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Transport of Potassium Ions across Planar Lipid Membranes by the Antibiotic, Grisorixin: I. The Equilibrium State and Self-Diffusion K^+ Fluxes

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Summary. $^{42}K^+$ tracer flux and steady-state conductance measurements were carried out with bilayer lipid membranes containing grisorixin, a carboxylic polyether antibiotic. When the membranes are placed between two bulk aqueous solutions of identical composition, the exchange or self-diffusion transmembrane flux of potassium is measured by a method which allows the characterization of the bilayer K^+ permeability at the equilibrium state. The K^+ self-diffusion flux increases with the pH in the range pH 6 to pH 9 and reaches a constant value for values above 9. This can be directly related to the increase of the surface concentration of the 1 : 1 complex formed by K^+ and the deprotonated polyether at both bilayer membrane interfaces. The transport model initially proposed by Pressman and coworkers (*Proc. Natl. Acad. Sci. USA* **58**:1949–1956, 1967) is again taken into consideration in the quantitative analysis of the flux data. The transmembrane transport of K^+ results from the translocation of its neutral complex with grisorixin and the association-dissociation of the antibiotic with either potassium or protons taking place at both interfacial space layers while the turnover of the mobile carrier is accomplished under asymmetrical conditions by a translocation process of the acidic grisorixin. Using the data of some previous studies for mixed ionophore-lipid monolayers at the air/water interface and the present results for the self-diffusion flux measurements, it was possible to propose an evaluation of the more important parameters characterizing the transport; namely, the total surface concentration of grisorixin, the interfacial pK and the translocation rate constant of its potassium neutral complex. The method proposed could be extended easily to other carboxylic polyethers, which would lead to an interesting comparison of their ionophoric properties using model membrane systems.

Key Words planar bilayers · grisorixin · carboxylic polyether ionophore · K^+ transport · radioactive tracer flux · self-diffusion flux · interfacial pK · diffuse layer

Introduction

Grisorixin belongs to the antibiotic class of carboxylic polyethers. Their original properties both in the fields of biology and chemistry have been reviewed recently in a monograph by Westley (1982, 1983). It is now well established that these carboxylic polyethers form with the alkaline and alkaline earth cation complexes of varying stoichiometry, by adopting macrocyclic-like conformations stabilized by hydrogen bonds. The formation of such cation-polyether complexes gives them the ability to transport alkaline and alkaline earth cations across lipid barriers, depending on the pH of the adjacent bulk phases. These ionophoric properties have been intensively studied in biological systems but less frequently in artificial lipid bilayers, black films and vesicles, and it would appear, that the main transmembrane transport mechanism involves a concomitant proton transport.

Besides their antibiotic activity, polyether ionophores have renal and cardiovascular effects and some of them are largely used in veterinary applications. They are frequently used in the field of cellular biology to modify the cation gradient in biological systems: cells and organites. As their cation transport properties reveal a certain specific selectivity, Na^+, K^+ and Ca^{2+} effluxes or influxes can be imposed and these are always coupled with a counter proton transport.

This paper deals with the ionophoric properties of grisorixin (*see* Fig. 1) in planar model lipid membranes using both $^{42}K^+$ tracer flux as well as electrochemical measurements. The experiments consist of determining both the fluxes of potassium and of electrical charges under different conditions (symmetrical or asymmetrical systems). The data can then be analyzed taking into account previous results obtained with mixed grisorixin-phospholipid monolayers at the air/water interface (Davion Van Mau et al., 1980; Amblard, 1983; Davion Van Mau & Amblard, 1983).

The ionophoric properties of some carboxylic polyether antibiotics (mainly X_{537A}, A_{23187}, nigericin and monensin) have up to now been extensively examined in the case of BLM's using essentially electrochemical methods (Mar & Pressman, 1972;

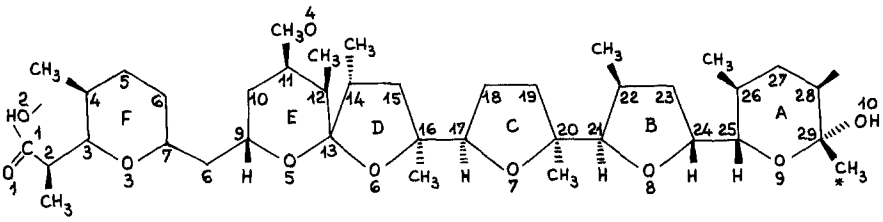

Fig. 1. Formula of the antibiotic grisorixin; the nigericin structure, it will be noted, differs from that of grisorixin only by the presence of an additional hydroxyl group on the carbon atom (*)

Celis et al., 1974; Markin, et al., 1975, 1977; McLaughlin & Eisenberg, 1975; Toro et al., 1976; Wulf & Pohl, 1977; Sandeaux et al., 1978; Kinsel et al., 1982; Amblard, 1983) and with spherical lipid bilayers either by radioactive tracers or spectroscopic measurements (Hyono et al., 1975; Degani & Elgavish, 1978; Hunt et al., 1978; Haynes et al. 1980; Kolber, 1980; Pohl et al., 1980; Kauffman et al., 1982).

On the contrary, little work has been published concerning their effects in black films using the radioactive tracer flux technique. This is due both to experimental difficulties and the low detection level of cationic fluxes through a small area membrane. The transport of alkaline earth cations by A_{23187} was thus first studied by Wulf et al. (1977) and Pohl et al. (1980), and that of Na^+ by monensin by Sandeaux et al. (1982); in the latter case a first series of experiments established that monensin carries Na^+ by an electrically silent transport process involving a counter-proton transport.

As opposed to the studies mentioned above, this paper deals with transmembrane K^+ fluxes across planar bilayers containing grisorixin, *at the equilibrium state*. As under such conditions there does not exist any difference between the composition of both bulk phases during the whole experiment, the addition of $^{42}K^+$ to one of the aqueous solutions makes a precise determination of the K^+ self-diffusion flux or exchange flux, $J^e_{K^+}$ while the electrical parameters of the membrane (capacitance and steady-state conductance) can be easily simultaneously measured.

Materials and Methods

Products

The bubble technique was used as a routine for the preparation of the membranes (area 1 mm²), from a lipidic solution of egg lecithin (SUPELCO) and cholesterol (MERCK) 2%: 2% wt/wt in *n*-decane (FLUKA purissimum), a composition which leads to suitably stable bilayers. The antibiotic grisorixin is introduced directly in the lipidic solution at the constant bulk concentration 0.01 M. The grisorixin sample was a gift from Dr. G. Jeminet (University Clermont-Ferrand, les Cézeaux, France), and had been highly purified by successive recrystallizations. The membranes were always formed using freshly prepared solutions. The water used was demineralized and twice distilled, once on alkaline permanganate. The KCl and Tris-methoxymethylaminomethane were, respectively, MERCK Suprapur and FLUKA puriss. samples. The latter was used as a buffer in the alkaline pH range.

The radioactive $^{42}K^+$ was obtained from the Radiochemical Center, Amersham, in the form of a concentrated stock solution of potassium chloride and then added to one of the aqueous solutions so that its final $^{42}K^+$ content is of the order of 5×10^{-3} M. The $^{42}K^+$ radioactive isotope emits γ rays and has a period of 12 hr 38 min and activity of 0.1 mCi/mg.

Methods

The detection limits of the K^+ fluxes are 10^{-11} mol cm^{-2} sec^{-1}. The cells were constituted by two cylindrical compartments. The inner one with a volume of approximately 1.5 cm³ was made of Teflon®. A hole of 1 mm diameter was punched into the side. The outer one was made of borosilicate glass with a volume of about 6.5 cm³. Aliquots (50 to 200 μl) of the "cold" aqueous solution were withdrawn every 10 to 15 min and the hydrostatic pressure difference compensated by the addition of an equal volume of aqueous solution. A correction is applied to compensate for the dilution effects of $^{42}K^+$. Experiments lasting as long as 2 hr were found to be possible.

The capacitance of the black films was measured by the charge pulse technique described elsewhere (Amblard et al., 1983), by means of an electrical setup including a Philips PM 5715 pulse generator and a Tektronix storage oscilloscope of the 5400 series. Stationary conductance measurements were determined with an applied voltage of 10 mV by recording the steady-state transmembrane current with a Keithley 427 current amplifier connected to a Tacussel TVED + EPLI recorder, two Ag/AgCl electrodes being used for these electrical measurements. Both aqueous solutions were stirred gently and continuously by means of small PVC stirrers driven by two electrical motors.

Results

The exchange K^+ flux and membrane conductance were measured simultaneously for each bilayer in order to distinguish electrically silent from charge transfer processes. The value of the self-diffusion flux $J^e_{K^+}$ was deduced from the variations with time of the number of $^{42}K^+$-labeled ions which cross the

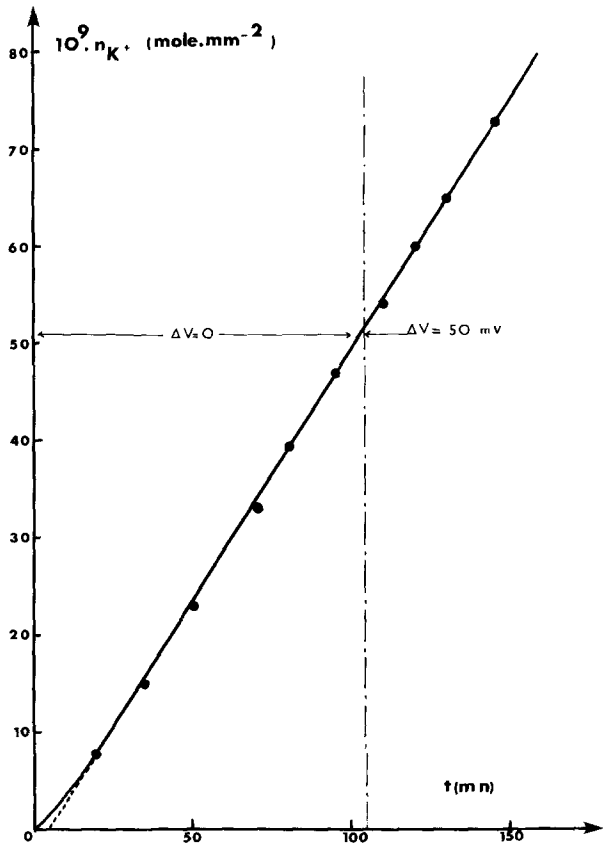

Fig. 2. Example of the experimental results obtained for the determination of the exchange flux $J^e_{K^+}$ (c_{KCl} 0.1 M; pH 7). After about 100 mn, a potential difference V_a = 50 mV was applied, which did not produce any detectable change in the slope of the n_K *vs.* t curve. n_K represents the total number of unlabeled potassium ions exchanged between both bulk aqueous phases as a time t of the experiment

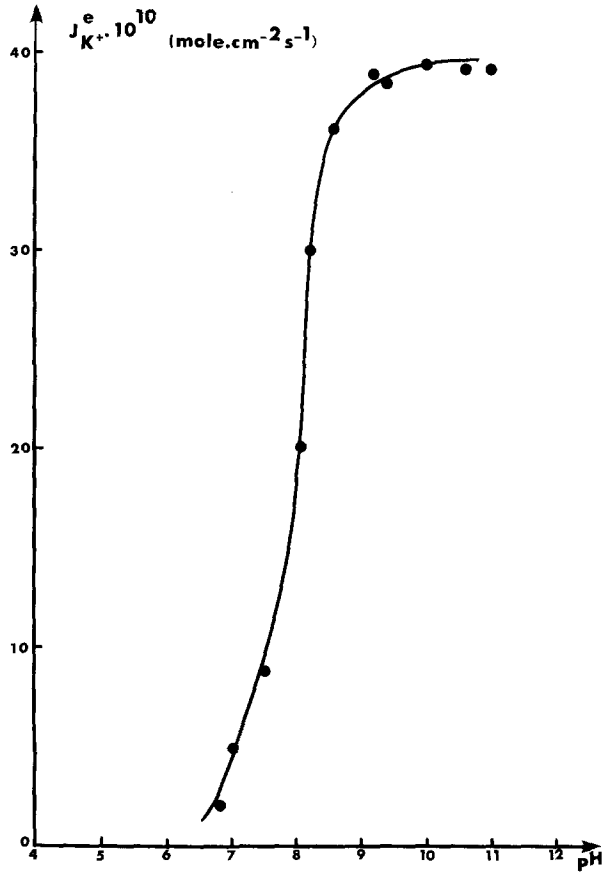

Fig. 3. Variations of the self-diffusion flux $J^e_{K^+}$ with the bulk symmetrical pH. (c_{KCl} 0.1 M)

bilayer under short-circuit conditions from the "hot" to the "cold" aqueous solution.

Figure 2 provides an example of the experimental curve n_{K^+} vs. time (n_{K^+} is the number of K^+ ions which have been transferred into the "cold" half-cell); the potassium chloride content of both aqueous solutions was 0.1 mole · liter^{-1} and their symmetrical pH 7. At the beginning of this experiment, the applied voltage ΔV was kept at zero (short-circuit conditions) except during short time lags where membrane conductance was measured (ΔV = 10 mV); at time t = 115 mn a 50-mV potential step was maintained until the end of the experiment; as appears from n_{K^+} variations, the transmembrane exchange flux of K^+ ions mediated by grisorixin remains unaffected by the transmembrane electrical field. About 5 min after the beginning of the measurements (steady state reaching mean time lag), the exchange flux (slope of the experimental curve) keeps a constant value. This very important experimental fact could be verified whatever the composition of the two aqueous solutions. It emphasizes the fact that the primary mechanism of transport of K^+ by grisorixin is electrically silent and does not depend on the transmembrane electrical field; however, a slight increase of the steady-state conductance of the grisorixin-enriched BLM's is revealed by the electrical results, the mean value of λ_{stat} for lecithin-cholesterol black films being of the order 10^{-8} Ω cm^{-2} under similar conditions (*see* Fig. 4).

Figure 3 shows the variations of the K^+ self-diffusion flux with the bulk pH; grisorixin concentration in the membrane-forming solution is 0.01 mole · liter^{-1} and the KCl concentration 0.1 mole · liter^{-1}. The shape of this curve is similar to a neutralization curve and in the basic pH range potassium fluxes keep constant values.

Figure 4 shows the variations with pH of electrical resistance of the bilayer, measured under similar experimental conditions as in Fig. 3. One will note that the pH value at which the conductance curve passes through a minimum, i.e. pH 8.0, is nearly the value corresponding to the inflection point of the curve in Fig. 3. When the potassium

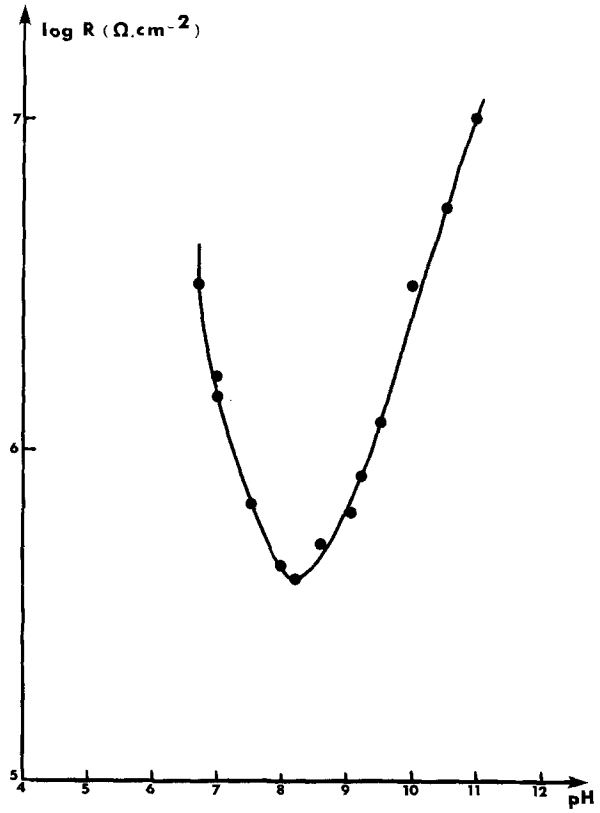

Fig. 4. Variations of the steady-state membrane resistance (semi-logarithmic plot) with the bulk pH. (Experimental conditions are the same as in Fig. 3)

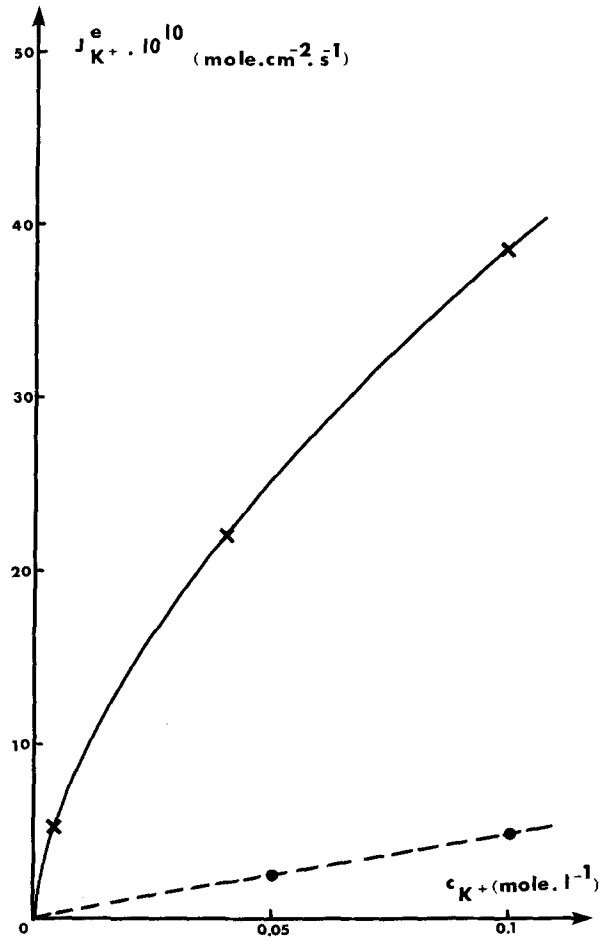

Fig. 5. Variations of the self-diffusion K^+ fluxes at pH 8.2 (×) and 7.0 (●) with the potassium symmetrical concentration

concentration of the two aqueous solutions is varied, the pH being kept constant, the values of $J^e_{K^+}$ and λ_{stat} show two different types of variations. At pH 7, $J^e_{K^+}$ varies linearly with c_{K^+} (*see* Figs. 5 and 6) while in the basic pH range (pH = 9.2 corresponding to the "plateau" mentioned above) the variations of $J^e_{K^+}$ are nonlinear and show a saturation-like shape. To sum up, these measurements of the radioactive tracer flux show that grisorixin markedly increases the K^+ permeability of planar bilayers and that the values of the self-diffusion transmembrane flux depends notably on the pH and the K^+ concentration but never on the transmembrane electrical field.

Discussion

The Transport Model

A quantitative analysis of the above data is now proposed on the basis of the simplest transport model first suggested by Pressman et al. (1967) within the framework of their studies of polyether antibiotics in mitochondrial systems. In this transport model only the three following forms of grisorixin are considered: (i) the protonated one GH, (ii) the dissociated one G^-, and (iii) the 1 : 1 complex GK. GK, GH, and G^- are assumed to remain located in the bilayer, more precisely at either of both membrane-solution interfaces (*see* Fig. 8). Proton exchange reactions take place between adsorbed G^- and the interfacial aqueous layers. Potassium ions are taken up by G^- or released from GK. Both neutral forms GH and GK of the ionophore are easily translocated through the membrane core, while the translocation rate of G^- is very low; this assertion is supported by the following experimental facts: at the air/water interface the dissociated G^- form is squeezed out of the mixed grisorixin-lecithin monolayers (Davion Van Mau et al., 1983), and is hence presumably situated just under the monomolecular layer as a result of its very slight water solubility; moreover, the translocation of this charged form across the central barrier is energetically less favored than that of both neutral GH and GK, which would explain the very low membrane con-

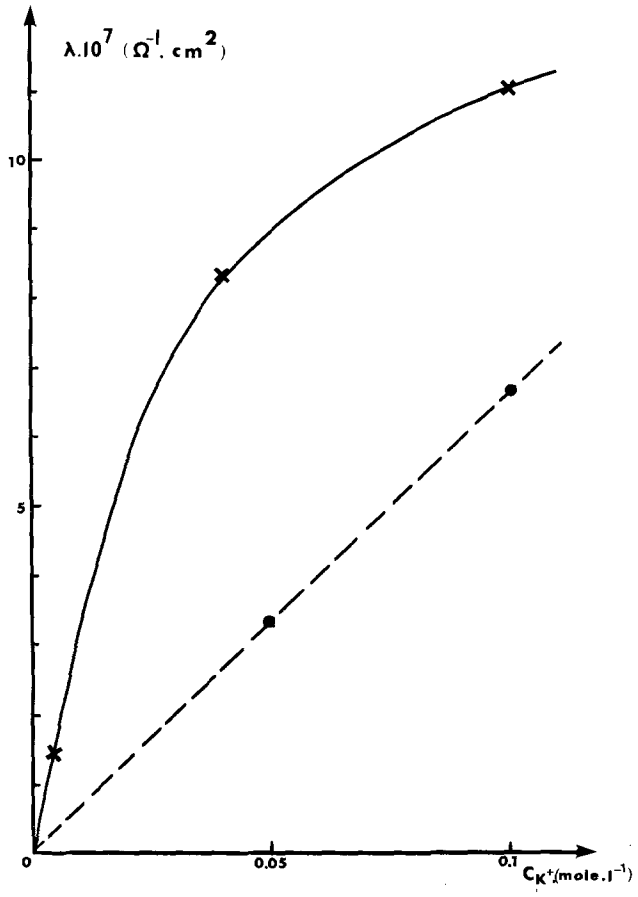

Fig. 6. Variations of the steady-state membrane conductance under similar conditions as in Fig. 5

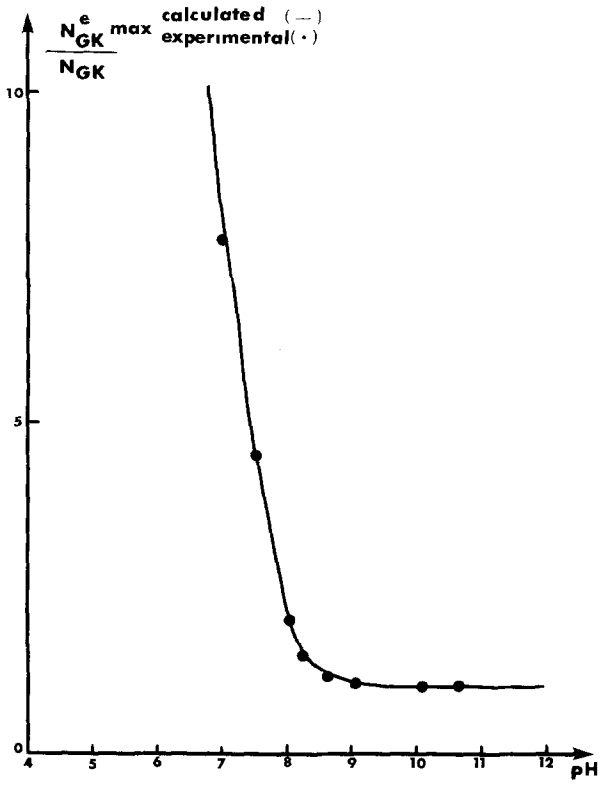

Fig. 7. Experimental (●) and theoretical (full line) values of the ratio N^e_{GK} max/N^e_{GK} as deduced either from the experimental data of Fig. 3 or from Eq. (7) of the text

ductance values obtained for basic pH's when $c_{K^+} \leq 0.001$ M (Amblard, 1983). To sum up the transport of one K^+ ion from each bulk aqueous phase to the other one is accomplished by the following steps:

Diffusion from the bulk to the closest approach plane 1′ of the aqueous side;

Complex formation reaction at the first interface;

Translocation of GK;

Dissociation of the complex at the second interface;

Diffusion in the second aqueous solution.

Similar steps are involved in the exchange process of protons from each aqueous solution to the other, involving translocation of GH in the core of the bilayer.

Expression of the Self-Diffusion Potassium Flux

It is now assumed that the translocation of GK constitutes the limiting step of the K^+ exchange process between the two bulk phases, which leads to the identification of $J^e_{K^+}$ and J^e_{GK}:

$$J^e_{K^+} = J^e_{GK} = k^T_{GK} \cdot N^e_{GK} \tag{1}$$

where J^e_{GK} is the exchange flux inside the bilayer, k^T_{GK} the rate constant of the GK translocation step and N^e_{GK} the surface concentration of the K^+-grisorixin 1 : 1 complex in the equilibrium state. The total surface concentration of the antibiotic at each BLM water interface will be referred to as N^e_T and N^e_T is given by expression (2):

$$N^e_{GH} + N^e_{GK} + N^e_{G^-} = N^e_T. \tag{2}$$

The constants K_{K^+} and K_{H^+} for both equilibria at the interface are now given by:

$$K_{K^+} = \frac{N^e_{GK}}{N^e_{G^-} \cdot c^i_{K^+}}$$

$$K_{H^+} = \frac{N^e_{GH}}{N^e_{G^-} \cdot c^i_{H^+}}. \tag{3}$$

$c^i_{M^+}$ represents the interfacial concentration of cation M^+ at the 1′ and 1″ planes. As the lipid used in this work has no net charge in the pH range under

study, the surface charge density σ (here expressed in μC cm^{-2}) can only be due to the anionic grisorixin G^- itself present on the membrane surface and hence can be given by Eq. (4) ($N^e_{G^-}$ is expressed in ion g cm^{-2}):

$$\sigma = -10^6 \cdot F \cdot N^e_{G^-}. \tag{4}$$

The interfacial concentrations $c^i_{M^+}$ are related to their bulk values c_{M^+} by the classical Boltzmann distribution equation

$$\begin{cases} c^i_{K^+} = c_{K^+} \exp(-f\,\Delta\Phi) \\ c^i_{H^+} = c_{H^+} \exp(-f\,\Delta\Phi) \end{cases} \tag{5}$$

where $\Delta\Phi$ is the diffuse layer potential and $f = F/RT$.

$\Delta\Phi$ in terms of the Gouy-Chapman theory is given by:

$$\Delta\Phi = \frac{2RT}{F} \sinh^{-1} \frac{\sigma}{11.74 c^{1/2}_{K^+}}. \tag{6}$$

If p is the correction term due to the diffuse layer charge distribution, i.e. equal to $\exp(-f\,\Delta\Phi)$, the above equations enable one to express the surface densities N^e_{GH} and $N^e_{G^-}$ and hence the self-diffusion flux J^e_{GK}:

$$N^e_{GK} = N^e_T \frac{K_{K^+} c_{K^+} p}{1 + (K_{H^+} c_{H^+} + K_{K^+} c_{K^+}) p} \tag{7a}$$

$$N^e_{GH} = N^e_T \frac{K_{H^+} c_{H^+} p}{1 + (K_{H^+} c_{H^+} + K_{K^+} c_{K^+}) p} \tag{7b}$$

$$N^e_{G^-} = N^e_T \frac{1}{1 + (K_{H^+} c_{H^+} + K_{K^+} c_{K^+}) p} \tag{7c}$$

$$J^e_{K^+} = k^T_{GK} \cdot N^e_T \frac{K_{K^+} c_{K^+} p}{1 + (K_{H^+} c_{H^+} + K_{K^+} c_{K^+}) p}. \tag{8}$$

Analysis of the Experimental Results

Considering now the data in Fig. 3 and the above Eq. (3), the pH at the inflection point already noticed on curve 3 is the apparent interfacial dissociation pK of the carboxylic function in the membrane-water interphase. Under these particular experimental conditions ($c_{K^+} = 0.1$ M), the parameter p can be assumed to be nearly equal to 1 as the Gouy potential is very small, so that:

$$K_{H^+} c_{H^+(\text{inflection})} = 1 + K_{K^+} c_{K^+}. \tag{9}$$

If one assumes that the equilibrium constant K_{K^+} is the same here as the one found at the air/water interface (Davion Van Mau et al., 1980), i.e. 5.4 mole^{-1} · liter, the value of K_{H^+} must lie between 1.0 and 1.6×10^8 mole^{-1} · liter, and that of the interfacial pK^i between 8.0 and 8.2. This value is considerably higher than the pK of the antibiotic in a bulk aqueous solution (around 5) but lower than the one found in a methanol solution (10.3) (Gachon et al., 1975).

Note that Kauffman et al. (1982) reported an interfacial pK^i of A_{23187} in the case of spherical bilayers corresponding to the bulk pK of the ionophore in a bulk mixture of water and methanol, 20%:80%. These remarks should provide some information on the dielectric constant of the interfacial water layer where the protonation-deprotonation reaction takes place.

A better comparison between the data on curve 3 and the theoretical expression (7a) is revealed by Fig. 7. Here, the values of the ratio $N^e_{GK(\text{plateau})}/N^e_{GK}$ are plotted against the bulk pH and as can be seen, there is excellent agreement between the experimental and the calculated curves.

The bulk concentration of grisorixin in the membrane-forming solution being 0.01 M, if the ionophore/lipid ratio is the same in the *n*-decane bulk phase as in each interfacial monomolecular layer then the molar fraction of the ionophore must be nearly equal to 0.1. Further, if one assumes that grisorixin occupies the same area per molecule here as it does near the collapse point of a lipidic monolayer at the air/water interface (Davion Van Mau et al., 1983), i.e. 140 Å^2 per molecule, then a value of about 10^{-11} mole · cm^{-2} can be deduced for its total surface concentration N^e_T. Returning now to the data of Fig. 5, a method for evaluating the true N^e_T value will be proposed and the results are gathered in Fig. 9. As k^T_{GK} is still an unknown parameter, the flux ratio for two different K^+ concentrations $c^{(i)}_{K^+}$ and $c^{(j)}_{K^+}$ can be referred to as $\theta^{(i,j)} = J^{e(i)}_{K^+}/J^{e(j)}_{K^+}$ and does not depend on the translocation constant.

$$\theta^{(i,j)} = \frac{p_{(i)} c^{(i)}_{K^+}}{p_{(j)} c^{(j)}_{K^+}} \cdot \frac{1 + K_{H^+} c_{H^+} + K_{K^+} c^{(j)}_K p_{(j)}}{1 + K_{H^+} c_{H^+} + K_{K^+} c^{(i)}_{K^+} p_{(i)}}, \tag{10}$$

In the calculation $c^{(j)}_{K^+}$ is arbitrarily chosen: 4×10^{-3} M corresponding to the smallest experimental concentration under study. Parameter θ is mainly dependent on N^e_T as it varies with both $p_{(i)}$ and $p_{(j)}$ and hence with the total negative charge density of anionic G^-. In brief, the higher N^e_T, the less $\theta^{(i,4\times10^{-3}\text{M})}$ increases with the potassium amount of the aqueous phases (*see* Fig. 9). Theoretical curves in Fig. 9 were obtained by iterative calculations

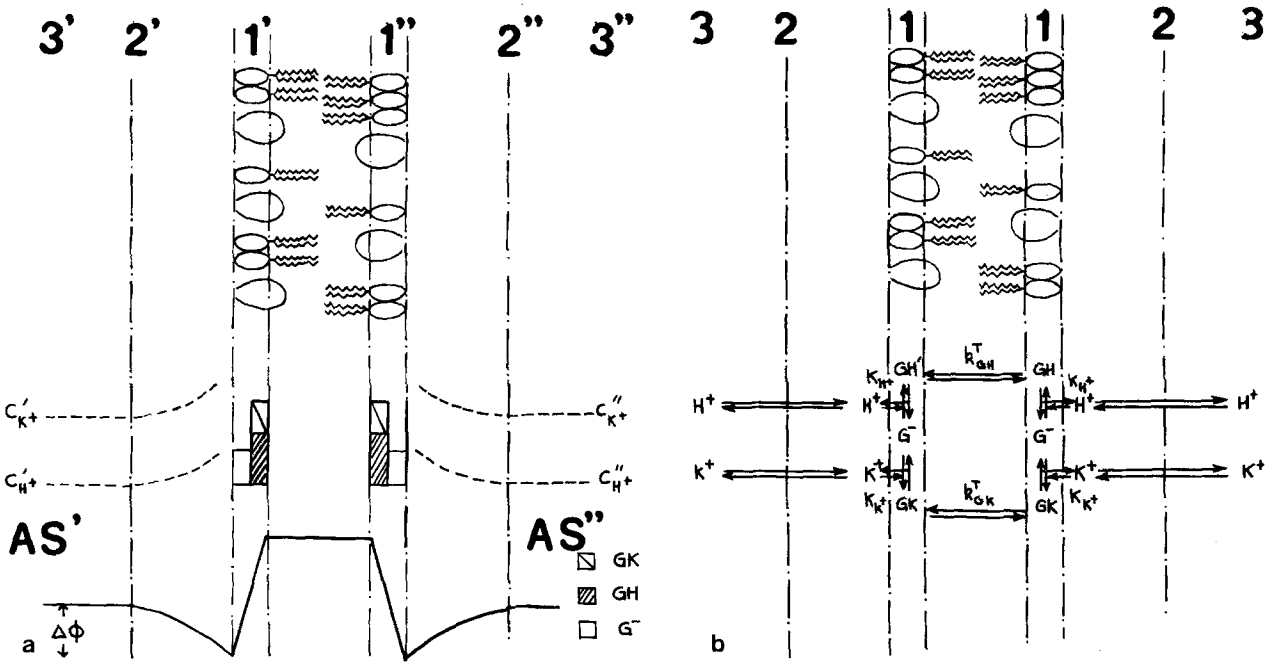

Fig. 8. Schematic representation of the transport model (*b*) and of the electrical transmembrane potential profile (*a*) in the whole membrane-aqueous solutions system

Table 1. Values of p and of the surface concentrations of the three grisorixin forms GK, G^- and GH calculated from Eqs. (4) to (10) for the value of N_T^e: 4.5 mole cm^{-2}

c_{K^+} (mole · liter^{-1})	4×10^{-3}	10^{-2}	4×10^{-2}	10^{-1}
$10^{13} \cdot N^e_{GK}$ (mole · cm^{-2})	3.8	3.4	9	17
$10^{13} \cdot N^e_{G^-}$ (mole · cm^{-2})	35	35	32	27
$10^{13} \cdot N^e_{GH}$ (mole · cm^{-2})	8.5	6.3	4	0.3
P	2.5	1.8	1.3	1.15

leading to successive determinations of parameters p, $N^e_{G^-}$, N^e_{GH}, N^e_{GK}, the first p value used for iteration being 1. The experimental dotted curve fits correctly for a value of N_T^e 4.5×10^{-12} mole cm^{-2} which is nearly half of the above evaluation.

These calculations make it possible to determine all the surface densities of GH, GK and G^- (*see* Table 1) and hence lastly that of the translocation rate constant k^T_{GK} (*see* Table 2). A nearly constant value k^T_{GK} of 2400 sec^{-1} illustrates the "turnover" of the 1 : 1 K^+-grisorixin complex. One notes that k^T_{GK} is about ten times as small as the translocation constants k_s, respectively 4 and 2.2×10^4 sec^{-1}, of free neutral valinomycin through monoolein and DMPC black films as reported in the review by Läuger et al. (1981); further, Benz and

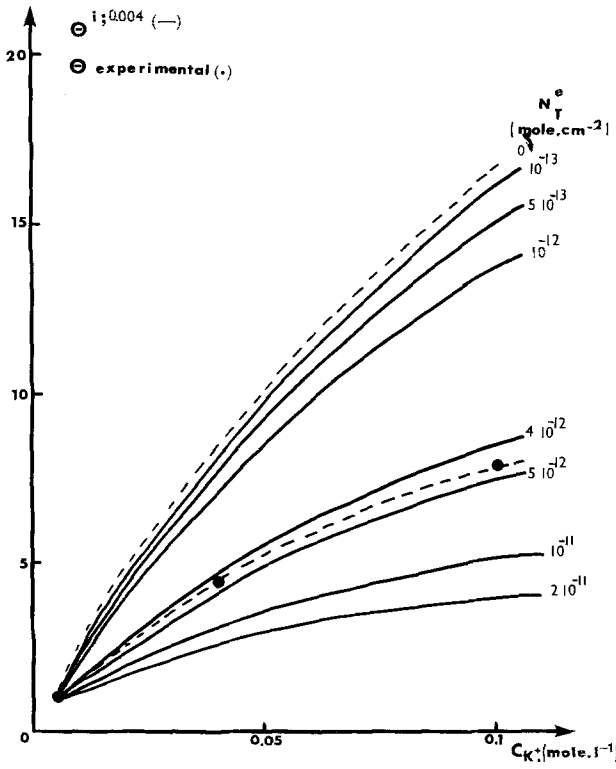

Fig. 9. Experimental (●) and theoretical (solid line) values of parameter $\theta^{i;0.004M}$ (see the text for the definition of θ) as deduced from Eqs. (4)–(10) for different arbitrary values of the grisorixin total surface concentration N_T^e at each BLM-A.S. interface

Table 2. Calculated values of k^T_{GK} (sec^{-1}) for different bulk pH values as deduced from the data shown in Fig. 3

pH	7	7.5	8.0	8.2	8.5	9	10
$10^{13} \cdot N^e_{GK}$ (mole · cm^{-2})	2.1	5	10	12.5	14.5	16	17
$k^T_{GK} = J^e_{K^+}/N^e_{GK}$ (sec^{-1})	2380	1800	1900	2500	2480	2400	2300

Cros (1978) noted a decrease of this translocation constant with an increase of the molar fraction of cholesterol in the membrane. So the translocation constant of GK is of the same order as that of free valinomycin.

Conclusion

The results reported above provide an example of the information on the behavior of carboxylic ionophores in model planar bilayers at the equilibrium state, which can be easily obtained by carrying out radioactive tracer cation flux measurements. The deduction of such equilibrium parameters is of basic importance for the study of asymmetrical systems, i.e. when there is a difference of pH or of the K^+ concentrations between the two bulk aqueous phases. It was shown here that the values of the self-diffusion K^+ fluxes are not at all negligible as they lie between 0.1 and 4×10^{-9} mole · cm^{-2} sec^{-1} in the basic pH range; in the second part of this flux study the data concerning such asymmetrical systems will be described and analyzed taking into account the above results. Moreover, the variations of the self-diffusion flux $J^e_{K^+}$ with pH can be compared with that of the surface potential of grisorixin monolayers at the air/water interface, spread out on a subphase of identical K^+ content (Davion Van Mau et al., 1980); as inflection points are observed for nearly the same bulk pH on both curves it thus appears that the apparent interfacial pK of grisorixin is the same at both BLM/water and air/water interfaces; as a consequence, the principal data obtained in monolayer studies can be correctly transposed to planar bilayer systems. The results reported in this first paper would benefit considerably from similar studies using other carboxylic ionophores and various alkaline and alkaline earth cations in order to establish correlations between their chemical structures and their different behaviors when present in bilayer model systems. Further investigations on the particular problem of the electrical properties of BLM enriched with grisorixin will be published elsewhere, these properties being due, as underlined above to a secondary transport mechanism of quantitatively minor importance.

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