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DETERMINATION OF RATE CONSTANTS IN CARRIER-MEDIATED DIFFUSION THROUGH LIPID BILAYERS

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SUMMARY

In this work data are presented on the relaxation current, under a voltage step, through soybean lipid bilayers in the presence of the carrier valinomycin. Measurements of voltage-dependent steady-state conductance have also been performed. These measurements are sufficient to calculate the full set of kinetic parameters determining the transport.

The data are analyzed according to the kinetic model, based on an Eyring treatment of the carrier-mediated diffusion. Complementary measurements of conductance as a function of antibiotic concentration have also been reported. These data allow one to calculate the membrane–solution partition coefficient of the carrier and the surface charge density of the membrane. The results are compared with those previously obtained with membranes of different lipid composition.

INTRODUCTION

Recent work[1,2] has indicated that stationary conductance measurements of lipid bilayer membranes in the presence of macrocyclic compounds, such as monactin or valinomycin, do not provide detailed information on the kinetics of carrier-mediated diffusion. We shall refer here to the kinetic model of transport, developed by Läuger and Stark[1], based on an Eyring treatment. An alternative procedure for the description of the transmembrane flux could be based on the model developed by Hall *et al.*[3], where a more specific shape for the energy barrier is considered.

For the determination of the rate constants of the kinetic model based on the Eyring treatment[1], additional electric relaxation experiments have to be performed[4,5]. Evaluation of the rate constants has already been obtained[5] in a system composed by phosphatidylinositol bilayer membranes, in the presence of valinomycin.

In this work we present data on the rate constants of ionic transport through bilayers formed from soybean lipid (which is a mixture of amphoteric and negatively charged phospholipids) in the presence of valinomycin. Stationary conductance measurements as well as electric relaxation measurements have been performed to determine the full set of kinetic parameters. Comparison of these values with those

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obtained by Stark *et al.*[5] can provide interesting insight into the effect of membrane lipid composition on carrier-mediated transport.

THEORETICAL CONSIDERATIONS

For the reader's convenience we shall summarize here some results through which the rate constants of transport can be evaluated. For more details the reader is referred to the original papers[1,2].

In this model translocation of the ion–carrier complex through the membrane is described as a jump over an activation barrier. The transport takes place in three steps:

(a) Ion-carrier association, characterized by the following heterogeneous reaction at the membrane solution interface between an ion M^+ from the aqueous phase (a) and a carrier S from the membrane (m):

$$M^+(a) + S(m) \underset{k_D}{\overset{k_R}{\rightleftarrows}} MS^+(m) \tag{1}$$

where k_R and k_D are the rate constants of the reaction.

(b) Translocation of the complex MS^+ to the other interface in a single step over a dielectric activation energy barrier. This process is characterized by the rate constant k_{MS}. At this stage the carrier S diffuses back and the cycle is closed.

The transmigration of the uncomplexed carrier across the membrane is described by the rate constant k_S. The whole process is schematized in Fig. 1, taken from Stark *et al.*[5], where

$$k'_{MS} = k_{MS} e^{-U/2} \tag{2}$$

$$k''_{MS} = k_{MS} e^{U/2} \tag{3}$$

and

$$U = \frac{FV}{RT} \tag{4}$$

V being the electrical potential applied to the membrane; F, R and T have the usual meaning.

This model is based on the approximation of negligible interchange of S and MS^+ across the interface. The concentration of the carrier S inside the membrane is determined by the partition coefficient γ_S, defined as:

$$\gamma_S = \frac{2N_S}{C_S d} \tag{5}$$

where d is the thickness of the membrane, N_S and C_S are interfacial and aqueous phase concentrations, respectively.

From the description of the model it is clear that four constants k_R, k_D, k_S and k_{MS}, are sufficient to describe the kinetics of the transport. Therefore, four independent experimental measurements involving transport parameters are required.

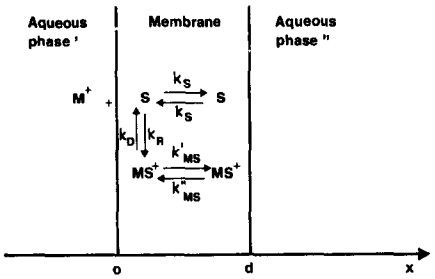

Fig. 1. Model of the carrier-mediated ionic transport. (From Stark *et al.*[5].)

Moreover, the determination of the partition coefficient of the carrier, γ_S, requires an additional measurement.

From the analysis of the kinetic model schematized in Fig. 1 the following relationships can be obtained[1,2] for the ratio of the specific conductance to the specific conductance in the limit of zero current λ_0:

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

where

$$A = \frac{2k_{MS}}{k_D} + \frac{k_R k_{MS}}{k_D k_S} C_M \tag{7}$$

C_M being the ionic concentration of the external aqueous solutions.

It has been shown[2] that the equilibrium constant K, for the ion–carrier association in the aqueous phase, in the case of valinomycin–potassium, is $K < 0.1\ M^{-1}$. Therefore, under our experimental conditions, we have $C_M K \ll 1$ and λ_0 reduces to[1,2]

$$\lambda_0 = \frac{F^2 d}{2RT} C_0 \gamma_s k_{MS} \frac{k_R C_M}{k_D(1+A)}, \qquad C_M K \ll 1 \tag{8}$$

where C_0 is the total carrier concentration in the aqueous solution.

The value of A is deduced from the best fit of λ/λ_0 (Eqn 6) to the experimental values at various applied potentials. Plotting A as a function of C_M one gets $2k_{MS}/k_D$ and $k_R k_{MS}/k_D k_S$.

Moreover, the plot of λ_0 as a function of C_0 allows one to calculate $\gamma_S\ k_{MS}\ k_R/k_D$.

These considerations show that the stationary conductance measurements give only three combinations of the five parameters to be determined. Therefore, additional relaxation experiments are required.

A voltage step applied to the membrane causes a relaxation current $I(t)$ due to a redistribution of the surface concentrations of complexed and uncomplexed carrier molecules, N_{MS} and N_S. One obtains[5]

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

where

$$\tau_1 = (a-b)^{-1} \tag{10}$$

$$\tau_2 = (a+b)^{-1} \tag{11}$$

$$a = \frac{1}{2}\left(C_M k_R + k_D + 2k_S + 2k_{MS}\cosh\frac{U}{2}\right) \tag{12}$$

$$b = \frac{1}{2}\left[\left(C_M k_R - k_D + 2k_S - 2k_{MS}\cosh\frac{U}{2}\right)^2 + 4C_M k_R k_D\right]^{\frac{1}{2}} \tag{13}$$

$$\alpha_1 = \frac{A}{2}\cosh\frac{U}{2} + B \tag{14}$$

$$\alpha_2 = \frac{A}{2}\cosh\frac{U}{2} - B \tag{15}$$

$$B = \frac{\cosh(U/2)}{4b}\left[A\left(C_M k_R + k_D + 2k_S - 2k_{MS}\cosh\frac{U}{2}\right) - 4k_{MS}\right] \tag{16}$$

$$I(\infty) = F(k'_{MS}\bar{N}'_{MS} - k''_{MS}\bar{N}''_{MS}) \tag{17}$$

$\bar{N}'_{MS}$ and $\bar{N}''_{MS}$ being the surface concentration of the ion-carrier complex when $t \to \infty$.

In many cases, because of experimental limitations of time resolution, only the larger relaxation time τ_1 and the relaxation amplitude α_1 can be detected.

However, the determination of τ_1 and α_1 are sufficient to provide the two additional combinations of constants necessary to calculate all four rate constants and the partition coefficient γ_S.

MATERIALS AND METHODS

The lipid employed was extracted from soybean (Carlo Erba–Milano), following the method (Type I) described by Szabo *et al.*[6]. Thin-layer chromatography gave the following lipid composition: phosphatidylcholine, 29%; phosphatidylinositol, 40%; phosphatidylserine and phosphatidylethanolamine, 31%. Gas chromatography, performed on the methyl esters gave the following fatty acid composition: palmitic acid, 19.5%; stearic acid, 5.5%; oleic acid, 8.2%; linoleic acid, 52.3%; linolenic acid, 6.7%. Small amounts of other fatty acids with a number of carbonium atoms smaller than 18:1% and with a number greater than 19:6.8%.

The soybean lipid was diluted with *n*-decane to form a solution of 1:20 by weight. Bilayer membranes were formed on a teflon diaphragm, with a circular hole of 1 and 1.5 mm of diameter in different experiments. Unless explicitly indicated the ionic strength of the external solution was held constant at 1 M in all experiments, by addition of LiCl. As already known[7] this cation has a negligible effect on the conductance in the presence of valinomycin.

Valinomycin was purchased from Calbiochem. (Los Angeles, Calif.). Con-

ductance measurements were performed using chloride coated silver plate electrodes with an area of about 5 cm^2.

The experimental arrangement for the electric relaxation experiments was similar to that described by Ketterer *et al.*[4]. A switch allows the selection between a constant voltage to be applied to the membrane (in the stationary conductance measurements), and a voltage step with a rising time smaller than 1 μs.

Two pulse generators are connected to the trigger of the oscilloscope and to that of the stimulator.

The oscilloscope is connected to an external resistance R_e in series with the membrane. The equivalent circuit is drawn in Fig. 2.

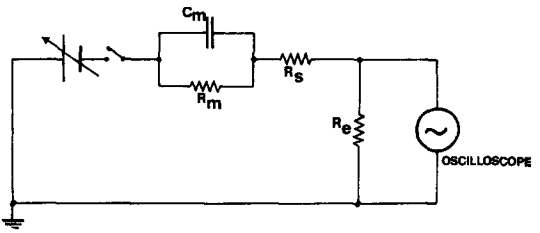

Fig. 2. Equivalent circuit of the cell, connected in series with an external resistance R_e, for the determination of the relaxation current. R_m and C_m represent the resistance and the capacitance, respectively, of the membrane; R_s is the combined resistance of electrodes *plus* solution.

The cell resistance R_s (electrodes *plus* solution) is about 200 Ω while the maximum value of the external resistance is $R_e = 440\,\Omega$. Under these conditions, we have $R_m \gg R_s + R_e$ and the charging time of the circuit $\tau_c \simeq C_m(R_e + R_s)$, where C_m is the membrane capacitance.

In the relaxation experiments we used cells with an opening of 1.5 mm in diameter so that $C_m \simeq 5$ nF. The same value was obtained with a direct capacitance measurement.

The maximum charging time of the circuit is therefore $\tau_c \simeq 3\,\mu$s.

RESULTS

Current–voltage characteristics were determined when the membrane was interposed between symmetrical ionic solutions in the presence of valinomycin at various concentrations (10^{-12}–10^{-8} M). Fig. 3 shows λ/λ_0 as a function of the applied potential U, at two different salt concentrations. As expected, the ratio proved to be independent of C_0. Each point is the mean value of at least three measurements using different membranes. The values of A fitting the experimental points are indicated. The experimental error of these points is of the order of 10%.

Fig. 4 shows values of steady-state conductance λ_0 as a function of valinomycin concentration at the indicated concentrations. The plot shows a linear dependence of λ_0 on C_0, as predicted by Eqn 8. The two measurements, performed at equal ionic strength, allow the calculation of γ_S once the rate constants of Eqn 8 have been evaluated. The value obtained is $\gamma_S = 10^4$.

Moreover, following the procedure indicated by Ciani *et al.*[8], the two curves at equal potassium concentration and different ionic strength, allow the calculation

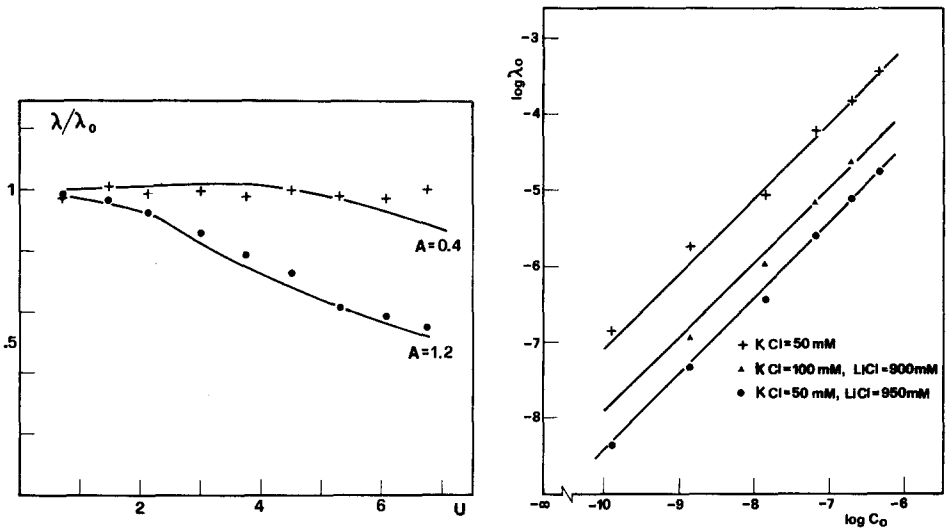

Fig. 3. Steady-state conductance ratio λ/λ_0, as function of the reduced potential U. The ionic strength is held constant at 1 M with the aid of LiCl. The full lines represent the theoretical curves calculated, *via* Eqn 6, with the indicated values of A. +, KCl 1 M; ●, KCl 10^{-2} M.

Fig. 4. Steady-state conductance in the limit of zero current, λ_0, as a function of valinomycin concentration, at the indicated values of ionic strength.

of the surface charge of the membrane. The value obtained is one negative elementary charge per 85 Å^2.

The electric relaxation measurements were performed in a 1 M KCl solution in the presence of valinomycin at concentration $C_0 = 2.5 \cdot 10^{-7}$ M. It has been shown[5] that the relaxation time and amplitude are independent of the carrier concentration, as expected from Relations 10–16.

A typical experimental run of the relaxation current at two different voltages is shown in Fig. 5.

Plotting $\ln [(I(t) - I(\infty))/I(\infty)]$ as a function of time t, for $t > \tau_C$ one obtains a straight line. This fact indicates that under such conditions, the relaxation process is determined by only one time constant.

Therefore we have:

$$\ln \frac{I(t) - I(\infty)}{I(\infty)} = \ln \alpha - \frac{t}{\tau} \tag{18}$$

The slope of Eqn 8 allows the calculation of the relaxation time, τ, and, extrapolating to zero, the relaxation amplitude, α, defined as

$$\alpha = \frac{I(0) - I(\infty)}{I(\infty)} \tag{19}$$

Eqns 10–16 show that both relaxation time and amplitude depend on the applied potential U. Therefore, we have performed relaxation measurements at different

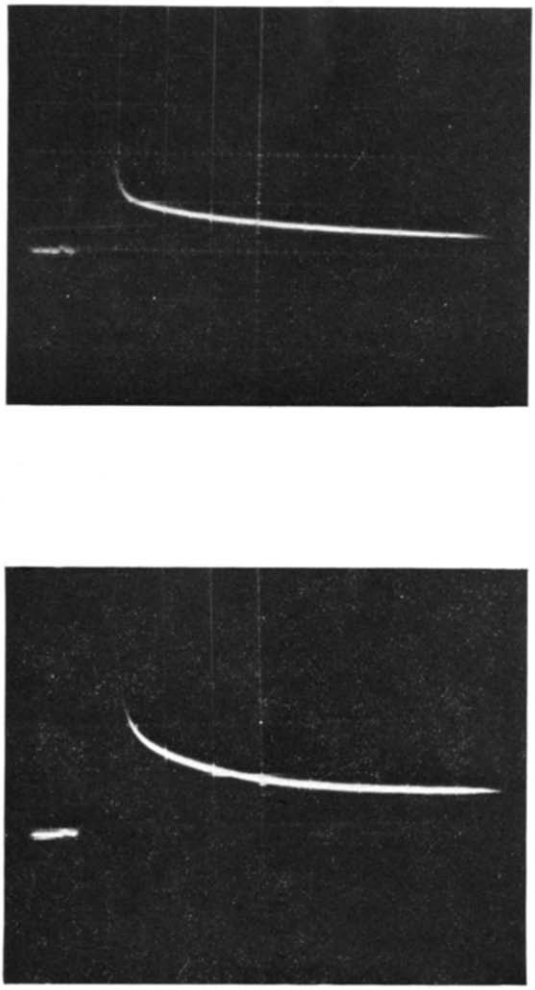

Fig. 5. Time course of the current after application of a voltage step. Upper curve: $U=1.9$ ($V=48$ mV); lower curve: $U=3.6$ ($V=90$ mV). Time scale: 10 μs/square; amplitude: 2.3 μA/square.

potential jumps. The results are shown in Figs 6 and 7. Each point is the mean value of at least three measurements on different membranes. At $U=1.9$ a greater number of measurements has been performed and the standard deviation of the observed values is of the order of 5%. Also reported in Figs 6 and 7 are the theoretical curves, calculated as explained below.

Determination of the rate constants

Fitting the experimental values of conductance (Fig. 3) to Eqn 6 one obtains $A=1.2$ ($C_M=1$ M) and $A=0.4$ ($C_M=10^{-2}$ M). Therefore the following values for the combinations of constants are deduced:

$$\frac{k_{MS}}{k_D}=0.2 \tag{20}$$

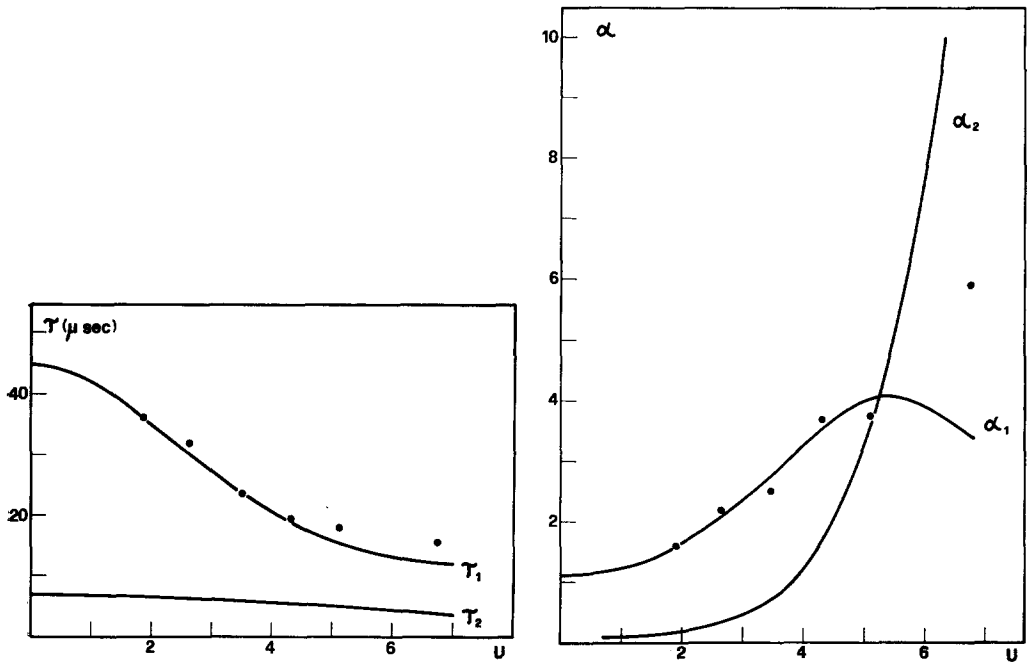

Fig. 6. Relaxation times as a function of the reduced potential U. The full lines represent the theoretical curves calculated *via* Eqns 10–13.

Fig. 7. Relaxation amplitudes as a function of the potential U. The full lines represent the theoretical curves calculated *via* Eqns 13–16.

$$\frac{k_{MS}k_R}{k_S k_D} = 0.8\ M^{-1} \tag{21}$$

Moreover, from the measurements of λ_0 as a function of C_0 as shown in Fig. 4, we have

$$\gamma_{MS}k_{MS}K = \left(\gamma_S k_{MS}\frac{k_R}{k_D}\right) = 1.3\cdot 10^4\ M^{-1}\cdot s^{-1} \tag{22}$$

The behavior of the relaxation current under a voltage step, for $t > \tau_C$, can be described by a single time constant, as previously observed. This fact means that the measured relaxation time coincides with the greater relaxation time τ_1, while the relaxation amplitude coincides with α_1 (*cf.* Eqn 9). This implies that either α_2 or τ_2, or both, are so small that they cannot be measured under the present experimental conditions. From Eqns 18 and 19 one gets for the mean value of τ_1 and α_1:

$$\alpha_1 = 1.6 \tag{23}$$

$$\tau_1 = 36\ \mu s \tag{24}$$

(values at $C_M = 1$ M, $V = 48$ mV)

From the data of Relations 20–24 and with the aid of Eqns 10 and 14 one can determine the constants characterizing the transport process. The values obtained are:

$$k_R = 7 \cdot 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$$

$$k_D = 4 \cdot 10^4 \text{ s}^{-1}$$

$$k_S = 2 \cdot 10^4 \text{ s}^{-1}$$

$$k_{MS} = 10^4 \text{ s}^{-1}$$

$$\gamma_S = 10^4$$

(The numerical values obtained by solving the system are $k_R = 6.56 \cdot 10^4$ M$^{-1} \cdot$ s^{-1}, $k_D = 4.22 \cdot 10^4$ s^{-1}, $k_S = 1.64 \cdot 10^4$ s^{-1}, $k_{MS} = 0.84 \cdot 10^4$ s^{-1}. These values have been employed in all the theoretical calculations.)

Substituting the values of the constants into Eqns 10, 11, 14 and 15, we obtain the trend of the theoretical curves of τ_1, τ_2, α_1 and α_2 as a function of voltage, to compare with the experimental points (see Figs 6 and 7). Notice that the theoretical curve τ_1 fits the measured relaxation times, while the values of τ_2 are much smaller. Fig. 7 shows that the curve α_1 fits the experimental points for voltages $U < 5$. At higher voltages the relaxation amplitude α_2 has a sudden increase and the experimental points do not agree anymore with α_1 or α_2. This fact means that at these values of voltage, the second relaxation process is no more negligible and the measured amplitude is a combination of α_1 and α_2.

We have determined the kinetic constants applying a voltage step $U = 1.9$, for which only one relaxation process is important. Therefore the hypothesis that the measured values of τ and α of Relations 23 and 24 coincide with τ_1 and α_1 is correct.

DISCUSSION

The values of the rate constants show that at $C_M = 1$ M KCl ($k_R C_M = 7 \cdot 10^4$ s^{-1}), all the rate constants are almost of the same order of magnitude (10^4 s^{-1}), the slowest process being the transmigration of the charged complex across the membrane.

At lower ionic concentrations, however, when $C_M \leqslant 10^{-2}$ M, the rate limiting step becomes the ion–complex association.

The fact that $k_{MS} < k_S$ can be attributed to the occurrence of an activation energy barrier for the charged complex, which is not experienced by the uncomplexed molecule.

If we compare the values of the constants obtained in the present system, with those determined by Stark *et al.*[5] with phosphatidylinositol membranes (see Table I), we observe that the rate constants, the partition coefficient and the surface charge σ, are of comparable magnitude.

However, while the relaxation amplitude is about the same as that determined by Stark *et al.*[5] we obtained a higher relaxation time.

Such behavior can be attributed to the lower value of the translocation ratio k_{MS} in the present system.

In fact, from Eqns 10, 12 and 13 one obtains $\partial \tau_1 / \partial k_{MS} < 0$, *i.e.* on decreasing k_{MS} the relaxation time increases.

TABLE I

COMPARISON OF THE RATE CONSTANTS, THE PARTITION COEFFICIENT AND THE SURFACE CHARGE DENSITY DETERMINED IN THE PRESENT SYSTEM WITH THOSE OBTAINED WITH PHOSPHATIDYLINOSITOL MEMBRANES[5]

	Soybean lipid	*Phosphatidylinositol*
k_R	$7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$	$5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$
k_D	$4 \cdot 10^4$ s^{-1}	$5 \cdot 10^4$ s^{-1}
k_S	$2 \cdot 10^4$ s^{-1}	$2 \cdot 10^4$ s^{-1}
k_{MS}	10^4 s^{-1}	$2 \cdot 10^4$ s^{-1}
γ_S	10^4	$6 \cdot 10^4$
σ	1 elementary charge per 85 Å^2	1 elementary charge per 60 Å^2

The finding that, even though the present system has a smaller surface charge density, nevertheless we obtain a greater relaxation time, suggests that the charge density is not the main factor in determining the relaxation process and possibly also uncharged membranes can exhibit measurable relaxation currents.

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REFERENCES

1 Läuger, P. and Stark, G. (1970) *Biochim. Biophys. Acta* 211, 458–466
2 Stark, G. and Benz, R. (1971) *J. Membrane Biol.* 5, 133–153
3 Hall, J. E., Mead, C. A. and Szabo, G. (1973) *J. Membrane Biol.* 11, 75–97
4 Ketterer, B., Neumcke, B. and Läuger, P. (1971) *J. Membrane Biol.* 5, 225–245
5 Stark, G., Ketterer, B., Benz, R. and Läuger, P. (1971) *Biophys. J.* 11, 981–994
6 Szabo, G., Eisenman, G. and Ciani, S. (1969) *J. Membrane Biol.* 1, 346–382
7 McLaughlin, S. G. A., Szabo, G., Eisenman, G. and Ciani, S. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 1268–1275
8 Ciani, S., Laprade, R., Eisenman, G. and Szabo, G. (1973) *J. Membrane Biol.* 11, 255–292
9 Shemyakin, M. M., Ovhinnikov, Yu. A., Ivanov, V. T., Antonov, V. K., Vinogradova, E. I., Shkrob, A. M., Malenkov, G. G., Evstratov, A. V., Laine, I. A., Melnik, E. I. and Ryabova, I. D. (1969) *J. Membrane Biol.* 402–430