Ion Transport Mediated by the Valinomycin Analogue \textit{cyclo}(L-Lac-L-Val-D-Pro-D-Val)\textsubscript{3} in Lipid Bilayer Membranes

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\textbf{ABSTRACT} \textit{Cyclo}(L-Lac-L-Val-D-Pro-D-Val)\textsubscript{3} (PV-Lac) a structural analogue of the ion-carrier valinomycin, increases the cation permeability of lipid bilayer membranes by forming a 1:1 ion-carrier complex. The selectivity sequence for PV-Lac is identical to that of valinomycin; i.e., Rb\textsuperscript{+} > K\textsuperscript{+} > Cs\textsuperscript{+} > NH\textsubscript{4}\textsuperscript{+} > Na\textsuperscript{+} > Li\textsuperscript{+}. The steady-state zero-voltage conductance, \(G(0)\), is a saturating function of KCl concentration. A similar behavior was found for Rb\textsuperscript{+}, Cs\textsuperscript{+}, and NH\textsubscript{4}\textsuperscript{+}. However, the ion concentration at which \(G(0)\) reaches a plateau strongly depends on membrane composition. The current-voltage curves present saturating characteristics, except at low ion concentrations of Rb\textsuperscript{+}, K\textsuperscript{+}, or Cs\textsuperscript{+}. The ion concentration at which the saturating characteristics appear depends on membrane composition. These and other results presented in this paper agree with a model that assumes complexation between carrier and ion at the membrane-water interface. Current relaxation after voltage-jump studies were also performed for PV-Lac. Both the time constant and the amplitude of the current after a voltage jump strongly depend on ion concentration and membrane composition. These results, together with the stationary conductance data, were used to evaluate the rate constants of the PV-Lac-mediated K\textsuperscript{+} transport. In glycerolmonooleate they are: association rate constant, \(2 \times 10^6\) \(\text{M}^{-1}\) \(\text{s}^{-1}\); dissociation rate constant, \(4 \times 10^5\) \(\text{s}^{-1}\); translocation rate constant for complex, \(5 \times 10^4\) \(\text{s}^{-1}\); and the rate of translocation of the free carrier \((k_s)\), \(55\) \(\text{s}^{-1}\). \(k_s\) is much smaller for PV-Lac than for valinomycin and thus limits the efficiency with which the carrier is able to translocate cations across the membrane.

\textbf{INTRODUCTION}

The lipid bilayer membrane represents an effective energy barrier to the passage of small ions. Ion carriers and ion channels are two efficient ways to lower this energy barrier (Parsegian, 1969), and both have been proposed as mechanisms to explain the numerous transport functions in biological membranes. In lipid bilayers, the highly selective ion carrier valinomycin has been...
extensively studied (Lev and Buzhinsky, 1967; Mueller and Rudin, 1967; Andreoli et al., 1967; Stark and Benz, 1971; Stark et al., 1971; Knoll and Stark, 1975; Benz et al., 1977). Valinomycin forms one-to-one complexes with alkali cations, and these complexes carry out the charge transfer across lipid bilayer membranes (for a review, see Läuger [1972]). Valinomycin-induced conductance in planar lipid bilayers is well described by a model composed of four transport steps (Fig. 1): (a) association of the ion with the carrier molecule (at the membrane surface), described by the rate constant $k_R$; (b) translocation of the ion complex through the membrane (i.e., $k_{MS}$); (c) dissociation of the complex, described by $k_D$; and (d) translocation of the free form of the carrier back through the membrane, $k_S$. Because the complexation reaction occurs at the membrane surface, this type of mechanism has been called an interfacial complexation (IC) mechanism (Läuger and Stark, 1970; Stark et al., 1971).

Valinomycin is able to transport $K^+$ in a highly efficient and selective manner. However, the relationship between the molecular structure of valinomycin (VAL), its high specificity, and its high rate of potassium transport are not fully understood. Such understanding requires insight into the relationship between the primary structure of the macrocyclic compounds and the rates and extent to which they bind and transport ions. To this end, we have previously reported the synthesis and transport properties of a cyclic

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**Figure 1.** Diagram of carrier-mediated transport. In the interfacial complexation mechanism (IC), the complexes are formed at the membrane-solution interface. In the solution complexation (SC) mechanism, the complexes are formed in the aqueous phases.
dodecapeptide, proline-valinomycin (PV) (Gisin and Merrifield, 1972a; Ting-Beall et al., 1974; Benz et al., 1976). In this paper, we report the synthesis and characteristics of the cation transport induced by the cyclic polypeptide cyclo(l-Lac-L-Val-d-Pro-d-Val)₃ (PV-Lac).

Fig. 2 shows the primary structures of the membrane-active complexones PV-Lac, VAL, and PV. The residues are exclusively aminoacids (PV), or both hydroxy and aminoacids (VAL and PV-Lac). By replacing all the hydroxy acid residues in VAL by D- or L-prolines, PV was obtained. On the other hand, in PV-Lac, just the hydroxyisovalerate residues in VAL were replaced by D-prolines (Koroshetz et al., 1977). Consequently, PV-Lac represents a hybrid ion carrier incorporating the “top” half of VAL and the “bottom” half of PV. Independently, PV-Lac was also synthesized by Fonina et al. (1976).

**Figure 2.** Comparison of the structures of the ion carriers, PV-Lac, PV, and VAL. One-third of each molecule representing a β turn is shown. Lac, L-lactate, D-Hyv, D-α-hydroxyisovalerate.

PV binds alkali cations with an affinity that is at least 10³ times greater than that of VAL (Gisin and Davis, 1973) in single- and two-phase bulk systems. The results obtained in lipid bilayer membranes have led to the conclusion that PV promotes K⁺ movement across bilayers by the solution complexation (SC) mechanism shown in Fig. 1 (Benz et al., 1976).

In this paper, we show that in two-phase bulk systems PV-Lac has a lower affinity for monovalent alkali cations than PV, but it binds cations more strongly than VAL. Further, through stationary conductance and current relaxation experiments, we show that PV-Lac-mediated cation transport is accomplished by means of an IC mechanism. The analysis of the data using a kinetic model previously developed for valinomycin (Stark et al., 1971)
suggests that ion transport through the bilayer is limited by the rate of return of the unloaded carrier.

MATERIALS AND METHODS

Membrane Formation

All membranes were formed by apposition of two monolayers spread at the air-water interface as described in detail by Alvarez and Latorre (1978) and Reyes and Latorre (1979). The membranes were made either from bacterial phosphatidylethanolamine (PE) (Supelco, Inc., Bellefonte, Pa.), glycerolmonoooleate (GMO) (Nu-Chek Prep., Inc., Elysian, Minn.), glycerolmonoeicosenoin (GME) (Nu-Chek), or a mixture of PE and bovine phosphatidylserine (PS) (Supelco) in a molar ratio of PE:PS of 1:1. Small amounts of a concentrated solution of PV-Lac in ethanol were added directly to the aqueous phases to obtain the desired final PV-Lac concentrations. They were unbuffered and had a pH of ~6. (Ultrapure salts were obtained from Alfa Div., Ventron Corp., Danvers, Mass.). When necessary, LiCl was used to maintain a constant ionic strength. After membrane formation and addition of PV-Lac, the aqueous phases were stirred for 20–30 min to ensure equilibrium.

Electrical Measurements

The system for measuring the electrical properties of the lipid bilayers has been described in detail by Alvarez and Latorre (1978). The zero-voltage conductance induced by PV-Lac was measured from steady-state, current-voltage curves recorded directly on an X-Y recorder. In some cases, current-voltage curves were obtained by applying a DC bias across the membrane and measuring the steady-state current. Both methods gave the same results. Current relaxation experiments were carried out with the voltage pulse technique described by Ketterer et al. (1971). The capacitative current surge due to the membrane capacitance was compensated as described by Alvarez and Latorre (1978) before the addition of PV-Lac. PV-Lac–induced instantaneous current (or conductance) was estimated from the current extrapolated at zero time. To improve the signal-to-noise ratio of the PV-Lac–induced current transients, several current wave forms were added and stored in digital form with the help of a Signal Averager model 1070 (Nicolet Instrument Corp., Madison, Wis.). The analog-to-digital conversion was performed by an eight-bit-transient recorder with a maximum conversion rate of 5 MHz. The digitized data from the curves were further analyzed with a Hewlett-Packard Co. (Palo Alto, Calif.) 9852A calculator coupled to a signal averager. The time constant of the current-measuring amplifier was 1 µs.

Zero-current potential measurements were done in the presence of symmetrical KCl and asymmetrical concentrations of PV-Lac. Initially, PV-Lac was present in both compartments at a concentration of 10^{-7} M. The concentration of PV-Lac was then raised in one compartment only by adding a known amount of PV-Lac from a concentrated stock solution. The potential was measured with a Keithley electrometer (Keithley Instruments, Inc., Cleveland, Ohio) model 602 through silver–silver chloride electrodes.

Two-Phase Extraction Experiments

Two-phase extraction experiments similar to those of Eisenman et al. (1969) and Haynes and Pressman (1974) were performed as described by Gisin et al., 1978. Typically, 1.5 ml of a 3 × 10^{-6} M solution of PV-Lac in chloroform (containing 0.75% ethanol) was agitated for 30 min with 1.5 ml of an aqueous picrate salt solution in a
vial sealed with a Teflon-lined screw cap. After centrifugation, the picrate uptake into
the chloroform layer was measured (ε375 = 18,000) against a chloroform sample not
containing PV-Lac but otherwise identically treated.

Synthesis of PV-Lac

1-butyloxy-carbonyl-(L-valyl-D-prolyl-D-valyl-L-lactyl)3-resin This depsipeptide resin
was synthesized according to the solid phase method (Merrifield, 1963). Whereas the amino acids were added in a stepwise manner, the didepsipeptide unit,
D-Val-L-Lac, was added by the segment condensation approach reported for the
synthesis of valinomycin (Gisin et al., 1969). Starting material was 3.5 g (2.6 mmol)
of 1-butyloxy-carbonyl-D-valyl-L-lactyl-oxymethyl polystyrene-co-1%-divinylbenzene
resin (Boc-D-Val-L-Lac-Res) (Gisin et al., 1969). Solvents and chemicals were reagent
grade; CH2Cl2, F3CCOOH, and diisopropylethylamine were distilled; and dimethylformamide was stored over "Molecular Sieves" (Chemical Dynamics Co., So. Plainfield, N. J.). Boc-L-Val was obtained from Vega-Fox Biochemicals Div., Newbery
Energy Corp. (Tucson, Ariz.). The synthesis of Boc-D-Pro (Gisin and Merrifield, 1972
a) and of Boc-D-Val-L-Lac (Gisin et al., 1969) has been described. The synthesis of the
dodecadepsipeptide resin was performed using F3CCOOH-CH2Cl2 (1:1, vol/vol) for
deprotection, diisopropylethylamine-CH2Cl2 (1:19, vol/vol) for neutralization, and
dicyclohexylcarbodiimide as a coupling agent (Gisin and Merrifield, 1972 b). The
protocol was similar to that used for the synthesis of related compounds (Gisin et al.,
1969; Gisin and Merrifield, 1972 a; Gisin et al., 1978). Double couplings with 1.5 eq
of Boc-D-Pro, Boc-L-Val, and Boc-D-Val-L-Lac were used, the first one for 90 min and
the second for 90 min or overnight. To suppress diketopiperazine formation, couplings
were performed in the reversed mode (Gisin and Merrifield, 1972 b).

The synthesis was monitored by determining, after coupling, the remaining amino
groups with picrate (Gisin, 1972). The starting material, Boc-D-Val-Lac-Res, showed
a picrate value (blank value) of 0.45 µmol/g, representing 0.6% of the amino groups
available after deprotection (740 µmol/g, 100%). After two couplings with Boc-D-Pro,
19 µmol (2.6%) remaining amino groups were found. This figure was reduced to 2.2
µmol/g (0.3%) by acetylation with acetic anhydride-pyridine-CH2Cl2 (1:1:3, vol/
vol/vol) for 10 min. At the tetradepsipeptide (Boc-L-Val double coupling) and
hexadepsipeptide (Boc-D-Val-L-Lac double coupling) stages, only 0.3% and 0.5%
amino groups, respectively, were measured and the synthesis was continued. At all
the other stages, the couplings were less complete (i.e., 1.5-2.5% titratable amine
remaining) and acetylations were performed. At the stages at which proline had been
added, a sample of the resin was hydrolyzed (Scotchler et al., 1970). Determination
of the Pro:Val ratio gave the following results: tridepsipeptide, 1.00:1.00 (calc. 1:1);
heptadepsipeptide, 1.97:3.03 (2:3); undecadepsipeptide, 2.67:5.34 (3:5); dodecadepsipeptide, 3.12:5.88 (3:6). The yield of Boc-dodecadepsipeptide resin, not corrected
for analytical samples taken, was 5.45 g (85%) with a substitution of 408 µmol/g by
amino acid analysis.

Cyclo(L-valyl-D-prolyl-D-valyl-L-lactyl)3, PV-Lac This depsipeptide was
cleaved from 4.4 g (1.8 mmol) of the resin with CF3COOH-30% HBr in acetic acid
(1:1, vol/vol) according to a published procedure (Gisin et al., 1978) to give, after
lyophilization from acetic acid, 2.3 g of crude depsipeptide hydrobromide. The
material was dissolved in 6 ml of CH2Cl2-CH3OH (9:1, vol/vol), neutralized with
pyridine (~0.5 ml) and chromatographed in three equal batches on a Bio-Beads SX-
1 (Bio-Rad Laboratories, Richmond, Calif.) column (2.5 × 180 cm; flow rate, 2.3 ml/
min; solvent, CH2Cl2). The depsipeptide that eluted after 440 ml as a sharp peak
(differential refractometer monitoring) was collected and lyophilized from acetic acid (total recovery, 1.44 g, 71% for cleavage and purification). This material ($R_t = 0.59$ by silica gel thin-layer chromatography with n-butanol–acetic acid–pyridine–water, 15:10:3:2, vol/vol/vol/vol) was used in the cyclization reaction according to Blaha and Rudinger (1965). Thus, 460 mg (0.41 mmol) depsipeptide, 310 mg (1.1 mmol) Woodward's Reagent K (Aldrich Chemical Co., Milwaukee, Wis.) and 60 ml dimethylformamide were stirred at 0°C for 3 h; 250 ml CH$_2$Cl$_2$ and 0.8 ml triethylamine were added, and the depsipeptide was allowed to cyclize for 10 d at room temperature. After evaporation to dryness, 20 ml of CH$_3$OH-water (8:2, vol/vol) was added, followed by mixed-bed ion-exchange resin (AG 501-X8, Bio-Rad) until thin-layer chromatography indicated absence of ninhydrin-positive components in the supernatant fluid. The supernatant fluid was collected and evaporated, and the residue was chromatographed in CH$_2$Cl$_2$ on a Bio-Beads SX-1 column (2.5 × 180 cm; flow rate, 1.7 ml/min). PV-Lac, eluting in a peak centered at 475 ml, was collected and lyophilized from t-butanol. Yield was 26 mg (6% for the cyclization step); amino acid analysis, Pro : Val = 2.87 : 6.17 (calc. 3 : 6); molecular weight by mass spectrometry, 1,101.6 ± 1 (calc. 1,102.53). Overall yield based on the starting material, BOC-D-Val-L-Lac-Res, was 3.6%. To produce 1 µmol of product, 96 µmol each of Boc-D-Pro and Boc-D-Val-L-Lac were consumed.

| Ion Carrier | Li$^+$ | Na$^+$ | K$^+$ | Rb$^+$ | Cs$^+$ | NH$_4^+$ | Tl$^+$ |
|-------------|-------|-------|-------|--------|--------|---------|-------|
| PV-Lac      | ~2×10$^{-2}$ | ~8×10$^{-3}$ | 1×10$^{-4}$ | 6.5×10$^{-5}$ | 1×10$^{-5}$ | 2.5×10$^{-4}$ | 2×10$^{-4}$ |
| PV$\ddagger$ | 2.1×10$^{-4}$ | 1.4×10$^{-4}$ | 2×10$^{-7}$ | 6×10$^{-7}$ | 5×10$^{-7}$ | 8×10$^{-7}$ | 1×10$^{-7}$ |
| VAL$\ddagger$ | --- | 2.8×10$^{-3}$ | 2.2×10$^{-4}$ | 5×10$^{-4}$ | 2.8×10$^{-3}$ | 1.8×10$^{-3}$ |

* Aqueous salt concentration (M) that converts 50% of the ion carrier in the organic phase into the cation complex (cf. Fig. 3).
† Values taken from Gisin et al. (1978) for comparison.

**RESULTS**

_Cation Binding Determined by Two-Phase Extraction Experiments_

The affinities of PV-Lac for monovalent cations are listed in Table I. These values are compared with those of VAL and PV determined in the same way. The dependence of picrate uptake on the log of the salt concentration was plotted to give a titration curve (Fig. 3) for each salt used. The half-saturation concentration read from these curves, $K_{50}$, is a measure of the two-phase binding constant (Table I). For all the cations studied, PV-Lac has an affinity about two orders of magnitude lower than PV. Conversely, it binds these cations 3–10 times more strongly than does VAL. Thus, in this assay, PV-Lac assumes a position intermediate between its structural parents, PV and VAL. The finding that K$^+$ and Cs$^+$ binding of PV-Lac is weaker than that of VAL (Fonina et al., 1976) possibly may be attributed to the different assays used (conductimetry and circular dichroism in aqueous ethanol).
The ionic selectivity sequence of PV-Lac is $\text{Rb}^+ > \text{K}^+ \sim \text{Cs}^+ \gg \text{Na}^+ > \text{Li}^+$ in the two-phase assay. It is very similar to that of PV ($\text{K}^+ > \text{Rb}^+ \sim \text{Cs}^+ \gg \text{Na}^+ > \text{Li}^+$) and of VAL ($\text{Rb}^+ \sim \text{K}^+ > \text{Cs}^+ \gg \text{Na}^+$), indicating a preference for the large alkali ions over the smaller ones. However, the preference of PV-Lac for potassium over sodium ($K_{\text{KNa}}/K_{\text{KOK}}$) is only 80, compared with ~700 for PV and >360 for VAL.

**Steady-State Conductance Measurements**

**Ionic Selectivity** We have measured the zero-voltage steady-state conductance induced by PV-Lac over a wide range of monovalent cation concentrations in GMO, GE, and PE lipid bilayer membranes. In all cases, the ionic strength was kept constant up to 1 M with LiCl.

Fig. 3 shows for each cation except Li$^+$ a region of linear dependence of membrane conductance, $G(0)$, on salt concentration. In this linear domain the slope is 1. The curves for the more permeable cations, i.e., Rb$^+$, K$^+$, Cs$^+$, and NH$_4^+$ all reach a plateau at the same value of $G(0)$, but they do so at different concentrations. In the linear domain the conductance ratios relative to Rb$^+$ are $\text{Rb}^+:\text{K}^+:\text{Cs}^+:\text{NH}_4^+:\text{Na}^+:\text{Li}^+ = 1:0.14:0.14:0.011:0.003:0.000002$. This selectivity sequence is qualitatively identical to the one obtained for VAL (Lev and Buzhinsky, 1967; Läuger, 1972; Szabo et al., 1973).

There are, however, important quantitative differences in the behavior of PV-Lac and VAL as the electrolyte concentration is increased. For example, $^1$ Ratios for Na$^+$ and Li$^+$ were determined by extrapolation (Fig. 4). These results agree with those reported by Enos and Eisenman (1978) for GMO-decane membranes.
PV-Lac-treated GMO membranes show a $G(0)$ vs. electrolyte concentration curve that reaches a plateau between $3 \times 10^{-4}$ and $10^{-3}$ M KCl (Fig. 5). On the other hand, for VAL in GMO-decane membranes, and under otherwise identical experimental conditions, $G(0)$ reaches a maximum at $\sim 10^{-1}$ M KCl (Knoll and Stark, 1975).

A log plot of conductance vs. PV-Lac concentration yields a straight line with a slope of 1 over the range of $10^{-8}$ to $10^{-5}$ M (data not shown). This proportionality and that between $G(0)$ and salt concentration suggest that the predominant transport unit is a single PV-Lac molecule.

Figure 4. Zero-voltage steady-state conductance of GME PV-Lac-treated membranes as a function of monovalent cation concentration. PV-Lac was added to a final concentration of $1.5 \times 10^{-6}$ M. Solid lines were calculated using Eq. 2 and the values for the rate constants and $\beta_0$ given in Tables II and III. Solid lines drawn assuming that $K_C M \ll 1$. For Na$^+$ (○) and Li$^+$ (▲) the solid lines have no theoretical meaning. At very low Rb$^+$ (●), K$^+$ (■), Cs$^+$, (□) or NH$_4^+$ (△) concentrations, the conductance values given in the figure were corrected by the membrane conductance obtained in the presence of LiCl and PV-Lac. Ionic strength was kept constant up to 1 M with LiCl.

**EFFECT OF MEMBRANE COMPOSITION** Fig. 5 shows that $G(0)$ strongly depends on membrane lipid composition (compare curves for PE and GMO). We note here that all the lipids shown in Fig. 5 are neutral under the experimental conditions described in Material and Methods. As discussed by several authors (Hladky and Haydon, 1973; Andersen et al., 1978; Reyes and Latorre, 1979), the higher conductance of GMO (or GME) membranes can be understood in terms of the differences in dipole potential between these two membranes relative to PE membranes. Because the difference between the measured dipole potential of PE and GMO bilayers spread at the air-
water interface is 140–180 mV (Hladky, 1974; Reyes and Latorre, 1979), one would expect \( G(0) \) to be 10³-fold larger in GMO membranes. Comparison of the linear portions of the PE and GMO curves of Fig. 5 gives a ratio of \( \frac{[G(0)]_{GMO}}{[G(0)]_{PE}} = 10^4 \). When phloretin is added to a PE membrane to a final concentration of 10⁻⁵ M, the steady-state conductance increases 12-fold when \([KCl] = 0.02\) M and threefold when \([KCl] = 0.5\) M (open triangles in Fig. 5). Phloretin acts by decreasing the positive dipole potential of PE membranes (Andersen et al., 1976). At 10⁻³ M phloretin, we found that the dipole potential of PE monolayers spread at the air-water interface is reduced by 100 mV. At the same phloretin concentration, the dipole potential change of PE bilayers calculated from changes in the conductance induced by three different ion probes (PV-K, tetraphenylborate, and nonactin-K⁺) is 94 ± 10 mV (Melnik et al., 1977; Latorre and Reyes, unpublished results).

Fig. 5 also shows results with two lipids differing only in the acyl chain length; i.e., GMO containing 18 carbon atoms vs. GME with 20. The PV-Lac–induced conductance is larger the shorter the hydrocarbon chain length. That \( G(0) \) increases linearly at low permeant ion concentrations and that it depends on membrane composition are difficult to explain in the framework
of a solution complexation mechanism. In terms of a solution complexation mechanism, the deviation from a linear conductance-concentration relation is explained as the complexation of a significant fraction of the carrier in the aqueous phases, leaving an increasingly smaller fraction of the free carrier as the ion concentration is increased. In a pure SC mechanism, the concentration of $M^+$ at which the concentration of $MS = S_{total}$ is solely a function of the dissociation constant for the complex in the aqueous phases, and it is independent of membrane composition. Therefore, it is expected that when the membrane composition is changed, the curves will saturate at the same ion concentration. Inasmuch as this is not the case, these results are not consistent with an SC mechanism.

Fig. 6 shows that the $G(0)$ vs. [KCl] curve for the negatively charged PE/PS membranes is shifted to the left when compared with that for the neutral PE membranes. This result gives further support to the notion that PV-Lac-induced change transfer through the membrane is brought about by an interfacial complexation mechanism. As discussed above, if the cation is transported via an SC mechanism, the $G(0)$ vs. [M] curve must plateau at the point of saturation of the carrier with cations in the aqueous phase, regardless
of the composition of the membrane. Changing the surface charge density of a membrane, however, will change the concentration of ion-carrier complexes (MS) at the membrane-solution interface according to

$$\text{MS}_{\text{surface}} = \text{MS}_{\text{bulk}} \exp (-e\psi_a/kT),$$

where $\psi_a$ is the double layer potential, $e$ is the electronic charge, and $k$ and $T$ have their usual meaning. Accordingly, if the surface charge is increased with negatively charged PS, the entire curve will be displaced upward along the conductance axis, but the maximum will be reached at the same salt concentration as in a neutral membrane. Contrary to these expectations, for PV-Lac-treated PE-PS membranes, the curve is shifted to the left along the concentration axis, and the level of the plateau remains unchanged.

**VOLTAGE DEPENDENCE OF THE STEADY-STATE CONDUCTANCE** In both GMO and GME membranes, we have found that the steady-state current-voltage ($I$-$V$) relationships are superlinear at K$^+$ concentrations below $10^{-4}$ M. The current-voltage curves saturate at higher concentrations, and the degree of saturation depends on K$^+$ concentration (Fig. 7). In PE membranes, on the other hand, the $I$-$V$ relationship is found to be superlinear, even at a concentration of $5 \times 10^{-2}$ M. These results further strengthen the hypothesis that PV-Lac-mediated ion transport is brought about by an IC mechanism (see, for example, Stark and Benz [1971]). On the other hand, an SC mechanism predicts that the $I$-$V$ relationship saturates quite generally. Furthermore, in those cases in which the steady-state current-voltage curve saturates at high voltages, the current does not change when the rate of stirring is varied. The absence of any effect of stirring on the steady-state current indicates that PV-Lac does not mediate transport of ions through an SC mechanism. In the SC mechanism, ion transport is limited by the unstirred layers and, therefore, changes in the steady-state current with stirring are expected.

**Interpretation of the Data Assuming a Simple IC Mechanism**

The IC mechanism of transport has been discussed extensively (e.g., Markin et al. [1969], Läuger and Stark [1970], and Hladky [1972]). This model accurately describes the ion transport induced in lipid bilayers by VAL (Stark and Benz, 1971, Knoll and Stark, 1975) and by the macrotetrolide actins (Ciani et al., 1973; Hladky, 1975). In the limit of zero applied potential, the conductance in the IC model is given by (Stark and Benz, 1971)

$$G(0) = \frac{F^2}{RT} \beta_s \frac{2k_tC_Mc_0}{(KC_M + 1)(1 + 2z + \nu C_M)},$$

where $\beta_s$ is the membrane-solution adsorption coefficient of the free form of the carrier (in centimeters), $c_M$ is the concentration of the permeant cation,
$C_0$ is the total carrier concentration in the aqueous phases, $K$ is the aqueous association constant for the ion and the carrier, $z = k_{MS}/k_{D}$, $v = k_R k_{MS}/k_{S}k_D$, and $F$, $R$, and $T$ have their usual meaning. According to Eq. 2, the conductance goes through a maximum and eventually declines as the electrolyte concentration is increased. Under the assumptions that the translocation rate constant for the complex, $k_{MS}$, is the only voltage-dependent rate constant, and that the complexes are "seeing" only a fraction, $\eta$, of the total applied voltage, the voltage dependence of the conductance is well described by (Benz, 1978)

$$\frac{G(V)}{G(0)} = \frac{2(1 + A) \sinh(\eta \mu/2)}{\eta \mu [1 + A \cosh(\eta \mu/2)]}$$

(3)

![Figure 7](image-url)
where $A = 2\varepsilon + vC_M$, $u$ is the reduced potential ($u = FV/RT$), and $V$ is the applied potential.

The best fit to experimental points shown in Fig. 7 and using Eq. 3 was obtained with $\eta = 0.73$ and the values of $A$ that make the best fitting of the curves are shown on the right side of the figure. Fig. 7 shows that the $G(V)/G(0)$ curves are superlinear only at very low $[K^+]$. This result indicates that the interfacial reaction is in equilibrium only at very low $K^+$ concentrations and that only at these concentrations is the rate-limiting step the translocation of the ion-carrier complexes through the membrane. For GME PV-Lac-treated membranes, $A$ is well described by the relation $A_{\text{GME}} = (0.065 \pm 0.03) + (2.900 \pm 500) C_{K^+}$. For GMO membranes, using the same procedure described above, we have found $\eta = 0.75$ and $A_{\text{GMO}} = (0.25 \pm 0.06) + (5.265 \pm 1.000) C_{K^+}$ and, for PE membranes, $\eta = 0.70$ and $A_{\text{PE}} = (0.15 \pm 0.05) + (5.92 \pm 1.0) C_{K^+}$.

It is clear that the large deviations from linearity of the $G(0)$ vs. $C_M$ curves are due to the large value of the parameter $v$. From the relations shown for $A$, we have obtained the values for the rate constant ratios $k_{Ms}/k_0$ and $k_{R}k_{Ms}/k_{D}k_{S}$.

Furthermore, from the value of $G(0)$ at low concentrations (i.e., $KC_M \ll 1$), the quantity $B_{c}k_{Ms}k_{R}/k_{D}$ can also be obtained.

**Voltage-Jump-Current Relaxation Measurements**

To study further the mechanism by which PV-Lac transports ions through the membrane, we have measured the relaxation of the membrane current after a voltage jump. In all instances, the observed current could be fitted with a constant plus a single exponential (Fig. 8). In the IC mechanism, the time-course of the current, $I(t)$, after a voltage jump is given by (Stark et al., 1971)

$$I(t) = I_0(1 + \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}),$$

(4)

where $I_0$ is the steady-state current, $\alpha_1$ and $\alpha_2$ are the relaxation amplitudes, and $\tau_1$ and $\tau_2$ are the relaxation times. $\alpha_1$, $\alpha_2$, $\tau_1$ and $\tau_2$ depend upon the four rate constants $k_R$, $k_D$, $k_{Ms}$, and $k_S$, upon ion concentration in the aqueous phases, and upon applied voltage. Therefore, if the complete current relaxation can be resolved, the four rate constants can be computed. With our experimental conditions, we have observed only one relaxation that we have assigned to the slower time constant, $\tau_1$, and therefore $\alpha$ corresponds to $\alpha_1$. We based this assignment on the following reasoning: $\tau_1$ and $\tau_2$ are given by the following expressions (Stark et al., 1971):

$$\tau_1 = (a - b)^{-1}$$

(5)

and

$$\tau_2 = (a + b)^{-1},$$

(6)

where $a = (1/2) [(k_R C_M + k_D + 2k_S + 2k_{Ms} \cosh (\eta u/2)]$ and $b = (1/2) [(k_R C_M - k_D + 2k_S - 2k_{Ms} \cosh (\eta u/2) + 4k_R k_D C_M]^{1/2}$. Therefore, in the limit of
FIGURE 8.
PV-Lac-induced currents in GMO, GME, and PE membranes after the application of a voltage step. The aqueous phases were unbuffered (pH ~6), and the membrane area was $7 \times 10^{-4}$ cm$^2$ in all cases. (A) GMO: 1 M KCl; $10^{-6}$ M PV-Lac; 10 mV. The “on” current relaxation is well fitted by a single exponential with $\tau_{on} = 25 \mu$s, and an instantaneous current, $I(0)$, equal to 2.1 $\mu$A. The charge translocated during the “on” response (i.e., $I(0)\tau$) is $5.25 \times 10^{-11}$ C. For the same record, $\tau_{off} = 28 \mu$s and $I(0) = 1.8$ $\mu$A. Therefore, $I(0)\tau = 5 \times 10^{-11}$ C. $I(0)$ and $\tau$ were found by performing log-linear regressions of the records shown in the figure. The correlation coefficients (that applied for at least three time constants) for both “on” and “off” current relaxations are >0.999. $I_\infty < 0.01 I(0)$. (B) GME: 1 mM KCl and 1 M LiCl; $1.2 \times 10^{-6}$ M PV-Lac; 25 mV. The inset shows a semi-log plot of the $I(t) - I_\infty$ vs. time of the experimental data. The data are well described by a single exponential (plus a steady-state component) with $I(0) - I_\infty = 1.65$ nA, $\tau_{on} = 11$ ms, and a regression coefficient, $r = 0.997$. $I_\infty = 0.8$ nA. (C) GME: 1 M KCl; $1.6 \times 10^{-6}$ M PV-Lac; 10 mV. The data are well described by a single exponential for both the “on” and the “off” responses. $[I(0)]_{on} = 166$ nA; $\tau_{on} = 170$ ms; $Q_{on} = 2.8 \times 10^{-11}$ C; $\tau_{on} = 0.999$. $I(0)_{off} = 164$ nA; $\tau_{off} = 175$ ms; $Q_{off} = 2.9 \times 10^{-11}$ C; $\tau_{off} = 0.9990$. $I_\infty = 1.5$ nA. (D) PE: 1 M KCl; $10^{-5}$ M PV Lac; 80 mV. Parameters describing the “on” current response: $I(0) - I_\infty = 15$ nA; $\tau = 5.7$ ms; $r = 0.996$; $I_\infty = 1.9$ nA. Parameters describing the “off” current response: $I(0) = 8$ nA; $\tau = 10.6$ ms; $r = 0.990$. 

Figure 8.
$C_M \to 0$, two solutions for Eqs. 5 and 6 are possible, i.e.,

\[
\lim_{C_M \to 0} \tau_{1,2} = \begin{cases} 
\frac{1}{2k_S} \\
\frac{1}{k_D + 2k_{MS} \cosh (\eta u/2)}
\end{cases}
\] (7)

(8)

Of these two solutions, 7 predicts a loss of voltage dependence of $\tau$ as the KCl concentration is decreased, and 8 predicts the opposite. Fig. 9 shows that $\tau$ is less dependent on voltage at low KCl concentrations. We conclude, therefore, that $\tau_{1,2} \approx 1/2k_S$ at very low KCl concentrations.

The predicted theoretical dependence of the relaxation amplitudes and of the relaxation times at low voltages ($V \leq 10$ mV) and KCl concentration can then be generated from the values of $k_{MS}/k_D$ and $k_R k_{MS}/k_D k_S$ obtained from steady-state conductance measurements and assuming various values of $k_{MS}$.
For any reasonable value of $k_{MS}$ (see below), the theoretical curves obtained using Eqs. 5, 6,

$$\alpha_1 = e + f,$$

and

$$\alpha_2 = e - f,$$

where

$$e = \frac{(2k_s + k_{RCM})2k_{MS} \cosh(\eta u/2)}{4k_Dk_s}$$

and

$$f = \frac{1}{2b}\{e(k_{RCM} + k_D + 2k_s - 2k_{MS} \cosh[\eta u/2]) - 2k_{MS} \cosh(\eta u/2)\}$$

in the limit of zero voltage ($u \ll 1$), the values theoretically obtained for $\alpha_2$ and $\alpha_2$ cannot fit the experimental data. For all the conditions tested, $\tau_2$ is too fast and $\alpha_2$ is too small to be resolved by our current-measuring systems (see, for example, Fig. 10 B). On the other hand, Fig. 10 A, B, and C shows that an excellent fit (solid lines) to the experimental data can be obtained if one assumes that $\tau$ is $\tau_1$ and $\alpha$ is $\alpha_1$. Fig. 10 A, B, and C also shows that although the relaxation times for the three different types of membranes tested at high $K^+$ concentrations are widely different, the relaxation times at very low $K^+$ concentrations are very similar.

$k_{MS}$ Can Be Obtained Independently at High $K^+$ Concentrations

At high $K^+$ concentrations, the “instantaneous” current ($I_0$) is much larger than the steady-state current ($I_s$). For example, in 1 M KCl, $I_s$ is less than 1% of the value of $I_0$ and is not resolved for the GMO and GME membranes in Fig. 8 A and C. On the other hand, at low concentrations, the steady-state current becomes an appreciable fraction of the total current (Fig. 8 B). This is also reflected in the value of the relaxation amplitude, $\alpha$ (Fig. 10 A). Fig. 8 A and C also shows that in 1 M KCl the total charge translocated during the “on” pulse is recovered during the “off” pulse; i.e., only a small fraction (<1%) had time to leave the membrane during the voltage pulse. Thus, the transfer of charge at 1 M KCl appears to be restricted to the membrane interior. This type of behavior has been observed with hydrophobic ions (Andersen and Fuchs, 1975) and for PV (Andersen et al., 1977). When the movement of charge during the initial transient is essentially confined to the membrane interior, the voltage dependence of the initial conductance, $G(0, 0)$, can be given by the empirical expression (Benz et al., 1976)

$$\frac{G(0, V)}{G(0, 0)} = \frac{2 \sinh(\eta u/2)}{\eta u},$$

where $G(0, 0)$ is the initial conductance at zero voltage. The relaxation time, $\tau$, at zero voltage, on the other hand, is reduced to the following relation
FIGURE 10.
Figure 10. Time constants, $\tau$, and relaxation amplitudes, $\alpha$, as a function of KCl concentration. (A) Membranes were formed with GMO. PV-Lac concentration was $10^{-6}$ M. Solid lines were drawn according to Eqs. 5 and 9 given in the text for $\tau_1$ and $\alpha_1$ with the parameters given in Table II. Each point is the mean value obtained with at least three different membranes. Bars are the standard deviation. Applied voltage was 10 mV. (B) Membranes formed from GME. Solid lines were drawn according to Eqs. 5 and 9 with the parameters in Table II. Dotted lines are the expected behaviors for $\alpha_2$ and $\tau_2$ from Eqs. 5 and 9. (C) Membranes formed from PE. Curves marked at 1, 2, and 3 were drawn using values of $k_{MS}$ of $10^2$, $10^3$, and $10^4$ s$^{-1}$, respectively, and Eq. 5. The solid line for $\alpha$ was drawn according to Eq. 9 and the parameters of Table II. PV-Lac concentration was $10^{-5}$ M. Applied voltage was 10 mV.

(Melnik et al., 1977):

$$\tau \approx \frac{1}{2k_{MS}}.$$  

Fig. 11 shows that the voltage dependence of the initial conductance induced by PV-Lac at 1 M KCl in GME membranes can be well described by Eq. 11 with $\eta = 0.7$. This value is in good agreement with the value of $\eta$ obtained by fitting Eq. 3 to the experimental voltage dependence of the PV-Lac-induced steady-state conductance in GME membranes (Fig. 7). For GMO membranes, a value of 0.75 for $\eta$ was found.

The values of $k_{MS}$ obtained using Eq. 12 at 1 M KCl were $2 \times 10^4$ s$^{-1}$ and $4.2 \times 10^3$ s$^{-1}$ for GMO and GME, respectively. These values can be compared...
with those obtained from the steady-state data and the fitting of $\tau_1$ and $\alpha_1$ (Fig. 10 A and B). This comparison indicates that both methods of calculating the value for the rate constant for the translocation of the complex give essentially the same results.

**Zero-Current Membrane Potential**

Ting-Beall et al. (1974) and Benz et al. (1976) have shown the presence of a zero-current potential when peptide PV is added to only one side of the membrane in the absence of a permeant ion concentration gradient. This observation has been taken as evidence for a charge transport mediated by PV mainly due to a solution-complexation mechanism (Benz et al., 1976). Zero-current potentials such as that found for PV are not present when PV-Lac is added to one side only and the KCl concentration is 1 M at both sides of the membrane. This experimental result further strengthens our hypothesis that PV-Lac is mediated by an IC mechanism.

**Summary of Results**

From the steady-state and from the kinetic data we have obtained the four rate constants of PV-Lac-mediated K$^+$ transport and the adsorption coefficient...
cient for the free form of the carrier in GMO, GME, and PE bilayers (Table II). The main conclusion that can be extracted from Table II is that regardless of the type of membrane used, the PV-Lac–mediated ion transport is rate limited by the slow rate of translocation of the free form of the carrier. In contrast to VAL, where \( k_s = 10^4 \text{ s}^{-1} \) in GMO membranes, \( k_s \) for PV-Lac is only \( 55 \text{ s}^{-1} \). Rate constants for PV-Lac–mediated transport for \( \text{Rb}^+ \), \( \text{Cs}^+ \), and \( \text{NH}_4^+ \) are given in Table III.

### Table II

**Influence of Membrane Lipid Composition on the Rate Constants of PV-Lac-Mediated K\(^+\) Transport and on the Adsorption Coefficient, \( \beta_s \), for PV-Lac**

| Lipid | \( k_R \) \( \text{M}^{-1} \text{cm}^2 \text{s}^{-1} \) | \( k_D \) \( \text{cm}^2 \text{s}^{-1} \) | \( k_{MS} \) \( \text{s}^{-1} \) | \( k_s \) \( \text{s}^{-1} \) | \( \beta_s \) |
|-------|------------------|------------------|------------------|------------------|------|
| GMO   | \( 2 \times 10^6 \) | \( 4 \times 10^6 \) | \( 5 \times 10^4 \) | \( 5.5 \times 10^1 \) | 0.0014 |
| GME   | \( 1.3 \times 10^6 \) | \( 1.5 \times 10^6 \) | \( 5 \times 10^5 \) | \( 1.5 \times 10^1 \) | 0.0011 |
| PE    | \( 1 \times 10^5 \) | \( 4 \times 10^5 \) | \( 3 \times 10^5 \) | \( 1.4 \times 10^1 \) | 0.005 |

\( \beta_s \) was determined with Eq. 2, the experimental value for \( G(0) \), and the rate constants of Table II.

### Table III

**Rate Constants and Adsorption Coefficient, \( \beta_s \), of PV-Lac-Mediated Transport Across GME Membranes for Various Permeant Cations**

| Cation | \( k_R \) \( \text{M}^{-1} \text{cm}^2 \text{s}^{-1} \) | \( k_D \) \( \text{cm}^2 \text{s}^{-1} \) | \( k_{MS} \) \( \text{s}^{-1} \) | \( k_s \) \( \text{s}^{-1} \) | \( \beta_s \) |
|--------|------------------|------------------|------------------|------------------|------|
| \( \text{Rb}^+ \) | \( 4.3 \times 10^6 \) | \( 1.0 \times 10^6 \) | \( 6.2 \times 10^3 \) | 15 | 0.0006 |
| \( \text{Cs}^+ \) | \( 1.7 \times 10^5 \) | \( 6.9 \times 10^4 \) | \( 5.4 \times 10^3 \) | 15 | 0.0011 |
| \( \text{NH}_4^+ \) | \( 1.3 \times 10^6 \) | \( 7.6 \times 10^5 \) | \( 5.7 \times 10^3 \) | 15 | 0.0005 |

\( \beta_s \) was determined as in Table II.

### Discussion

PV-Lac–Mediated Ion Transport Is Accomplished Through an IC Mechanism

The results that are presented in this paper strongly suggest that, similar to VAL, PV-Lac acts by an interfacial complexation mechanism. This conclusion is supported by the following findings. First, we found that the steady-state conductance strongly depends on membrane composition. Both the results obtained with membranes with different dipole potential (PE vs. GMO) and with membranes with different surface charge densities (PE vs. PE-PS) can be explained in the framework of an IC mechanism, but not in terms of an SC mechanism. Second, we found that the stationary current-voltage curves are superlinear or sublinear, depending on the concentration of the permeant ion. Within the framework of an IC mechanism, the shape of the current-voltage
relationship depends on the parameter $A$ of Eq. 3 (Benz et al., 1973). We found superlinear curves only at very low concentrations of permeant ion, and we concluded that this is due to a very large value of the rate constants ratio, $\frac{k_{MSkR}}{k_Dk_S}$, obtained by plotting $A$ versus $C_M$. The fact that the shape of the current-voltage relationship is changed by changes in $C_M$ is strong evidence in favor of an IC mechanism (Stark and Benz, 1971; Laprade et al., 1975). Third, the SC mechanism shown in Fig. 1 predicts the presence of a steady-state electrical potential difference when the concentration of the carrier is different in the two sides of the membrane that separates otherwise identical solutions. We did not find such potentials even at KCl concentrations as high as 1 M. Fourth, Stark and Benz (1971) calculated the maximum current flux for the case in which the complex is formed in the aqueous phase. In this case, the maximum current flux, $I_{SC}^{\text{max}}$, is given by:

$$I_{SC}^{\text{max}} = F \frac{D_{MS}}{\delta} [\text{MS}],$$

where $F$ is Faraday's constant, $D_{MS}$ is the diffusion coefficient of the complex in the aqueous phase, and $\delta$ is the thickness of the un stirred layers. Assuming that all $S$ is in the MS form at a concentration of $10^{-6}$ M, $D = 3 \times 10^{-6}$ cm$^2$/s, and $\delta = 5 \times 10^{-3}$ cm, $I_{SC}^{\text{max}}$ is $6 \times 10^{-6}$ A/cm$^2$. Under the same experimental conditions, PV-Lac promotes a cation flux in GMO membranes equivalent to $10^{-5}$ A/cm$^2$. This value is 167-fold larger than the one calculated for a pure SC mechanism.

To What Extent Does PV-Lac Complex Cations in Aqueous Solution?

The equilibrium constant for the complex formation in the aqueous phase, $K$, is given by (Läuger and Stark, 1970)

$$K = \frac{\beta_S k_R}{\beta_{MS} k_D},$$

where $\beta_S$ and $\beta_{MS}$ are the membrane-solution adsorption coefficients for the free form of the carrier and the complex, respectively. For VAL, $K$ has been reported to be $\leq 0.1$ M$^{-1}$ (dioleoy phosphatidylcholine-decane membranes) (Stark and Benz, 1971) and 0.8 M$^{-1}$ (dioleate-decane membranes) (Laprade et al., 1975). For PV-Lac, a lower limit for $\beta_{MS}$ can be inferred from current-relaxation measurements at high salt concentrations.

At high electrolyte activities, if a sufficiently large portion of PV-Lac is complexed, it is possible to sweep a large fraction of the complexes across the membrane by applying a large voltage so that they could almost all be at the far side of the membrane. In this condition, the rate of carrier return will limit the flux of the complexes. Table IV shows that $k_S$ for PV-Lac is about 1,000-fold smaller than that of VAL and, therefore, at concentrations of K$^+$ high enough and large enough voltages, we can in principle translocate all the complexes from one membrane interface to the other.$^4$

$^4$ In the following, we assume that the rate of adsorption, $k_S^{\text{max}}$, of PV-Lac and the rate of adsorption of the complexes are slow compared with the rate of translocation of the complexes.
Fig. 12 shows that for PV-Lac the amount of charge translocated, $Q$, reaches a limiting value at high potentials. The result is the same whether one takes the "on" or the "off" relaxation to calculate $Q$. This can be interpreted as a complete depletion of PV-Lac complexes from one of the membrane interfaces and opens the possibility for calculating a lower limit for $\beta_{MS}$. In this case, $\beta_{MS}$ is approximately given by the expression

$$\beta_{MS} = \frac{Q_m}{F(C_{MS})_a},$$

where $Q_m$ is the maximum charge translocated per unit area; $F$ is Faraday's constant, and $(C_{MS})_a$ is the concentration of the complexes in the aqueous phases expressed in moles per cubic centimeter. To obtain a lower limit for $\beta_{MS}$, we assume that all $S$ in the aqueous solution is in the MS$^+$ form. With this proviso, we obtain $\beta_{MS} \geq 5 \times 10^{-3}$ cm, and, using the values given in Table IV and Eq. 14, we have $K \leq 1.4 \text{ M}^{-1}$. The $K$ thus obtained is higher than the one reported for VAL but much lower than that found for PV (Table IV). This result is in agreement with the affinities of PV-Lac for monovalent cations measured in two-phase bulk systems (Table I). PV-Lac assumes, then, an intermediate position between PV and VAL, but the half-saturation concentrations are more similar to those of VAL than those of PV.

### Table IV

|        | $k_{IR}$ | $k_D$ | $k_{MS}$ | $k_S$ | $\beta_S$ | $\beta_{MS}$ | $K_A$ | Reference |
|--------|----------|-------|----------|-------|-----------|--------------|-------|-----------|
| VAL    | $2.9 \times 10^4$ | $2.7 \times 10^6$ | $2.1 \times 10^4$ | $3.8 \times 10^4$ | $0.0019$ | $-$ | $>0.1$ | §, § |
| PV-Lac | $2.1 \times 10^4$ | $4 \times 10^6$ | $5 \times 10^4$ | $5.5 \times 10^3$ | $0.0014$ | $0.005$ | $\leq 1.4$ | § |
| PV     | $-$      | $-$   | $5 \times 10^3$ | $-$ | $0.003$ | $8$ | $-$ | § |

* Glycerolmonooleate membranes.
† Stark and Benz (1971).
§ Benz and Lüuger (1976).
¶ Present results.
‖ Benz et al. (1976).

Low $k_S$ Limits the Efficiency of the Ion Transport Rate of PV-Lac

The turnover number, $f$, defined as the number of ions that may be transported per second by a single carrier molecule, is a characteristic parameter for the efficiency of ion transport rate of carrier molecules. A simple expression for $f$ in the limit of high permeant ion concentration has been given by Lüuger (1972):

$$f = \left(\frac{1}{k_S} + \frac{1}{k_{MS}} + \frac{2}{k_D}\right)^{-1}.$$  

Strictly, the adsorbed charge density is equal to $Q_m$ divided by the fraction of applied potential that is effective in moving complexes through the membrane (Andersen et al., 1978).
Taking the values of the rate constant for the VAL system (Table IV), one obtains $f = 2.9 \times 10^4 \text{ s}^{-1}$. Because of the low value of $k_S$ for PV-Lac, Eq. 16 is reduced to $f \approx k_S = 55 \text{ s}^{-1}$. This value of $f$ is 500-fold smaller than the value obtained for VAL.

Assumptions of the Model

An exact theoretical description of the current-voltage curve requires an exact knowledge of the voltage dependence of the interfacial reaction as well as of the internal free barrier for translocation of the complex. More sophisticated forms of Eqs. 2 and 3 have been given in the literature (e.g., Ciani [1976]). In this case, the steady-state electrical properties are described in terms of a model that allows for voltage dependence of the interfacial reaction (see also Hladky [1974]). The model also considers a trapezoidal free-energy barrier for the translocation of the complex (Hall et al., 1973; Hladky, 1974). In the theoretical treatment presented here, the shape of the barrier is considered to a certain extent by introducing the parameter $\eta$ in Eq. 3. The voltage dependence of $k_R$ and $k_D$ (i.e., the voltage dependence of the interfacial complexation reaction) will depend on how far into the hydrocarbon region the ions must move to form the complexes and on the fractional membrane concentration.
thickness at which the complexes are located once they are formed. Because the voltage dependence of $k_R$ and $k_D$ was not considered in the present paper, the values given in Table II must be taken as a first approximation of the true values for the different rate constants. There is no a priori reason to think that $k_R$ and $k_D$ are independent of voltage (the assumption made to calculate the ratios $k_M / k_D$ and $k_R k_M / k_D k_S$). Knoll and Stark (1975) found that the experimental data for VAL is better explained by assuming a small voltage dependence of the interfacial reaction, and that $k_R$ decreases as the Rb$^+$ concentration is increased.

Membrane Composition and PV-Lac-mediated Transport

Effects of Membrane Thickness and Viscosity

The translocation of the complexes is limited by the image force barrier, which in turn is a function of the dielectric constant, $\varepsilon_m$, and the membrane thickness, $d$. Accordingly, if two membranes of dielectric thicknesses $d_1$ and $d_2$ with a dielectric constant, $\varepsilon_m$, are considered, the difference in energy, $W(d)$, of a charged complex located in the central plane of the membrane is given by (Parsegian, 1969):

$$\Delta W(d) = \frac{(e)^2}{4\pi \varepsilon_0 \varepsilon_m} \left( \frac{1}{d_2} - \frac{1}{d_1} \right) \ln 2,$$

where $e$ is the electronic charge and $\varepsilon_0$ is the permittivity of free space. The value of $d$ for the different membranes can be calculated from the values of the specific capacitance, $C_s$, assuming a value for $\varepsilon_m$ of 2.1. The specific capacitances of GMO and GME are 0.79 $\mu F/cm^2$ and 0.70 $\mu F/cm^2$, respectively. These values give dielectric layer thicknesses of 2.35 and 2.66 nm for GMO and GME bilayers, respectively. Therefore, $\Delta W(d)$ between GMO and GME is found from Eq. 17 to be 0.87 $kT$. This means that if only the image force barrier is taken into account, $k_M$ for GMO and GME membranes should be different by a factor of 2.5. Experimentally, we found that $k_M$ for GMO membranes is 10-fold larger than $k_M$ for GME membranes (Table II). $k_M$ also depends on the mobility of the complex in the membrane. It is possible, therefore, that some of the observed decrease in $k_M$ when $d$ is increased by changing the membrane composition from GMO to GME can be due to an increase in membrane viscosity. That $k_S$ is 2.7-fold less in GME vs. GMO membranes (Table II) gives support to this hypothesis. Similarly, $\Delta W(d)$ between PE ($C_s = 0.68 \mu F/cm^2$, $d = 2.73$ nm) and GMO is 1.06 $kT$, which means that the effect of the image force barrier on $k_M$ for PV-Lac in these two lipids should change $k_M$ by a factor of 3. However, Table II shows that $k_M$ in a PE membrane is 166-fold smaller than the $k_M$ in a GMO membrane. It is unlikely that a difference in membrane viscosity can account for this result inasmuch as $k_S$ decreases by only 3.7-fold when the membrane lipid composition is changed from GMO to PE.

Dipole Potential

GMO and PE membranes differ considerably in all four rate constants, but the larger effects are on $k_R$, $k_D$, and $k_M$ (Table II). $k_R$

*Benz et al. (1977) found a similar effect on the $k_M$ and $k_S$ for VAL in decane-containing membranes when the hydrocarbon chain length of monoglycerides was varied.*
decreases $2 \times 10^2$-fold, $k_D$ decreases $10^2$-fold, and $k_{MS}$ decreases $1.7 \times 10^2$-fold when the membrane lipid composition is changed from GMO to PE. It is also important to note that the stability constant for the complex, $K_h = k_R/k_D$, is 20-fold larger in the case of GMO as compared with PE. Thus, the differences between GMO and PE seen in the steady-state data are due to both an increase in the translocation constant for the complexes and an increase in $K_h$.

Lipid bilayer experiments indicate that the interior of GMO membranes is less positive than the interior of PE membranes by about 120–130 mV (Andersen et al., 1978; Reyes and Latorre, 1979). As discussed by Krasne and Eisenman (1976), the effect of the difference in dipole potential between these two types of membranes will depend on where the complexation reaction plane is located. Our finding that $k_R$, $k_D$, $k_{MS}$, and $K_h$ are larger in GMO than in PE membranes is consistent with a picture in which the plane of reaction is located between the hydrocarbon interior and the surface of the membrane. However, we must be very cautious in attributing any physical meaning to this plane for several reasons. First, the reaction plane for different types of membranes may be located at different positions. Second, it is not clear where the dipole potential drop occurs. As pointed out by Krasne and Eisenman (1976), some fraction of the potential may drop across the polar head region and may extend to the aqueous phases. Third, the carrier molecule is of dimensions comparable to those of the membrane and, therefore, the term “plane” can be meaningless.

That $k_R$ and $k_D$ are much smaller when measured in PE instead of GMO can be due to structural differences (other than differences in dipole potential) between PE and GMO polar head groups. It is not clear at present what would be the effect of this factor on the complexation reaction.

**Structural Considerations**

The three-dimensional structure of the valinomycin-potassium complex (Pinkerton et al., 1969; Ivanov et al., 1969; Ohnishi and Urry, 1969) is well understood. It shows the cation in the center of a regular octahedron, the corners being occupied by the six ester carbonyl oxygens of the molecule. The six amide groups form intramolecular hydrogen bonds and stabilize the complex. All of the hydrophobic side chains point outward, shielding the hydrogen bonds and the potassium ions from the solvent. The complexes of PV (Gisin and Merrifield, 1972 a; Gisin and Davis, 1973; Davis et al., 1976; Gisin et al., 1978) are essentially isostructural with the VAL-K⁺ complex, both in solution (Davis et al., 1976) and in the crystalline state (Hamilton et al., 1979). Most likely, the structures of the PV-Lac complexes are very similar.

The relative values for the cation affinities of PV-Lac, VAL, and PV depend on the free-energy differences between the complexed and the free forms. Such differences between VAL and PV have been discussed in electrostatic and in structural terms (Davis et al., 1976; Davis and Gisin, 1979; Hamilton et al., 1979). Both types of consideration lead to the conclusion that PV should bind cations more strongly than VAL. In VAL all of the carbonyl
oxygens that interact with the cation are in ester bonds, whereas in PV they are in amide bonds. The dipole moment of an amide ligand is two fold larger than that of an ester ligand (McClellan, 1963); this leads to stronger complexation in PV. In PV-Lac, three ester carbonyls and three amide carbonyls interact with the cation. Not unexpectedly, therefore, the cation affinity of PV-Lac is intermediate between VAL and PV (Table I). Furthermore, the cation is not expected to occupy the center of the PV-Lac complex. Rather, it should be displaced toward the three amide carbonyl oxygens at the “bottom” of the molecule.

The ester ligands of VAL have considerable rotational freedom. Therefore, capture or release of a cation is accompanied by only moderate conformational changes in the backbone (Davis and Tosteson, 1975). In contrast, the proline rings in PV prevent independent rotation of the amide ligands, and significant distortion in the backbone must occur during opening and closing of the binding cavity. Thus, the differences in the cation affinities of VAL and PV may also derive from differences in potential energy of the uncomplexed forms and in the rate of cation dissociation. For PV the complexed states are more stable and the uncomplexed states are less stable than for VAL (Davis and Gisin, 1979; Hamilton et al., 1979). PV-Lac as a hybrid between PV and VAL, again, must assume an intermediate position.

The parameter $k_{MS}$ describes the rate at which a complex, after its formation at one interface, is transported to the opposite interface of the membrane. Before this process takes place, the hydrophobic ion is balanced near the interface in a minimum of the potential energy profile (Ketterer et al., 1971). This profile is the sum of an electrostatic term (image force barrier) and a term that accounts for hydrophobic interactions. Since the complexes of VAL, PV-Lac, and PV are isostructural, identical in charge (plus one), dipole moment (approximately zero) and similar in size, they all will experience the same electrostatic forces. However, due to the differences in their surface structure, their hydrophobic terms will differ and determine both the relative depth of the potential well and its distance from the center plane of the membrane. Thus, a more hydrophobic ion is expected to start the translocation step described by $k_{MS}$ from a location closer to the membrane center. This point is at a lower potential energy level than that for a less hydrophobic ion, but the height of the energy barrier in the membrane center will be reduced to an even greater extent. Consequently, a more hydrophobic ion will experience a relatively smaller barrier, i.e., have a larger $k_{MS}$. The hydrophobicity of the side chains of K$^+$-VAL (nine isopropyl and three methyl groups) is greater than that of K$^+$-PV-Lac (six isopropyl, three methyl, and three propylene groups). In agreement with reasoning described above, the experiments show that for PV-Lac, $k_{MS}$ is five times smaller than for VAL but 10 times larger than for PV (Table IV).

Though related to that described above, the structural basis for the large difference in $k_S$ (the translocation rate of the uncomplexed peptide) between VAL and PV-Lac is more complicated. This is because the free forms, unlike the complexes, can assume different conformations, depending on the envi-
For instance, VAL has a "closed" hydrophobic conformation in cyclohexane and an "open" conformation in the more polar ethanol (Grell et al., 1979). Likewise, cyclo(L-Pro-L-Val-D-Ala-D-Val)₃, PVAV (Davis and Gisin, 1979), and PV (Davis et al., 1976) favor a conformation with less intramolecular hydrogen bonding in methanol than in chloroform. In the open conformation, more polar groups (hydrogen bond donors and acceptors) are exposed to the solvent, whereas in the closed form these groups are intramolecularly saturated. Consequently, a more polar configuration will be located, on the average, farther away from the membrane center. This will result in a relatively smaller $k_s$. The ratio $k_s^{VAL}/k_s^{PV-Lac}$ is 640 (Table IV), corresponding to a 3.9 kcal difference in the relative barrier height. Such a small difference can be accounted for by the assumption that PV-Lac molecules at the aqueous phase–membrane interface are capable of more polar interactions (fractional hydrogen bonds) than VAL.

An alternative explanation is based on the observation that the interconversion rate between different conformers is very slow for PV; i.e., $\ll 1$ s⁻¹ (Davis et al., 1976) but fast for VAL. If only a closed, hydrophobic conformer can pass the hydrocarbon interior, the rate at which this conformer is formed can become limiting for the translocation step. PV-Lac may also have the slow interconversion rate from its structural parent, PV, and may have a small $k_s$ for that reason.

The support of this work by Dr. R. B. Merrifield and the technical assistance of Mr. R. K. Miller are gratefully acknowledged. We want also to acknowledge the comments on the manuscript by Drs. O. S. Andersen, D. J. Benos, and D. G. Davis. This project received U. S. Public Health Service support through grants HL-12157, HL-24047, GM-27833, and GM-25277.

Received for publication 8 September 1980.

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