. Lipid films as transducers for detection of antigen-antibody and enzyme-substrate reactions. *Science.* **153**:185.
28. BARFORT, P. 1966. A versatile antigen-antibody indicator. *Proc. 19th Ann. Conf. Eng. Biol. Med.* 207. (Abstr.)
29. LÄUGER, P., W. LESSLAUER, E. MARTI, and J. RICHTER. 1967. Electrical properties of bimolecular phospholipid membranes. *Biochim. Biophys. Acta.* **135**:20.

30. ROBINSON, R. A., and R. H. STOKES. 1955. Electrolyte Solutions. London, Butterworth & Co. (Publishers) Ltd. 121.
31. MULLINS, L. J. 1959. The penetration of some cations into muscle. *J. Gen. Physiol.* **42**:817.
32. STEIN, W. D., and J. F. DANIELLI. 1956. Structure and function in red cell permeability. *Discussions Faraday Soc.* **21**:238.
33. WILBRANDT, W., and T. ROSENBERG. 1961. The concept of carrier transport and its corollaries in pharmacology. *Pharmacol. Rev.* **13**:109.