

The Effects of Macrocyclic Compounds on Cation Transport in Sheep Red Cells and Thin and Thick Lipid Membranes

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INTRODUCTION

For some years, work in this laboratory has been directed toward elucidation of the mechanism of selectivity of high-potassium (HK) and low-potassium (LK) sheep red cell membranes for Na^+ and K^+ (1-4). One recent approach has been to compare the ionic permeability of the intact cell membranes with thin artificial bilayer membranes prepared from lipids extracted from these cells (5). It was found that the thin lipid membranes have ionic permeability properties which are vastly different from those of intact HK and LK sheep red cell membranes. For example, the DC electrical resistance of the bilayers is about 2×10^8 ohm cm^2 , while that of the red cell membranes is estimated to be from 10 to 100 ohm cm^2 . Furthermore, the bilayers are more permeable to both Na^+ and K^+ than they are to Cl^- , while the ratio of Cl^- to K^+ or Na^+ permeability of red cells is of the order of 10^6 (6). Finally, the thin lipid membranes do not distinguish between Na^+ and K^+ , and the ionic transport properties of such membranes prepared from HK and LK sheep red cell lipids are identical. The intact cell membranes do distinguish between these alkali metal ions, and, furthermore, the Na^+ and K^+ transport properties of the two genetic types of sheep red cells are significantly different. Apparently, the thin lipid membranes lack essential components which are responsible for the distinctive cation permeability properties of the intact cell membranes.

In the course of continuing attempts to identify such components, we became aware of reports of the effect of the macrocyclic depsipeptide antibiotic, valinomycin, on the ionic permeability of lecithin (7) and mixed brain lipid (8) bilayer membranes. This compound had previously been reported to stimulate the respiration of mitochondria in the presence of K^+ but not in the presence of Na^+ (9). Valinomycin was found to produce specific permeability of intact HK and LK sheep red cell membranes, as well as thin artificial membranes prepared from lipids extracted from these cells, to K^+ but not to Na^+ (10, 11). In the presence of K^+ (10^{-4} M) and valinomycin (5×10^{-7} M), the electrical resistance of the lipid bilayer membranes is about 10^8 ohm cm^2 , which is in the range of biological membranes.

The significance of these observations derives from the unusual structure of valino-

mycin (L-lactate—L-valine—D-hydroxyvalerate—D-valine)₃. All amino nitrogens (of valine) and hydroxyl oxygens (of lactate and hydroxy valerate) are in peptide or ester linkage, respectively. All side chains consist entirely of saturated hydrocarbon. The compound is uncharged and almost insoluble in water. In hydrophobic environments, e.g., lipid membranes, it is likely that the molecule is maintained in an open conformation due to interactions between the side chains and the adjacent aliphatic hydrocarbon. Under such circumstances, the diameter of the central hole is between 6 and 7 Å. It is reasonable to suspect that valinomycin can substitute for water in the hydration shell of appropriately sized cations (8, 10). The smaller size of the hydrated K⁺ may permit it to enter the hole in valinomycin more readily than the larger hydrated Na⁺. The experiments reported in this paper were directed toward evaluation of this and other hypotheses regarding the mechanism by which macrocyclic compounds modify the alkali metal cation permeability of sheep red cell and artificial thin lipid membranes. Two complementary lines of investigation will be described: first, the relation between molecular structure and Na⁺-K⁺ selectivity of macrocyclic compounds,¹ and, second, the interactions of active compounds, valinomycin² and nonactin¹ with bulk lipid phases and thick lipid membranes.

RELATION BETWEEN MOLECULAR STRUCTURE AND Na⁺-K⁺ SELECTIVITY OF MACROCYCLIC COMPOUNDS

The effect of various natural and synthetic macrocyclic compounds on the Na⁺ and K⁺ permeability of HK and LK sheep red cells was measured as previously described (10). HK cells were suspended in a low-K medium while LK cells were incubated in a high-K medium in the presence of the test compound. All incubations of red cells were carried out at 37°C and pH 7.4. The action of these compounds on thin lipid membranes was evaluated by measuring the membrane resistance and potential when the two bathing solutions were as follows: (a) KCl (10⁻¹ M), NaCl (10⁻² M):KCl (10⁻¹ M), NaCl (10⁻² M); (b) KCl (10⁻² M), NaCl (10⁻¹ M):KCl (10⁻¹ M), NaCl (10⁻² M); (c) KCl (10⁻² M), NaCl (10⁻³ M):KCl (10⁻¹ M), NaCl (10⁻² M). All such measurements were made at room temperature (about 22°C) in unbuffered solutions at pH 5.6.

Table I summarizes results obtained with compounds which were observed to have some activity toward Na⁺-K⁺ transport across sheep red cell and thin lipid membranes. Table II presents some characteristics of these molecules and of related substances which were found not to display such activity. The only compounds which simulated in most details the action of valinomycin were the macrocyclic polyester nonactin and a mixture of its methylated derivatives monactin and dinactin (12). One of the linear hexapeptides tested (Merck L-606, 470-1-1) showed comparable selectivity for K⁺ over Na⁺ but was considerably less potent in reducing the resistance of the membranes. Gramicidin B was observed to decrease membrane resistance dramatically but to show relatively little selectivity for K⁺ over Na⁺. All other compounds listed in Table II failed to alter the rate or selectivity of Na⁺ and K⁺ transport in the assay systems used in these experiments.

¹ Tieffenberg, M., P. Cook, and D. C. Tosteson. Paper in preparation.

² Andreoli, T. E., and D. C. Tosteson. Paper in preparation.

It is instructive to compare the molecular structure of valinomycin and nonactin (and its methylated derivatives), since the two compounds have such similar actions on K^+ and Na^+ permeability. The nonactin series, like valinomycin, are uncharged,

TABLE I
EFFECT OF ACTIVE COMPOUNDS ON K^+ AND Na^+ PERMEABILITY OF HK AND LK SHEEP RED CELLS AND THIN LIPID MEMBRANES

Compound	Conc.	Red cells				Bilayers	
		LK		HK		Resistance in KCl (0.1 M)	$\frac{T_{Na}}{T_K}$
		ΔK	ΔNa	ΔK	ΔNa		
	<i>M</i>	<i>mM (original 1. cells)</i>		<i>mM (original 1. cells)</i>		<i>ohm cm²</i>	
Valinomycin	10^{-7}	+28	-2	-37	+1	5×10^3	<0.01
Nonactin (Squibb NC-1-84-R3)	10^{-7}					2×10^6	<0.01
	10^{-6}	+22	+5	-45	-1		
Monactin (70%)–dinactin (30%) (Squibb EJB-V-14A)	10^{-7}	+18	-4	-19	-1	4×10^4	<0.01
Linear hexapeptides (Merck L-606, 470-1-1)	10^{-5}					5×10^6	<0.01
	10^{-4}	0	+2	0	-5		
Gramicidin B	10^{-7}	+41	-57	-84	+73		
	10^{-6}					3×10^2	0.4
Nigericin	3×10^{-8}	+21	-6	-36	+18		
	10^{-6}					5×10^6	<0.1

The effects of the compounds on red cell permeability to cations are expressed as the difference between the Na^+ and K^+ content of cells exposed to the test substance and the Na^+ and K^+ content of control cells incubated for the same length of time (usually 2–3 hr for HK and 1–2 hr for LK cells) in the absence of the compound. HK cells were incubated in a medium containing Na^+ (165 mM), K^+ (5 mM) while LK cells were in a medium containing Na^+ (20 mM), K^+ (150 mM). More prolonged incubation of LK cells in a high-K medium in the presence of an active compound produced excessive colloid osmotic lysis. The changes in cell cation content (ΔK and ΔNa) are expressed as millimoles per that number of cells which at the start of the experiment occupied a volume of 1 liter. The term $T_{Na/K}T$ is the ratio of the transference number for Na^+ to that for K^+ . The compounds studied were obtained from the following sources: valinomycin from J. C. McDonald, Prairie Regional Laboratories, Saskatoon, Saskatchewan, Canada; nonactin, monactin–dinactin from E. Dutcher, Squibb Institute for Medical Research, New Brunswick, N.J.; valinomycin and linear hexapeptides from R. Denkwalter and F. Holly, Merck, Sharp and Dohme Research Laboratories, Rahway, N. J.; gramicidin B from L. C. Craig, The Rockefeller University.

are poorly soluble in water, have exclusively aliphatic side groups, and contain different optical isomers of the monomers. They differ from valinomycin in that they have 32 instead of 36 ring atoms, contain no nitrogen and thus have no peptide bonds, and have only 4 instead of 12 carbonyl groups, all in ester linkage. Corey–Pauling models of these compounds reveal the hole in the center of the molecule to be

TABLE II
SOME PROPERTIES OF MACROCYCLIC COMPOUNDS TESTED FOR
CATION SELECTIVITY

Compound	Maximum conc. tested	Approx. mol wt	Ring atoms	Ester bonds	Peptide bonds	Free —NH ₂	Free —COOH	Free —OH	Free —N—H
<i>M</i>									
Compounds with high selectivity for K ⁺ over Na ⁺									
Valinomycin		1111	36	6	6	0	0	0	0
Nonactin		750	32	4	0	0	0	0	0
Monactin (70%)— dinactin (30%)		765-780	32	4	0	0	0	0	0
Linear hexapeptides (Merck L-606, 470-1-1)		650	0	0	5	2-3	0-1	0-1	0-1
Compounds with moderate to low selectivity for K ⁺ over Na ⁺									
Gramicidin A		2000	0	0	16	0	0	1	4
Gramicidin B		2000	0	0	16	0	0	1	3
Gramicidin C		2000	0	0	16	0	0	2	3
Nigericin		713							
Inactive cyclic polypeptide antibiotics and toxins									
Gramicidin S	10 ⁻⁵	1240	30	0	10	2	0	0	0
Viridogrisein	10 ⁻⁵	850	22	1	7	0	0	2	0
Vernamycin	10 ⁻⁵	867	19	1	6	1	0	1	0
Polymixin B	10 ⁻⁵	1220	21-26	0	11	5	0	2	0
Bacitracin A	10 ⁻⁵	1411	23	0	6	2	1	0	0
			30	0	9	1	1	0	0
Subtilin	10 ⁻⁵	3300	15-18	0		4	2	0	1
Streptogramin	10 ⁻⁵	500							
γ Amanitin	10 ⁻⁴	1072							
Phalloidin	10 ⁻⁴	890	15	0	3	0	0	1	0
			25	0	4	0	0	3	1
Inactive synthetic peptides									
L-606, 518-1-1	10 ⁻⁴	650	0	0	5				
L-607, 237-1-1	10 ⁻⁴	650	0	0	5	2-3	0-2	0-1	0
L-607, 237-1-1	10 ⁻⁴	650	0	0	5	2-3	1-2	0	0
L-607, 238-1-1	10 ⁻⁴	650	0	0	5	2-3	1-2	0	0
L-608, 967-1-1	10 ⁻⁴	1300	0	0	11	1	0	0	0
L-607, 933-0-2	10 ⁻⁴	650	18	0	6	0	0	0	0
Cyclic hexapeptide	10 ⁻⁵	600	20	0	5	1	1	0	0

The compounds tested were obtained from the following sources: viridogrisein, polymixin B, bacitracin A, subtilin, and synthetic peptides from R. Denkwalter and F. Holly, Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.; streptogramin and vernamycin from E. Dutcher, Squibb Institute for Medical Research, New Brunswick, N.J.; the cyclic hexapeptide from K. Brendel, Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N.C.; amanitin and phalloidin from Professor T. Wieland, University of Frankfurt, Frankfurt (am Main), Germany. The sources of other compounds are cited in the note to Table I. Blank spaces in the table indicate that we were unable to obtain the information about that particular compound.

slightly (0.5–1.0 Å) smaller in diameter than is the case in valinomycin when both molecules are in their most open conformation. These similarities and differences in structure and action between valinomycin and the nonactin series establish that the presence of peptide bonds is not necessary for high selectivity of K^+ over Na^+ . Furthermore, small differences in the diameter of the ring in the range between 5.5 and 7.5 Å are not of crucial importance in determining this property of the molecules.

The fact that none of the other cyclic compounds tested, e.g. vernamycin, gramicidin S, etc., produced either increased permeability or selectivity for K^+ of Na^+ may be related to the smaller ring sizes (19–30 ring atoms) of these molecules. However, other factors such as lipid solubility, the presence of charged and/or hydrophilic side groups, etc. must also be considered and evaluated before this conclusion can be accepted.

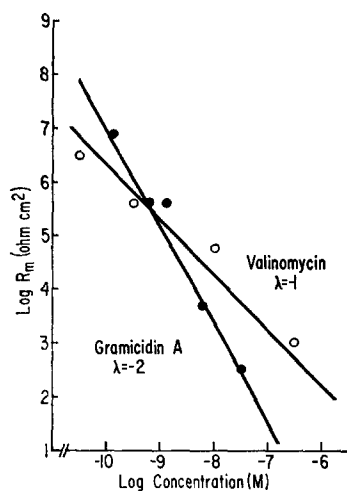


FIGURE 1. Relation between concentration of gramicidin and valinomycin and electrical resistance of thin lipid membranes. R_m , membrane resistance; λ , slope.

The results with the one active set of synthetic linear hexapeptides (L-606, 470-1-1) are difficult to interpret at present. They were found to be inactive on red cells but to produce substantial increases both in conductance and particularly K^+ - Na^+ selectivity in bilayers. The active preparation of linear hexapeptides contains 48 different sequences of the amino acids Ileu, Pro, Glu, Leu, Ala, Gly, Val, Tyr, Try, Ser, Gln. All the hexapeptides contained carboxy-terminal amides. It was the only preparation of linear hexapeptides tested (including some containing amides) which showed any activity on red cells or bilayers. It is possible that these compounds could form macrocyclic rings by interactions of the terminal amino and amide groups with negative groups on the membrane surface or interaction between two molecules of the hexapeptides to form dimers. Such dimerization has been shown to occur with gramicidin A, which is also a linear peptide (13). The data shown in Fig. 1 also support the idea that gramicidin A acts on thin lipid membranes as a dimer. The slope of the relation between log membrane resistance and log gramicidin A concentration is about -2 in contrast to the slope of -1 for the analogous relation with valinomycin (12).

INTERACTIONS OF MACROCYCLIC COMPOUNDS WITH
BULK LIPID PHASES AND THICK LIPID MEMBRANES

In order to characterize the effect of macrocyclic compounds on the electrical conductance of thin lipid membranes, it is necessary to know the concentration of the compound in the bilayer. Because of the small volume of the bilayer itself (about 10^{-8} ml), direct measurements of the concentration are technically difficult. We have attempted to estimate the concentration of valinomycin in such thin lipid membranes indirectly by measuring the partition coefficient of the compound between aqueous solutions of KCl (0.1 M) on the one hand, and decane alone or decane containing sheep red cell lipids on the other.² The results of such measurements in the KCl: pure decane system are shown in Table III. 15 ml of a KCl solution (0.1 M) containing valinomycin (1.25×10^{-6} M) was equilibrated by vigorous mixing with a small volume

TABLE III
PARTITION COEFFICIENT OF VALINOMYCIN
BETWEEN 0.1 M KCl AND DECANE

No. of measurements	Volume	0.1 M KCl		Decane		Partition coefficient	
		(Valinomycin)		Volume range	(Valinomycin) range	Mean	SD
		Initial	Final range				
	<i>ml</i>	10^{-4} M	10^{-4} M	<i>ml</i>	10^{-4} M		
10	15	1.25	1.7-9.5	1.0-2.5	7.5-19	3400	± 500

(1.0-2.5 ml) of pure *n*-decane. The phases were separated by centrifugation, and the concentration of valinomycin remaining in the aqueous phase was estimated by measuring the electrical resistance of a bilayer membrane formed in the presence of this solution. The electrical resistances of standard membranes formed on the same day in KCl (0.1 M) containing known concentrations of valinomycin were also measured and the concentrations of the compound in the unknown solutions were computed by interpolation. The partition coefficient of valinomycin between decane and KCl (0.1 M) was found to be 3400 ± 500 . The value was the same when the aqueous phase was NaCl (0.1 M) or pure H₂O.

Assuming that this partition coefficient between KCl (0.1 M) and decane also describes the relation between the concentrations of valinomycin in KCl (0.1 M) and a thin lipid bilayer, it is possible to compute that about 2×10^{11} valinomycin molecules are present in 1 cm² of such a membrane when the concentration of the compound in the aqueous phase is 10^{-7} M. If these molecules were uniformly distributed over the membrane area, there would be one valinomycin molecule in each square of membrane surface 200 Å on a side, or about 2000 molecules per μ^2 . The electrical resistance of a thin lipid membrane in the presence of valinomycin (10^{-7} M) and KCl (0.1 M) is about 10^8 ohm cm². By comparison, it has been estimated that there are no more than 13 tetrodotoxin-sensitive sodium channels per μ^2 of membrane surface in lobster axons

which have a resting resistance of about 10^8 ohm cm^2 and a very much lower active resistance (18). Apparently, the conductance per channel in the active lobster axon membrane is at least two and probably more than three orders of magnitude greater than the conductance per valinomycin molecule in thin lipid bilayer membranes.

In order to understand the mechanism by which macrocyclic compounds selectively reduce membrane resistance to monovalent cations, it is important to know whether these agents act only on lipid bilayers or on bulk lipid phases as well. To explore this question, we have formed thick lipid membranes of pure decane and decane containing various concentrations of sheep red cell lipids and macrocyclic compounds. The electrical properties of these membranes were evaluated in chambers of the type previously described (5). The membranes were about 0.7 cm^2 in area and between 1 and 15 mm thick. The interfaces of these thick lipid membranes with the two aqueous

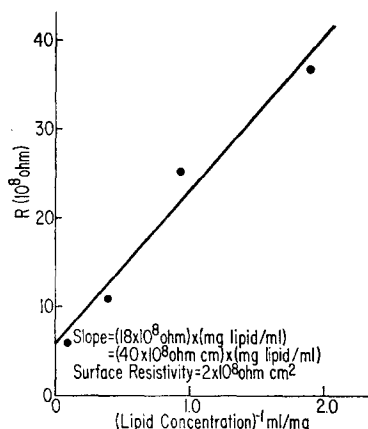


FIGURE 2. Effect of lipid concentration on electrical resistance of thick lipid membranes. R , resistance.

bathing solutions were formed by cellophane dialysis tubing membranes. The electrical resistance of such a membrane 3 mm thick is shown in Fig. 2. The electrical resistance is plotted on the ordinate as a function of the reciprocal of the concentration of red cell lipids in decane on the abscissa. The specific resistivity of pure decane in this system is $> 10^{16}$ ohm cm . The resistance falls greatly upon addition of a small concentration (< 0.5 mg/ml) of red cell lipids. At higher lipid concentrations, the relation between resistance (R) and lipid concentration (C_L) is described by the equation

$$R = \frac{\lambda}{C_L} + 2R_s \quad (1)$$

where R_s is a constant. For the experiment shown in Fig. 1, λ is about 40×10^8 (ohm $\text{cm}) \times (\text{mg lipid per ml})$, and R_s , computed from the zero intercept at infinite lipid concentration, is 7×10^8 ohm cm^2 . It is interesting to note that this value is about the same as the measured DC resistance of bilayer membranes made from lipids dissolved in decane (5, 14).

In order to test the hypothesis that the resistance of thick lipid membranes is determined, in part, by limiting monolayer resistances of about 10^8 ohm cm^2 , the resistances of lipid membranes of different thicknesses were measured. The results of two such experiments with membranes containing different concentrations of lipid are shown in Fig. 3. Both the curves can be described by the equation

$$R = \frac{R_b L}{A} + \frac{2R_s}{A} \quad (2)$$

L is the thickness and A the area of the thick membrane. The value of R_b is approximately reciprocally related to the concentration of lipid, whereas the value of R_s is relatively independent of this parameter. The values of R_s (which may be thought of as the resistance of one lipid-water interface) computed from these data agree quite well with that derived from equation 1 and the data in Fig. 2 as well as with

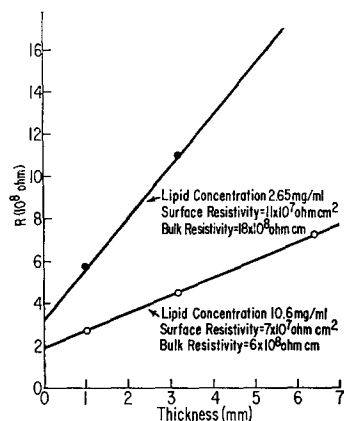


FIGURE 3. Effect of thickness on electrical resistance of thick lipid membranes. R , resistance.

the measured DC resistance of bilayer membranes. Thus, at least with regard to electrical resistance, the properties of bilayer membranes can be accounted for as the sum of two aqueous-lipid interfaces separated by a considerable, and to some extent arbitrary, thickness of lipid. The distinctive resistance of the bilayer is apparently referable to the molecular arrangements in each monolayer, presumably the energetic restrictions on the movement of both polar and aliphatic parts of the phospholipid molecules.

The effect of the macrocyclic polyesters monactin and dinactin on the DC electrical resistance of thick lipid membranes is shown in Fig. 4. The preparation used in these experiments contained monactin (70%) and dinactin (30%). The two curves shown in Fig. 4 were obtained with sheep red cell lipids dissolved in decane (30 mg/ml) with and without mon-dinactin (10^{-4} M) dissolved directly in the lipids. Note that the macrocyclic compound markedly decreases the zero intercept. Following the interpretation of Fig. 3, it is reasonable to conclude that this effect is due to a reduction in the electrical resistance of the interface between the lipid and aqueous phases. It should be noted that the accuracy of the estimate of R_s in this case is poor because the

intercept is small compared to the slope of the relation between resistance and thickness. R_s could have any value less than about 10^6 ohm cm^2 . Estimates of the slope of the curve describing the experiments with red cell lipids alone are unreliable owing to the scatter in the data. The length of the lines intersecting the upper curve represents the range of individual measurements in the experiment. This scatter may be related to the extreme sensitivity of the resistance to small changes in lipid concentration.

Fig. 5 shows the ionic selectivity of a lipid membrane 3 mm thick made of sheep red cell lipids dissolved in decane (30 mg/ml) containing mon-dinactin (10^{-4} M). The solution in the rear chamber contained KCl (0.1 M) and NaCl (0.01 M). The potential difference of the rear chamber with respect to the front chamber is plotted on the ordinate as a function of the concentrations of KCl and NaCl in the front chamber

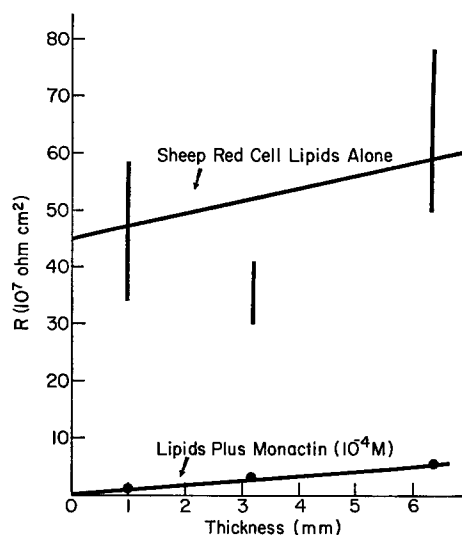


FIGURE 4. Effect of mon-dinactin on electrical resistance of thick lipid membranes. R , resistance.

plotted on the abscissa. The ionic strength of the solutions in the front chamber was maintained equal to that in the rear chamber by substitution of NaCl for KCl. Note that the potential difference across these thick lipid membranes is dominated by K^+ over the concentration range 10^{-1} – 10^{-4} M. The reason for the fact that the slope of the line relating $\log \text{K}^+$ concentration (in the range 10^{-2} – 10^{-4} M) to membrane voltage is 43 mv with these thick lipid membranes containing mon-dinactin, whereas it is indistinguishable from the theoretical value for a K^+ electrode in bilayer membranes exposed to these compounds, remains to be elucidated.

On the basis of these and other observations, we propose the following picture of the ionic permeability of thin and thick lipid membranes. In the absence of macrocyclic compounds, the high electrical resistance of bilayer membranes is referable to the properties of the monolayers of phospholipid on each surface. It appears to make relatively little difference whether the hydrophobic phase between the monolayers is thin, e.g. 30–50 Å as in bilayers, or thick, e.g. 1–10 mm as in the thick lipid membranes described above. This high resistance is partly due to restriction of movement

of the phospholipid molecules secondary to interactions of the polar heads with water and ions in the aqueous phase and of the aliphatic chains with each other in the interior of the membrane, i.e., to the forces responsible for the lamellar structure. It is also due to the fact that ions which do penetrate into the membrane interior, e.g. KCl or NaCl directly from the aqueous phase or phosphoryl groups of phospholipids with gegenions such as K^+ or Na^+ , do so mainly as ion pairs because of the low dielectric constant in this region. The macrocyclic compounds, because of the highly polarizable carbonyl groups in the ester and, in the case of valinomycin, also peptide bonds, serve to raise the dielectric constant in the immediate vicinity of monovalent cations like K^+ which can fit into the central hole. In this sense, these compounds appear to provide K^+ with a "hydration shell" which, like water, has a high dielectric constant but, unlike water,

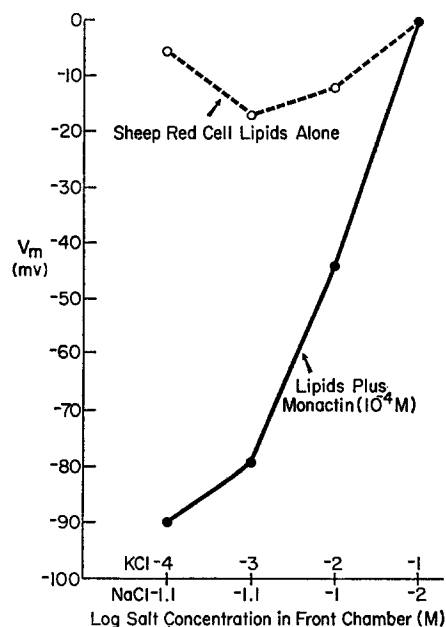


FIGURE 5. Effect of potassium concentration on electrical potential difference across thick lipid membranes. For ordinate and abscissa, see text. V_m , membrane voltage.

is readily soluble in the hydrophobic regions of the membrane. This effect permits more complete dissociation and independent movement of cations and anions and thus a lower electrical resistance in the membrane interior. The selectivity of the macrocyclic compounds for cations compared to anions presumably derives from the anionic character of the carbonyl oxygens. The selectivity of these agents for monovalent cations could be mainly steric, as suggested above. It may also relate to the relative field strengths of the ions. If the field strength is too high, the complex of macrocyclic compound and cation may itself form ion pairs with neighboring anions in the membrane interior and thus be unavailable for carrying electrical current. Experimental attempts to distinguish between these possibilities are in progress in our laboratory. Clearly, many of these ideas derive from much early work on permeability of biological membranes which emphasized the importance of the concept that the

surface of cells is an interface between aqueous and nonaqueous phases (e.g., 15–17). The relevance of this proposed mechanism of cation selectivity produced by macrocyclic compounds in thin and thick lipid as well as HK and LK sheep red cell membranes to the naturally occurring cation selectivity of biological membranes remains a problem for future exploration.

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