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Carrier-Mediated Ion Transport

Electrical relaxation experiments give insight into the kinetics of ion transport through artificial lipid membrane.

P. Läuger

Biological membranes have a thickness of about 100 angstroms, and they are made up of lipids and proteins in a more or less ordered arrangement. The lipid molecules are oriented in such a way that their polar head-groups are in contact with the aqueous phases, whereas the hydrocarbon chains form the interior of the membrane. Because hydrocarbons have a low dielectric constant, the energy required to bring a small ion, such as sodium or potassium, from the aqueous medium into the membrane is many times the mean thermal energy. This means that the lipid portions of the membrane represent an extremely high barrier for the passage of these ions.

Nevertheless, biological membranes are permeable to alkali ions, and therefore one must assume that mechanisms exist by which the activation energy of the ion transport is drastically reduced. One possible mechanism is represented by a mobile carrier molecule that binds the ion at one membrane-solution interface, then migrates to the opposite interface and releases the ion into the aqueous solution. The concept of a carrier that facilitates the transport of ions and small hydrophilic molecules such as sugars and amino acids across the cell membrane dates back to the experiments performed by Osterhout (*1*) in 1933. This concept has since been worked out in great detail (*2*), but the existence of ion carriers in biology remained hypothetical until such compounds as valinomycin, monactin, and enniatin B were isolated and characterized [for reviews see (*3, 4*)]. These compounds are produced by certain microorganisms and possess antibiotic activity. Valinomycin and enniatin B are depsipeptides, that is, they are built up by α-amino acids and α-hydroxy acids in alternating sequences (Fig. 1). Monactin and the other macrotetrolides (nonactin, dinactin, trinactin) are cyclic compounds which contain four ether and four ester bonds. All these substances share a common property: they are macrocyclic molecules in which one side of the ring is hydrophilic, the other strongly hydrophobic. These compounds form complexes with alkali ions in organic solvents with a high degree of specificity (*3–5*); for instance, the stability constant of the potassium complex of valinomycin in methanol is larger by a factor of about 10^4 than the stability constant of the sodium complex (*6*). Valinomycin and the macrotetrolides may be used to extract alkali ions from water into an organic phase (*7, 8*).

The structure of the valinomycin-K^+ complex has been studied by spectroscopic methods (nuclear magnetic resonance, infrared spectroscopy, optical rotatory dispersion) as well as by x-ray diffraction (*3, 9*). The oxygen atoms of

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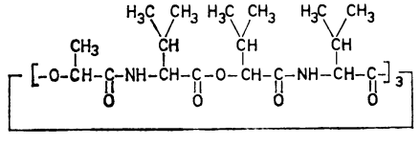
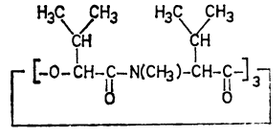
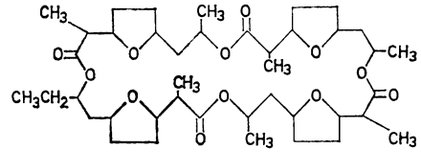
the six ester carbonyl groups form an octahedral cage around the potassium ion (Figs. 2 and 3). In this way the interior of the complex offers to the cation an environment which is similar to the hydration shell of the ion in aqueous solution. On the other hand, the exterior of the complex is strongly hydrophobic. The conformation of the molecule is stabilized by hydrogen bonds which are formed between neighboring amid groups. These hydrogen bonds are essential for the complex formation: if the optical configuration of only one acid in the molecule is changed, so that not all hydrogen bonds can be formed, the stability of the complex is greatly reduced (*3*).



Valinomycin



Enniatin B



Monactin

Fig. 1. Chemical structures of macrocyclic ion carriers.

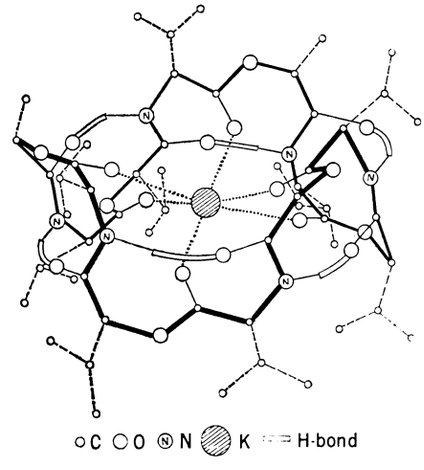


Fig. 2. Structure of the valinomycin-K^+ complex (*3*).

Artificial Lipid Membranes

The ability of valinomycin to increase the potassium permeability of biological membranes was first demonstrated in experiments with mitochondria and erythrocytes (*7, 10*). Similar results were obtained with the macrotetrolides (*11*). To interpret these findings it was proposed that the macrocyclic antibiotics act as carriers for K^+. Because of the great complexity of biological membranes, however, detailed information on the transport mechanism could not be obtained from these experiments. For this reason, artificial model membranes are now used in many studies with macrocyclic antibiotics.

Artificial lipid membranes of macroscopic area and with a thickness of less than 100 Å may be formed by a technique originally developed by Mueller and his co-workers (*12*). A small amount of a lipid solution, such as lecithin in *n*-decane, is put over the hole in a Teflon diaphragm which is completely immersed in an aqueous solution (Fig. 4). In this way a lamella forms which gradually becomes thinner and, in an intermediate stage, shows bright interference colors, corresponding to a thickness of less than 1 μm. After some time a discontinuous transition to a much thinner structure takes place at localized spots in the lamella where the light reflection drops to almost zero. These "black" spots expand until nearly the whole area of the hole is covered with a uniform membrane. The thickness d of this membrane may be measured by electrical (*13*) and optical (*14*) methods. For a lecithin with chains containing 18 carbon atoms a value of $d \simeq 70$ Å is found, which is nearly equal to two times the length of a fully extended lecithin molecule. This and other findings support the conclusion that the membrane consists essentially of a bimolecular layer (bilayer) of oriented lipid molecules. The structure of the bilayer membrane is shown in Fig. 4. The polar head groups of the lipid molecules point toward the aqueous medium, whereas the fatty acid chains form the interior of the membrane. Some hydrocarbon solvent remains dissolved in the film, but otherwise the structure of the artificial bilayer closely resembles the arrangement of the lipid molecules in biological membranes. Recent experiments with artificial lipid membranes have indicated some of the basic mechanisms by which ions may cross biological membranes.

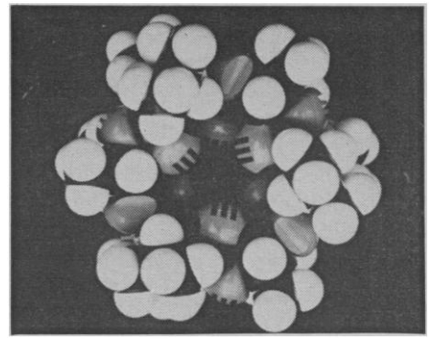


Fig. 3. Molecular model of valinomycin. The size of the central cavity nearly equals the diameter of an unhydrated potassium ion. [Courtesy Dr. B. F. Gisin]

To measure the electrical properties of bilayer membranes, a Teflon cell is used which is divided into two compartments by a diaphragm (Fig. 5). The membrane is formed at a circular hole in the diaphragm and may be observed through a glass window which is inserted into the outer wall of the cell. Calomel or silver–silver chloride electrodes are used to apply a voltage and to measure the electrical current across the membrane.

Ion Permeability of Lipid Bilayer Membranes

In solutions of potassium chloride the electrical resistance of an unmodified bilayer membrane is very high, a typical value being 10^8 ohms per square centimeter. If small amounts of monactin or valinomycin are added either to the aqueous phase or to the film-forming solution, the resistance of the bilayer drops by several orders of magnitude (*3, 15–17*). This increase in the electrical conductivity comes about by

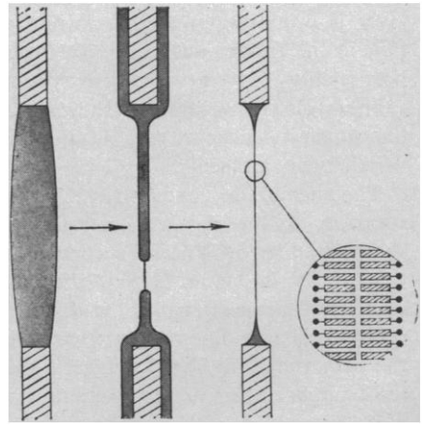


Fig. 4. Formation of optically "black" lipid membranes.

a selective increase in the K^+ permeability of the membrane. This may be shown by an experiment in which a difference in the KCl concentration is maintained across the membrane. A membrane potential then develops, the dilute solution becoming positive with respect to the concentrated solution. The magnitude of the membrane potential very nearly reaches the thermodynamic limiting value of 58 millivolts for a concentration (or activity) ratio of 10 : 1, corresponding to a nearly ideal cation selectivity.

Valinomycin is highly specific for certain alkali ions (*18*). In Fig. 6, the membrane conductance λ_o (referred to 1 cm^2 of the bilayer) which has been measured in $10^{-2}M$ solutions of different alkali ions is plotted as a function of ion radius. The data show, for example, that K^+ gives a conductivity that is higher by a factor of 10^3 than the conductivity in the presence of Na^+. The reason for this high specificity is not fully understood. One possible explanation may lie in the steric constraints of the macrocyclic ring, which preclude an optimal electrostatic interaction between the relatively small sodium ion and the carbonyl oxygen atoms of the ring (*4, 19*). Alternatively, the selectivity may reflect differences in ion-ligand interactions of the type found in glass electrodes and certain organic solvents (*20*).

The increase in the membrane conductivity is strictly proportional to the antibiotic concentration in the aqueous phase over several orders of magnitude. This is shown in Fig. 7 for valinomycin in the presence of $1M$ KCl. For low concentrations of valinomycin it may be assumed that a simple distribution equilibrium exists between the aqueous phase and the membrane—that is, that the valinomycin concentration in the bilayer is proportional to the concentration in the aqueous solution. This proportionality between conductivity and valinomycin concentration shows that the smallest transport unit is a single valinomycin molecule.

The membrane conductivity λ_o as a function of the K^+ concentration is shown in Fig. 8. When the concentration of K^+ is below $10^{-1}M$ the conductivity is proportional to the K^+ concentration. This finding, together with the data shown in Fig. 7, suggests that the charge carrier in the membrane is a 1 : 1 complex of K^+ and valinomycin. At higher K^+ concentrations a deviation from linearity occurs in the sense that the conductivity increases more slowly than the rate at which the K^+ concentration increases. This saturation behavior is characteristic for carrier systems at high concentrations of the transported particle where a substantial fraction of the carrier is in the complexed form.

These observations, together with similar results obtained in experiments with monactin and its homologs, support the hypothesis that valinomycin and the macrotetrolides act as mobile carriers (*15, 16, 21*). In particular, the finding that the membrane conductivity is proportional to the valinomycin concentration over a large range of concentrations excludes the possibility that several antibiotic molecules together form an aggregate which bridges the membrane and may act as a pore. However, the assumption that a single valinomycin molecule forms a fixed pore in the membrane is extremely unlikely for geometrical reasons (the bilayer membrane has a thickness of about 70 Å, whereas the valinomycin molecule is a flat cylinder of 12 by 16 Å). Further evidence for the mobile-carrier mechanism has been obtained from recent experiments with membranes made from a mixture of glyceryldipalmitate and glyceryldistearate which undergo a phase transition from a "liquid" to a "solid" state on cooling below 40°C (*22*). In solutions containing valinomycin (or nonactin) and K^+, the membrane conductance is high above 40°C, but shows a sharp decrease at the point of transition to the "frozen" state of the membrane.

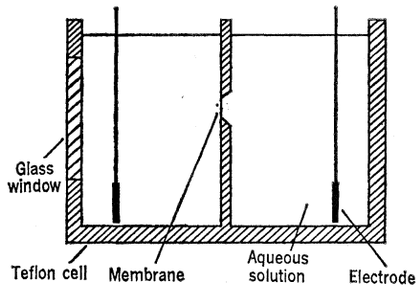


Fig. 5. Teflon cell used for measuring the electrical properties of artificial lipid membranes.

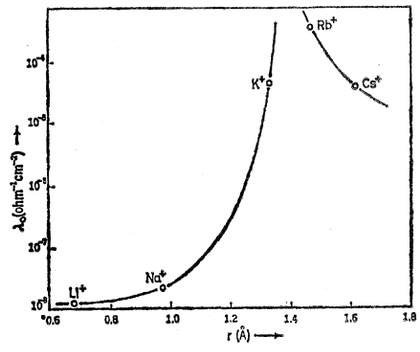


Fig. 6. Ion specificity of valinomycin (*18*). The membrane conductivity λ_o as measured in $10^{-2}M$ solutions of different alkali ions (lithium, sodium, potassium, rubidium, cesium) at a constant valinomycin concentration of $10^{-7}M$ is plotted as a function of ion radius r. Dioleoyllecithin membrane, 25°C; λ_o is the ohmic limit of the conductance and is obtained by extrapolation to zero voltage.

Kinetic Analysis of Ion Transport

If valinomycin or monactin act as carriers in the classical sense, then the ion transport mediated by these compounds occurs in four distinct steps (Fig. 9). In the first step, the carrier S in the membrane combines with a metal ion M^+ from the aqueous solution. This association reaction takes place at the membrane-solution interface and may be described by a rate constant k_R. If c_M is the aqueous concentration of M^+ and N_S is the concentration at the interface of the carrier S (expressed in moles per square centimeter) then the number of complexes MS^+ which are formed per square centimeter of the interface per second is equal to $k_R c_M N_S$. In the second step the complex MS^+ migrates to the opposite interface. The transport of MS^+ through the membrane is not a simple diffusion process; it may be more adequately described by a jump over a barrier of high activation energy. This is because of the peculiar shape of the potential energy profile of large hydrophobic ions such as that represented by the complex MS^+. It may be shown (*23*) that the interaction of a hydrophobic ion with the membrane leads to an energy minimum at the membrane-solution interface. The two minima are separated by a broad energy barrier which is determined by the electrical image forces acting upon the ion near the boundary between media of different dielectric constants (*24*). The jump of MS^+ over the barrier may be characterized by a translocation rate constant k_{MS}; k_{MS} then gives directly the jump frequency of the complex. In the third step the complex dissociates and releases the ion M^+ into the aqueous phase. This reaction is described by a dissociation rate constant k_D. In the

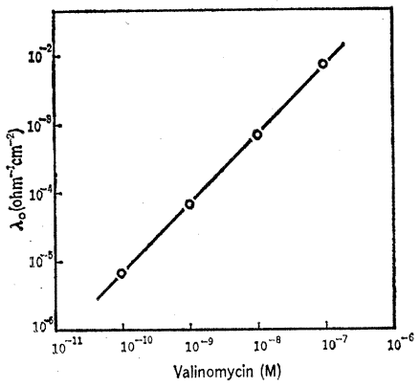


Fig. 7. Electrical conductance λ_0 of a dioleoyllecithin membrane as a function of the valinomycin concentration in the aqueous phase (25°C). The aqueous solution contains 1*M* KCl (*17*).

fourth step, the cycle is closed by the back transport of the free carrier S.

To simplify the mathematical analysis of the system, the transport of S, too, is treated as a rate process which may be characterized by a translocation rate constant k_S. (It may be shown that both descriptions, diffusion or rate process, are correlated by the relation $k_S \simeq D_S/d^2$, where D_S is the diffusion coefficient of S in the membrane and d is the membrane thickness.)

From this description of ion transport several interesting questions arise. Is there a rate-limiting step in the overall reaction sequence or do all reactions take place at comparable rate? Is the ion specificity of the carrier determined by thermodynamic factors alone (stability constant of the complex MS^+), or also by kinetic parameters (rate constants)? To answer these questions a detailed kinetic analysis of the carrier system must be made. Such an analysis appears difficult at first because of the necessity to determine not only the four rate constants, k_R, k_D, k_S, and k_{MS}, but also the concentration of the carrier in the bilayer. The analysis becomes possible, however, by combining measurements of steady-state conductance with results obtained from electrical relaxation experiments (*17, 18, 25, 26*).

Useful information on the transport kinetics may be obtained simply by measuring the current-voltage characteristic of the bilayer membrane in the presence of the carrier. This may seem surprising because in most systems in which the conductance is ionic, the current-voltage characteristic is linear and therefore does not contain much information. In the case of the carrier system, however, the shape of the current-voltage curve is influenced by the relative rates of the individual steps in the transport process.

For instance, if the rates of association and dissociation of the complex MS^+ are very high, the chemical reaction at the interface is always very close to equilibrium even if a current flows through the membrane. The overall transport is then limited by the translocation of MS^+ across the membrane. As mentioned above, the translocation of the complex may be treated as a jump over an activation barrier. In the presence of an external voltage, V, the shape of the barrier is modified by the electrostatic energy; as a consequence, the rate constants for the jump from left to right (k'_{MS}) and from right to left (k''_{MS}) become unequal. This may be expressed, to a first approximation, by the relations (*25*):

$$k'_{MS} = k_{MS}e^{u/2} \quad (1)$$

$$k''_{MS} = k_{MS}e^{-u/2} \quad (2)$$

where k_{MS} is the jump frequency in the absence of an external voltage, and u denotes the reduced voltage:

$$u = V/(RT/F) \quad (3)$$

(R is the gas constant, T the absolute temperature, and F the Faraday constant). Equations 1 and 2 predict an exponential increase of the current with voltage at higher values of V. In the limiting case where the overall transport rate is determined by the translocation of the complex, the current-voltage curve is bent toward the current axis.

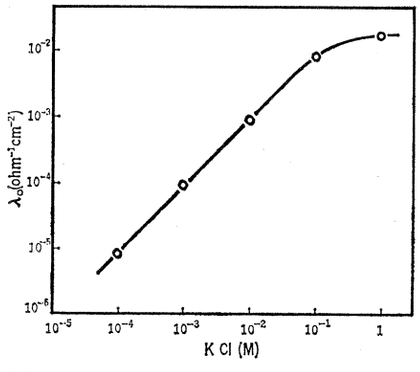


Fig. 8. Membrane conductance λ_0 of a phosphatidylinositol membrane as a function of potassium concentration (25°C) at a valinomycin concentration of $10^{-7}M$ in the aqueous phase (*17*). The ionic strength has been held constant at 1*M* by the addition of LiCl (the contribution of LiCl to the conductivity may be neglected).

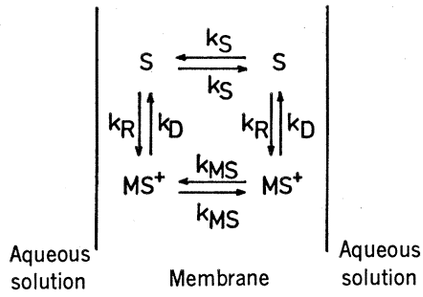


Fig. 9. Transport of the cation M^+ by the carrier S, where k_S and k_{MS} are the translocation rate constant, k_R is the rate constant of the association reaction, and k_D is the dissociation rate constant.

Conversely, if k_D is small, that is, if the dissociation step is rate limiting, the complex MS^+ will accumulate at one interface at large values of the voltage. The current J is then determined by dissociation rate constant k_D and becomes independent of voltage. In this case a saturating current-voltage characteristic is expected, that is, $J(V)$ is bent toward the voltage axis. [It should be mentioned that a saturating $J(V)$ curve is not only observed for $k_D \ll k_{MS}$, but also occurs at large concentrations of the transported ion. Then the concentration of S in the membrane becomes small compared with the concentration of MS^+, with the consequence that the current is limited by the back transport of free carrier molecules.]

Two examples of current-voltage curves are shown in Fig. 10. With a phosphatidylserine membrane and monactin as the carrier, the current increases faster than the voltage. This means that k_D is of the same order or larger than k_{MS}. In the presence of valinomycin, however, a saturating $J(V)$ characteristic is observed.

It is useful to summarize briefly the results of the mathematical analysis of the carrier system in the steady state (*17, 25, 26*). The specific membrane conductance λ is defined as the ratio of current density and voltage:

$$\lambda = J/V \quad (4)$$

In the limit of small voltages, λ reduces to the ohmic conductance λ_0:

$$\lambda_0 = (J/V)_{V \approx 0} \quad (5)$$

The formal treatment of the carrier model then yields a rather simple relation for the ratio λ/λ_0:

$$\frac{\lambda}{\lambda_0} = \frac{2}{u}(1 + A)\frac{\sinh(u/2)}{1 + A\cosh(u/2)} \quad (6)$$

The ratio λ/λ_0 depends on the reduced voltage u and on a parameter A which is given by a combination of the ion concentration c_M and the rate constants:

$$A = \frac{2k_{MS}}{k_D} + c_M \frac{k_R k_{MS}}{k_D k_S} \qquad (7)$$

The parameter A can be determined from the experimental values of λ/λ_0; if λ/λ_0 is measured as a function of ion concentration c_M, the two terms in A may be obtained separately. A third combination of rate constants follows from the theoretical expression for λ_0:

$$\lambda_0 = \frac{F^2}{RT} N_S k_{MS} \frac{c_M k_R/k_D}{1+A} \qquad (8)$$

where N_S is the interfacial concentration of free carrier molecules in the equilibrium state ($J = 0$). Thus, steady-state conductance measurements can yield only three independent combinations of the five unknown parameters k_R, K_D, k_{MS}, k_S, and N_S. The additional information which is needed for a complete kinetic analysis of the carrier system is obtained by electrical relaxation experiments.

The Electrical Relaxation Method

Relaxation techniques have been widely used in chemical kinetics for the evaluation of rate constants. This method is not restricted to homogeneous chemical systems but may also be used for the kinetic analysis of transport processes in membranes (*23, 26*). The principle of the method is simple. The system is disturbed by a sudden displacement of an external parameter such as temperature or pressure and the time required by the system to reach a new steady state is measured. In experiments with bilayer membranes a suitable external parameter which may be suddenly changed is the electrical field strength within the membrane (Fig. 11). For this purpose the cell with the membrane is connected to a voltage source and an electronic switch which permits an increase in the field strength to be made in less than 1 microsecond. The current through the membrane is measured with an oscilloscope as a voltage drop across the external resistor R_e. Immediately after the voltage jump a current spike is observed which results from the charging of the membrane capacitance. This capacitive spike limits the time resolution of the method; under favorable conditions, the resolution is about 1 microsecond. After the disappearance of the capacitive transient, the current, in general, does not immediately become constant, but approaches the stationary value J_∞ with a characteristic time course. The origin of this relaxation process may be explained as follows. At the first moment after the voltage jump, the concentrations of the complex MS^+ in the two interfaces retain their equilibrium values and are the same on both sides. Under the influence of the electrical field, charged complexes jump across the membrane, resulting in a certain initial current J_0. In the steady state, however, the concentrations of MS^+ at the two interfaces have become unequal, and, accordingly, the stationary current J_∞ is different from J_0.

A measurement of the membrane current J as a function of time t contains very useful information about the rate constants of the system. An analytical expression for the function $J(t)$ may be obtained by a mathematical treatment of the carrier model in the nonstationary state (*26*). In general, the time course of J is governed by two relaxation times τ_1 and τ_2:

$$J(t) = J_\infty(1 + \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}) \qquad (9)$$

The "amplitudes" α_1 and α_2 as well as the relaxation times τ_1 and τ_2 are complicated functions of the four rate constants, the ion concentration, and the voltage, and are described elsewhere (*26*). The essential point is that if any two of the four parameters, α_1, α_2, τ_1, τ_2, can be measured in addition to the steady-state conductance parameters, a complete kinetic analysis of the carrier system can be performed.

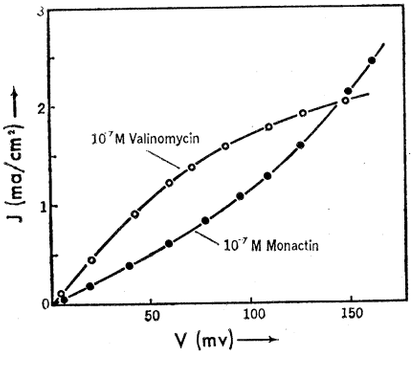


Fig. 10 Current density J as a function of voltage V for a phosphatidylserine membrane in the presence of monactin or valinomycin (25°C). The aqueous solution contained 1*M* KCl (*17*).

The Valinomycin-K^+ System

An oscillogram from a relaxation experiment which has been carried out with a phosphatidylinositol membrane in the presence of 1*M* KCl and $10^{-7}M$ valinomycin is shown in Fig. 12. If log $(J-J_\infty)$ is plotted as a function of time t, a straight line is obtained for $t > t_c$, where t_c is the width of the capacitive transient. This means that the relaxation process may be described by a single time constant $\tau \simeq 30$ μsec. As theory predicts, τ is found to be independent of the carrier concentration in the aqueous phase. The relaxation time is slightly dependent on the concentration of K^+ and strongly dependent on voltage.

To account for the observation that there was only one time constant in the experiment, there are three possible interpretations: (i) τ is the longer time constant τ_1, the second relaxation process being masked by the capacitive transient; (ii) τ is the shorter relaxation time τ_2, but the amplitude α_1 is too small to be detected; (iii) both time constants are approximately equal ($\tau_1 \approx \tau_2 \approx \tau$). The quantitative analysis shows that only the first interpretation is applicable to all the experiments (for the second interpretation some of the rate constants assume negative values, whereas the third interpretation gives the wrong sign of the concentration dependence of τ).

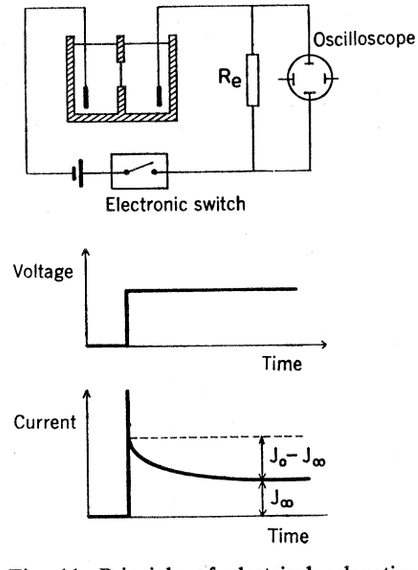


Fig. 11. Principle of electrical relaxation measurements with lipid bilayer membranes. The current through the membrane is measured as a voltage drop across the external resistor R_e.

Together with the stationary conductance data, the additional information contained in the experimental values of the relaxation time and amplitude is sufficient to evaluate the single rate constants of the carrier system. For valinomycin-K+ with a phosphatidylinositol membrane the results are

$$k_R \simeq 5 \times 10^4 M^{-1}\ \text{sec}^{-1}$$
$$k_D \simeq 5 \times 10^4\ \text{sec}^{-1}$$
$$k_S \simeq 2 \times 10^4\ \text{sec}^{-1}$$
$$k_{MS} \simeq 2 \times 10^4\ \text{sec}^{-1}$$

Furthermore, the distribution coefficient of the free carrier S may be obtained from the experiments. If the distribution coefficient is defined as the ratio N_S/c_S, where N_S is the concentration of S at the interface and c_S is the concentration of S in the aqueous solution, then

$$\frac{N_S}{c_S} \simeq 2 \times 10^{-2}\ \text{cm}$$

This means that at an aqueous concentration of $c_S = 10^{-7} M$ (the highest concentration used in these experiments) the interfacial concentration becomes equal to 1.2×10^{12} valinomycin molecules per square centimeter, corresponding to a mean separation of about 100 Å.

Discussion

From the numerical values of the rate constants a number of interesting conclusions may be drawn. First, the data show that k_D, k_S, and k_{MS} are of the same order of magnitude. This means that the translocation of free carrier and complex across the membrane as well as the dissociation of the complex at the interface occur at comparable rates (10^4 to 10^5 per second). From $k_S \simeq 2 \times 10^4\ \text{sec}^{-1}$ the mean time between successive jumps of the carrier becomes equal to $1/k_S = 50$ μsec. (For comparison, the diffusion time of a molecule of the size of valinomycin over a distance of 70 Å in water is about 0.2 μsec.)

The finding that k_S and k_{MS} are equal within the limits of error is surprising. Of course, if the carrier transport mechanism were a simple diffusion process in a viscous medium, we would expect the two translocation rates to be similar because the free carrier and the complex are of similar size. But, as shown previously, such a diffusion mechanism is unlikely, because the

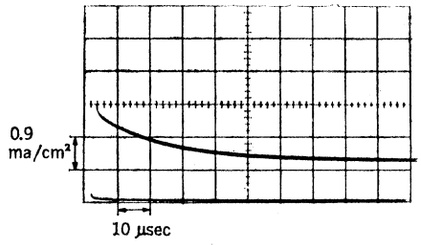


Fig. 12. Oscillogram from a relaxation experiment performed with a phosphatidylinositol membrane in the presence of 1*M* KCl and $10^{-7}M$ valinomycin (25°C). The capacitive transient (0 to 4 μsec) is not completely shown (*26*).

charged complex has to overcome a high electrostatic energy barrier in the center of the membrane. Such a barrier, however, does not exist for the transport of the neutral carrier molecule, and therefore we would expect k_S to be considerably larger than k_{MS}. A possible explanation may lie in the fact that valinomycin is highly surface active (*3*). It is therefore probable that valinomycin is adsorbed at the membrane-solution interface. In the adsorbed state some of the carbonyl groups of the molecule may point toward the aqueous phase whereas the apolar side chains may be oriented toward the hydrocarbon interior of the membrane. This means that energy is required when the molecule is released from (desorbs from) the interface and enters into the hydocarbon core of the membrane. By contrast, when the carrier molecule binds the alkali ion, the carbonyl groups are turned toward the interior of the complex and the surface activity of the molecule is lost. If this picture is correct, then one may conclude from $k_S \simeq k_{MS}$ that the activation energy for the desorption of the free carrier into the interior of the membrane is incidentally equal to the height of the electrostatic energy barrier for the charged complex.

The rate of formation of the complex at the interface is described by the rate constant k_R. It is important to note that k_R depends on the surface charge of the membrane. This is simply a consequence of the definition of k_R: the number of associations per square centimeter per second is set equal to $c_M k_R$, where c_M is the bulk concentration of the cation in the aqueous phase. Now, as a consequence of the negative charge of the phosphatidylinositol membrane, the cation concentration $\bar{c}_M$, at the membrane surface is larger than the bulk concentration by a Boltzmann factor exponent ($-\varphi$), where φ is the electrical potential (expressed in units of RT/F) at the membrane surface (*27*). Thus, we may introduce a "true" association rate constant $\bar{k}_R$:

$$c_M k_R = \bar{c}_M \bar{k}_R = c_M e^{-\varphi} \bar{k}_R \qquad (10)$$

$$k_R = \bar{k}_R\, e^{-\varphi} \qquad (11)$$

The Boltzmann factor $e^{-\varphi}$ is of the order of 20 for a phosphatidylinositol membrane under the conditions of these experiments (this can be shown by conducting experiments at different ionic strengths). The true association rate constant then becomes $\sim 3 \times 10^3 M^{-1}$ sec^{-1}. This value is by four orders of magnitude smaller than the rate constant for the association of valinomycin and K+ in methanol (*6*). This finding indicates that the conditions for the reaction in solution and at the membrane are rather different. In solution the association takes place in a homogeneous medium; on the other hand, the complex formation at the interface is a heterogeneous reaction in which the ion comes from the aqueous phase and combines with a carrier molecule which is bound to the membrane. The detailed mechanism of this heterogeneous reaction is not clear, however, and therefore we can not explain why the reaction at the membrane is so much slower than in solution. It is possible that the carrier molecule at the interface is stabilized in a conformation which is less favorable for complex formation.

To conclude, it is interesting to compare the action of a carrier molecule with the function of an enzyme. An enzyme reduces the energy barrier which separates the reactants from the products of a chemical reaction. In an analogous way, the function of a valinomycin molecule consists in reducing the extremely high activation barrier for the transport of an alkali ion across the hydrocarbon interior of a lipid membrane. The activity of an enzyme may be characterized by two parameters: the half-saturation concentration and the turnover number. In the case of the carrier molecule the half-saturation concentration corresponds to that ion concentration in the aqueous phase at which half of the membrane-bound carrier molecules are in the complexed form. This concentration $\bar{c}_M$ is equal to $k_D/\bar{k}_R \simeq 20M$ [in methanol the corresponding $\bar{c}_M$ value is much smaller, about 25 μM, see (*6*)]. Thus, under most conditions, the membrane-bound valinomycin is far from saturation.

By analogy with an enzyme, the rate of turnover, or the turnover number of a carrier, may be defined by the following fictitious experiment. Consider a lipid membrane which separates two aqueous phases and which contains a fixed number, N, of carrier molecules. The solution on the left side contains ions of concentration c_M, which are transported by the carrier; the ion concentration in the solution on the right side is assumed to be zero. If the aqueous phases are electrically short-circuited, a carrier-mediated ion flux of magnitude Φ occurs through the membrane. At low ion concentration c_M, the flux increases linearly with c_M. At high concentrations, however, the carrier becomes gradually saturated, and Φ finally approaches a maximal value Φ_{max} (in a similar manner an enzyme-catalyzed reaction approaches a maximal rate in the limit of high substrate concentration). The turnover number f is then defined as the maximum number of ions which may be transported per second by a single carrier molecule:

$$f = \frac{\Phi_{max}}{N} \qquad (12)$$

This theory leads to the following simple expression for the turnover number:

$$f = \left(\frac{1}{k_S} + \frac{1}{k_{MS}} + \frac{2}{k_D}\right)^{-1} \qquad (13)$$

With the above values of the rate constants, one finds $f \simeq 10^4$ sec^{-1}, which means that a single valinomycin molecule is able to transport 10^4 K+ ions per second across the membrane. This number is much higher than the turnover number of most enzymes.

Thus, we may state that valinomycin has a surprisingly low affinity for K+ in the heterogeneous system membrane-water. In spite of this low affinity, valinomycin is an effective ion carrier; obviously, the reason is the high turnover number of the molecule.

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Fermat's Mathematics: Proofs and Conjectures

Fermat's working habits as a mathematician shed new light on the mystery of his famous "last theorem."

Michael S. Mahoney

One of the comforts of investigating the work of Pierre de Fermat (1601–1665), one of the very few comforts, lies in not having to explain to nonhistorians of science who he was. Anyone who has studied mathematics remembers, at least vaguely, the legend connected with the name Fermat, the famous "last theorem." "Wasn't he the man who didn't have room in the margin of some book for a proof he had, a proof which no one since has been able to find?" "Yes," runs the answer, "that's the one." To be more precise, in the margin of his copy of Claude Bachet de Méziriac's 1621 edition of Diophantus of Alexandria's *Arithmetica*, next to Proposition II,8 (To split a square into two squares), Fermat wrote (*1*, p. 291):

> But one cannot split a cube into two cubes, nor a quadratoquadrate [that is, fourth power] into two quadratoquadrates, nor in general any power *in infinitum* beyond the square into two like powers. I have uncovered a marvelous demonstration indeed of this, but the narrowness of the margin will not contain it.

That is, Fermat claimed he had found a proof of the theorem that the equation $x^n + y^n = z^n$ has no rational solution for integer n greater than 2, but did not have room to set it down. Apparently he did not set it down elsewhere, nor has anyone since been able to prove the theorem; not Euler, nor Gauss, nor