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Increase in Lipid Microviscosity of Unilamellar Vesicles upon the Creation of Transmembrane Potential

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Summary. Diffusion potential of potassium ions was formed in unilamellar vesicles of phosphatidyl choline. The vesicles, which included potassium sulfate buffered with potassium phosphate, were diluted into an analogous salt solution made of sodium sulfate and sodium phosphate. The diffusion potential was created by the addition of the potassium-ionophore, valinomycin. The change in lipid microviscosity, ensuing the formation of membrane potential, was measured by the conventional method of fluorescence depolarization with 1,6-diphenyl-1,3,5-hexatriene as a probe. Lipid microviscosity was found to increase with membrane potential in a nonlinear manner, irrespective of the potential direction. Two tentative interpretations are proposed for this observation. The first assumes that the membrane potential imposes an energy barrier on the lipid flow which can be treated in terms of Boltzmann-distribution. The other interpretation assumes a decrease in lipid-free volume due to the pressure induced by the electrical potential. Since increase in lipid viscosity can reduce lateral and rotational motions, as well as increase exposure of functional membrane proteins, physiological effects induced by transmembrane potential could be associated with such dynamic changes.

Key words lipid fluidity · membrane potential · fluorescence polarization

Introduction

Membrane potential can be modulated by ambient physical factors like temperature [15] and pressure [56]. The effects can be mediated via changes in the membrane proteins [2, 21, 36, 37] or in the lipid bilayer [18, 35, 52]. While the effect on protein structure is specific for each case, the presumed effect on the lipid bilayer should be more general and less specific.

It is commonly agreed that the observed effect of hydrostatic pressure P on electrical potential of excitable membranes [17, 27, 57] can be related to changes occurring in the lipid bilayer which result in slowing down the rate of ion translocation, k, as expressed in Eq. (1) [25]:

$$k_P = k_o e^{-P\Delta V^{\ddagger}/RT} \tag{1}$$

where $\Delta V^{\ddagger}$ is the activation volume of the process. Similarly, one can ascribe this effect to changes in the lipid microviscosity $\bar{\eta}$ which by simulation to a macroscopic system should obey [19, 38]:

$$\eta_P = \eta_o e^{P\Delta V^{\ddagger}/RT}. \tag{2}$$

Experimentally, hydrostatic pressure [7, 53], as well as osmotic pressure [4], were found to affect membrane lipid fluidity. Moreover, the well-established lipid fluidizing effect of general anesthetics can be reversed by pressure, presumably via a similar mechanism [24, 26, 28].

If one assumes that the observed effects of pressure on membrane potential, on the one hand, and on lipid fluidity on the other hand can be internally coupled, then membrane potential may be expected to affect lipid fluidity and *vice versa*. Except for some preliminary observations [13, 33], this type of coupling has not yet been analyzed. The following study was undertaken to elucidate the coupling between membrane potential and lipid fluidity and its quantitative aspects, in artificial membrane vesicles.

Materials and Methods

Egg phosphatidylcholine (PC) grade 1 was obtained from Lipid Products (Nutfield, England). Dicetylphosphate (DCP), L-α-dipalmitoyl phosphatidylcholine (DPPC), cholesterol, valinomycin, gramicidin of highest purity were purchased from Sigma Co. (St. Louis, Mo). The fluorescent phospholipid, dansyl-phosphatidyl ethanolamine (DNS-PE) was prepared from egg phosphatidyl ethanolamine grade 1 (Lipid Products) and dansyl chloride as described [55]. The product was purified by preparative thin-layer chromatography. 1,6-Diphenyl-1,3,5-hexatriene (DPH), Puriss, was obtained from Fluka (Buchs, Switzerland). All inorganic substances were of analytical grade. Nigericin was kindly donated by Dr. Y. Shahak.

Unilammellar Vesicles

Sealed vesicles, composed of 95% PC (or 95% DPPC) and 5% DCP, used as models for biological membranes, were prepared by

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the conventional procedure of ultrasonic irradiation [22, 23]. PC and DCP in chloroform/methanol 1:1 (vol/vol) total weight of 10 mg were mixed with 4–20 µl of 2×10^{-3} M DPH in tetrahydrofuran in a sonication vessel. The mixture was then evaporated under nitrogen to complete dryness. Four ml of potassium salt solution (225 mM K_2SO_4, 50 mM K_2HPO_4, pH 6.7) were placed on top of the dried lipid [5] and the mixture was then sonicated at 90 watts for 20 min with a Branson sonifier model 160 at ~4 °C under nitrogen atmosphere. These conditions were previously verified to result in over 90% unilammelar sealed vesicles [34]. After sonication, the lipid suspension was centrifuged for 10 min at $30{,}000 \times g$ to remove undispersed residual material. 0.5 ml of the sonicated vesicles were passed through a G-25 sephadex column equilibrated and eluted with the same potassium salt solution. Only the middle portion (1 ml) of the eluted liposomes (2 ml) was used in the experiments. In a few experiments sodium, instead of potassium was included in the vesicles and both the procedure and the materials were analogous to the above. Similar vesicles were prepared analogously with 1% (wt/wt) DNS-PE instead of DPH.

Membrane Potential

The selected portion of sealed vesicles (~0.6 mg/ml) in the potassium salt was diluted into a sodium salt of exactly the same molarity and pH. The fluidity of the lipid layer was measured (*see below*) after about 30 sec and checked for stability up to 5 min later. Valinomycin was then added by a 1:1000 (vol/vol) dilution of a 1 mM stock solution in ethanol. At this final concentration of 10^{-6} M the number of valinomycin molecules per vesicles, for most experiments, was estimated to be around 100.

The presence of valinomycin facilitates the transport of potassium ions across the vesicles in both directions [12]. Assuming that sodium ions, as well as the anions, do not take part in this exchange process and are completely segregated, the net membrane potential E created by the valinomycin gating is due only to the concentration difference of potassium inside and outside the vesicle. We have therefore, estimated E from the simple form of Nernst equation:

$$E = \frac{RT}{F} \ln \frac{[K^+]_{in}}{[K^+]_{out}}. \tag{3}$$

At 25 °C, where most experiments were carried out $RT/F = 59$ mV.

Verification of the estimated E was performed by fluorescence quenching of the potential probe diS-C_3-(5) present at a concentration of 10^{-6} M as described [50, 54].

Lipid Fluidity

Steady-state fluorescence depolarization of the probe DPH was used for evaluation. The eluted DPH-labeled vesicles were highly fluorescent and even at the maximal dilution of 1:1000 into the sodium salt the fluorescence signal was sufficient for accurate determination of lipid microviscosity $\bar{\eta}$ [48]. Since the lifetime of excited state was found to be unaffected by the membrane potential (8.8 nsec at 25 °C) the parameter $2.4\left(\frac{r_o}{r}-1\right)^{-1}$ was used as an approximate scale for lipid microviscosity [48]. In this expression r is the fluorescence anisotropy, which is directly measured, and r_o is the limiting r value. At 366 nm (Hg band), which was used for DPH excitation, $r_o = 0.362$ [48].

For fluorescence measurements with DNS-PE the same instrumental setup was used and the lipid viscosity was presented by the relative scale of $\left(\frac{r_o}{r}-1\right)^{-1}$ [48]. The value for r_o was taken as 0.316 [47].

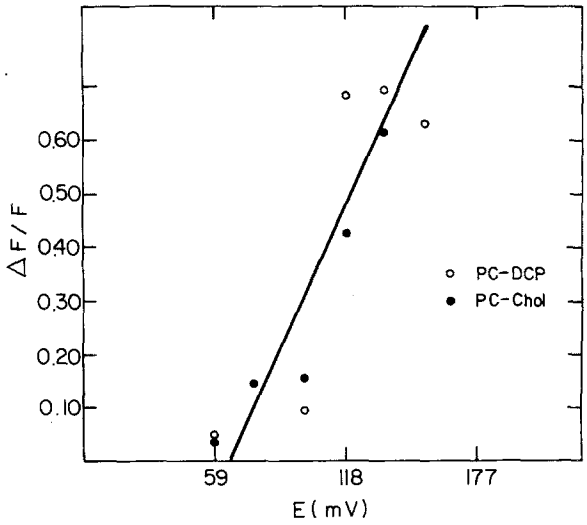

Fig. 1. Relative decrease in fluorescence intensity of the probe diS-C_3-(5) (10^{-6} M) as a function of membrane potential in PC-DCP (o) and PC-Chol (1.5:1, mole/mole) (•) vesicles

Results

Sealed vesicles including 275 mM potassium salt were diluted into 275 mM sodium salt followed by the addition of 10^{-6} M valinomycin (*see* Materials and Methods). The formation of membrane potential due to potassium gradient was verified by fluorescence quenching of diS$-C_3$-(5) and the results are given in Fig. 1. Above ~50 mV a linear dependence between the fluorescence quenching and membrane potential was observed [54]. For evaluation of the effect of valinomycin on the lipid fluidity, vesicles containing potassium salt were first diluted into identical potassium salt and the degree of fluorescence polarization was measured. Valinomycin was then added and the fluorescence polarization measurements were repeated. The effect of valinomycin on $\bar{\eta}$ was in all cases smaller than 1% increase. Nevertheless, for each measurement with membrane potential this control experiment was carried out and the results were corrected accordingly.

Preliminary observations have shown that upon the formation of membrane potential in the sealed vesicles by potassium gradient the fluorescence anisotropy of DPH slightly increased, irrespective of the potential direction. Since the excited state lifetime of DPH did not change with potential formation, the increase in DPH fluorescence polarization indicates an increase in rotational hindrance [48] presumably due to decreased lipid fluidity [48].

The results of a large series of experiments, presented as the percent change in $\bar{\eta}$ as a function of the potassium gradient or the corresponding E, are summarized in Fig. 2. Analogous experiments with

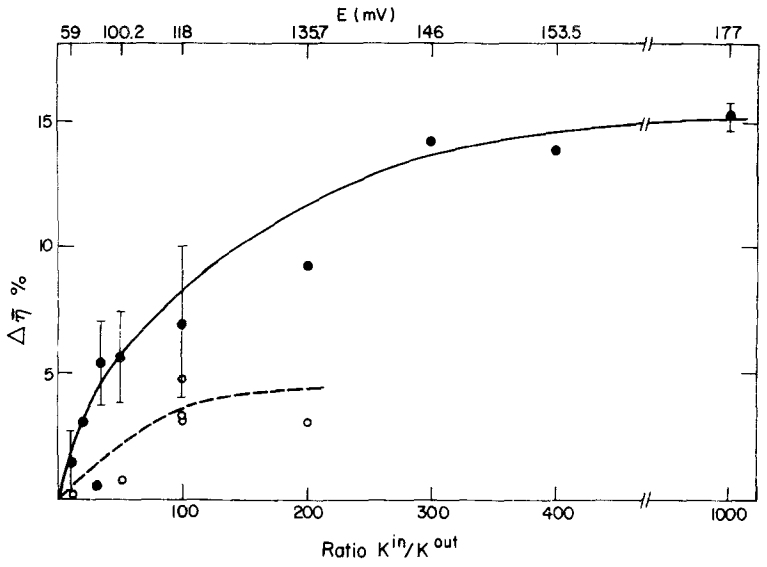

Fig. 2. Dependence of lipid microviscosity (presented as percentage change in $\bar{\eta}$) on diffusion potential of potassium ions across PC-DCP (●) and PC-Chol (1.5:1 mole/mole) vesicles (○)

vesicles containing cholesterol (1 mole per 1.5 moles of phospholipids), showed about twofold smaller increase in $\Delta\bar{\eta}$ with membrane potential (*see* Fig. 2). This suggests that the effect of membrane potential on lipid fluidity increases with the increase of the absolute value of $\bar{\eta}$.

Parallel experiments to the above were carried out with vesicles including 275 mM sodium salt which diluted into 275 mM potassium salt containing 10^{-6} M valinomycin. In these experiments as well, increase in $\bar{\eta}$ was observed presumably due to the penetration of a few potassium ions into the vesicles and the formation of membrane potential. This assumption was verified with sodium including vesicles which contained 1% potassium salt. The results obtained with these vesicles were similar to the converse experiment where potassium-including vesicles were diluted 1:100 into sodium salt (*data not shown*). To test whether the potassium transport itself could affect the lipid fluidity, experiments with the ionophore nigericin, which exchanges potassium and hydrogen ions [14, 42], were carried out at different K^+_{in}/K^+_{out} levels. In all experiments there was no effect on the apparent $\bar{\eta}$ value.

Attempts to abolish the potential and thus to return to the original lipid microviscosity were inconclusive due to technical difficulties. The addition of the ionophore gramicidin, which can translocate sodium as well as potassium across the vesicle wall [14, 42], was found to markedly affect the $\bar{\eta}$ value by itself and thus to mask the reversal of the relatively smaller effect due to the membrane potential. Upon the addition of potassium thiocyanate, which is expected to abolish the membrane potential by translocation of the thiocyanate ion, only partial reduction of $\bar{\eta}$ was detected. It was found that in the absence of membrane potential thiocyanate itself affects the lipid fluidity.

The effect of membrane potential on lipid fluidity at a region closer to the headgroup was measured with identical vesicles including 1% DNS-PE as a fluorescence probe instead of DPH (*see* Materials and Methods). The fluorescence spectrum of the dansyl moiety of this phospholipid, which is sensitive to the local dielectric constant [6, 47], exhibited a maximum at 522 nm, and band width of 90 nm similarly to that observed in 70% ethanol. These spectral characteristics indicate that in these vesicles the dansyl is located almost exclusively at a region of a dielectric constant of about 40, which is presumably the hydrocarbon-water interface. The effect of membrane potential on the degree of fluorescence polarization of DNS-PE indicated an increase in $\bar{\eta}$ which was much smaller than that observed with DPH in all dilutions tested. In the common experiment of $K_{in}/K_{out}=100$ the average increase in $\bar{\eta}$ with this probe was about 5% as compared to 8% obtained with DPH (*see* Fig. 2).

The small increase in $\bar{\eta}$ due to membrane potential observed in egg-PC vesicles labeled with DPH, suggested that in vesicles prepared from saturated phospholipid (e.g. DPPC) a resolvable shift in the liquid-crystalline to gel-state phase transition may be expected. Results of temperature dependence of lipid microviscosity $\left(\text{expressed in the relative units of } \left(\frac{r_o}{r}-1\right)^{-1}\right)$ in DPPC-DCP vesicles are shown in Figs. 3 and 4. As shown in Fig. 3 in the whole range of temperature (25–48 °C) the membrane potential

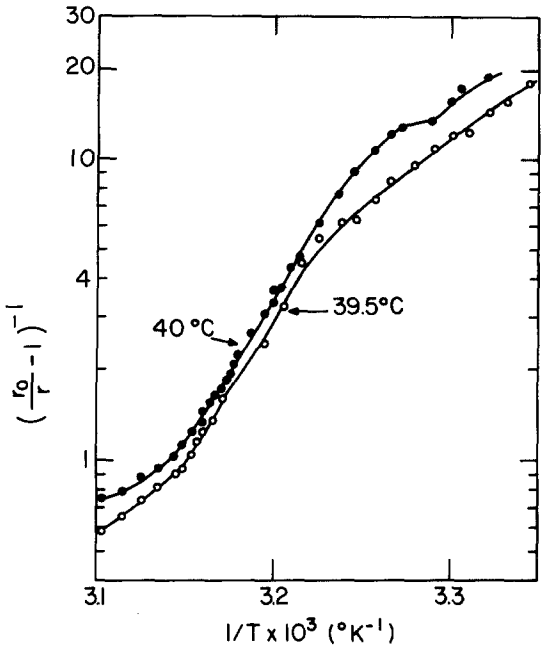

Fig. 3. Temperature profile of microviscosity $\bar{\eta}$ of DPPC vesicles labeled with DPH given as $\ln\bar{\eta}$ *vs.* $1/T$ in the presence (●) and absence (○) of a transmembrane potential of 118 mV *(see text)*

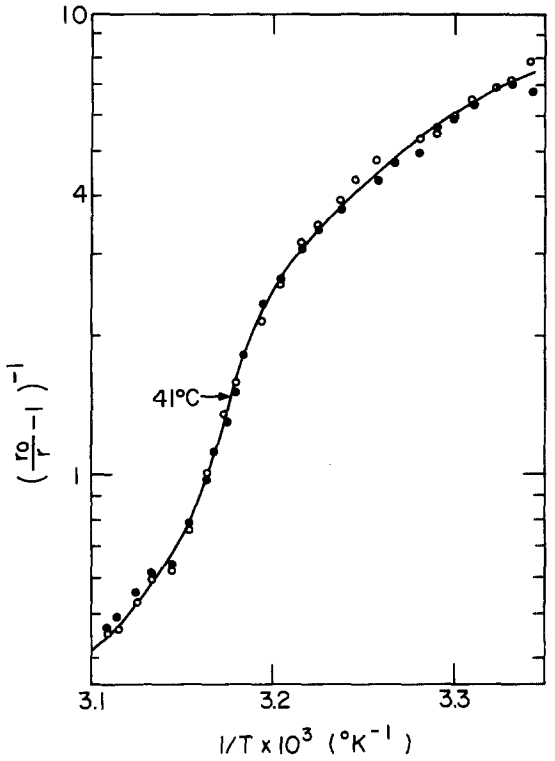

Fig. 4. Temperature profile of the microviscosity parameter $\left(\frac{r_0}{r}-1\right)^{-1}$ of DPPC vesicles labeled with DNS-PE in the presence (●) and absence (○) of 118 mV transmembrane potential *(see text)*

(118 mV) caused an increase in $\bar{\eta}$. Above the lipid phase transition the increase was around 14% similar to what was observed in the PC-DCP vesicles (*see* Fig. 1). Below the phase transition the increase in $\bar{\eta}$ was more pronounced (around 30%), while around the phase transition (~40 °C) the increase in $\bar{\eta}$ was the smallest. This experiment was repeated several times and in all cases an increase in the temperature of the phase transition of about 0.5 °C, could be resolved (*see* Fig. 4).

The temperature profiles of DPPC-DCP vesicles labeled with 1% DNS-PE (Fig. 4) revealed only a very small effect on the membrane potential. This demonstrated that in the fully saturated lipid region as well, the dynamics of the hydrocarbon-water interface is less sensitive to membrane potential than the hydrocarbon region.

Discussion

The formation of membrane diffusion-potential by a gradient of potassium ions was shown in this study to induce a small, but consistent, increase in lipid microviscosity. Most of the experiments were carried out with a higher potassium concentration inside PC vesicles and the increase in microviscosity was calculated as

$$\Delta\bar{\eta}=\frac{\bar{\eta}_{K-Na+Val}}{\bar{\eta}_{K-K+Val}}\cdot\frac{\bar{\eta}_{K-K}}{\bar{\eta}_{K-Na}}-1$$

where K−Na stands for vesicles with potassium inside and sodium outside, K−K for vesicles with potassium inside and outside and Val for addition of valinomycin. In this way most of the possible errors could be eliminated.

In principle the apparent increase in DPH fluorescence polarization could be interpreted as a result of alignment of the probe molecules along the created electric field. It can be shown, however, that the electric field of a magnitude of 10^5 volts/cm, which is associated with the membrane potential formed in our experiments, is too small to cause any significant alignment of DPH. The average kinetic energy U, of a field F, imposed on a molecule with a dipole moment of μ is given by

$$U=\mu F\int_0^{2\Pi}\cos\theta\,d\theta\approx\tfrac{1}{2}\mu F \qquad (4)$$

where θ is the angle between the dipole vector and the direction of the electric field. Assuming that DPH is highly polarizable in the excited state (in the ground state it is presumably totally apolar) and possesses $\mu^*=1D=10^{-18}$ esu/cm, for 100 mV of

membrane potential and membrane thickness of 50 Å, $F = 2 \times 10^5$ volt/cm $= 667$ statvolts/cm and therefore $U \sim 3 \times 10^{-16}$ ergs. For a distinct alignment this energy should be much greater than the thermal energy KT. Yet the latter at 25 °C equals $\sim 4 \times 10^{-14}$ ergs, namely more than two orders of magnitude higher than U. Furthermore, if one assumes that DPH is randomly oriented in the lipid bilayer, which is well above its phase transition [16, 32], then the net effect of U will be towards accelerating the DPH rotation rather than hindering it, namely to decrease its degree of fluorescence polarization.

The effect of increase in $\bar{\eta}$ by membrane potential seems to be confined to the hydrocarbon core as suggested from the experiments with DNS-PE. This is supported by NMR studies [58] showing that the phospholipid headgroups are not affected by membrane potential. The increase in $\bar{\eta}$ with potential is also reflected in a small increase in the melting temperature of DPPC. A similar effect on DPPC phase transition was also observed by X-ray diffraction upon application of pressure [51].

Elucidation of the mechanism involved in increasing lipid viscosity by membrane potential can be attempted by applying classical expression of viscosity to the hydrocarbon core of the lipid layer. This type of simulation is in fact used in evaluation of lipid microviscosity by fluorescence depolarization [48] and avoids the formidable complexity imposed by the anisotropic structure of lipid bilayers.

Viscosity of a Newtonian fluid obeys an interesting empirical analogy to reaction rate constant [19] which can be neatly resolved by the absolute reaction rate theory [31]. As such, it is affected by changes in thermodynamic parameters, like temperature or pressure, in a statistical fashion which obeys a Boltzmann distribution:

$$\eta = \eta_o e^{\Delta E^{\ddagger}/RT} \tag{5}$$

where η is the viscosity after a change in one of the thermodynamic parameters and $\Delta E^{\ddagger}$ is the corresponding activation energy per mole. $\Delta E^{\ddagger}$ therefore determines the magnitude of η/η_o when all other thermodynamic parameters remain constant. Accordingly, the dependence of η on temperature is determined by $\Delta E_t^{\ddagger}$, the thermal flow-activation energy, which is approximately the change in enthalpy in the process of flow-activation. Changes in η due to pressure (P) are determined by $\Delta E_P^{\ddagger} = P\Delta V^{\ddagger}$ where $\Delta V^{\ddagger}$ is the activation volume of the flow process, namely the difference in volume of the flow unit (which in simple Newtonian fluids is the effective volume of the molecule itself) in the activated and the resting states [*see* Eq. (2)]. In noncompressible fluids $\Delta V^{\ddagger} \sim 0$ and therefore pressure does not affect η.

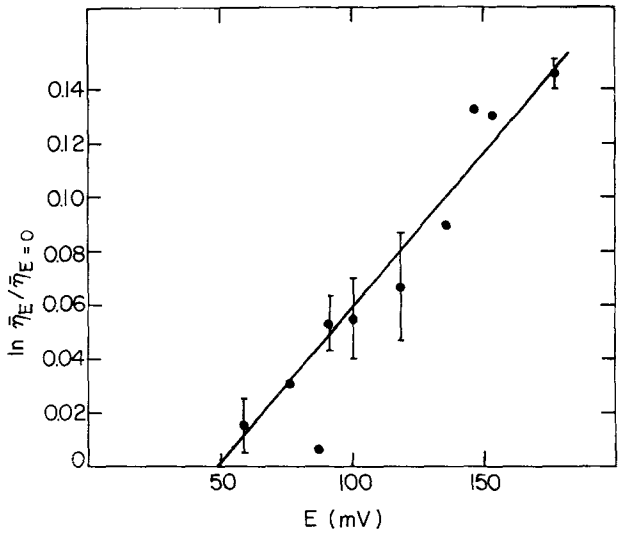

Fig. 5. Replot of the data given in Fig. 2 as $\ln(\bar{\eta}_E/\bar{\eta}_{E=0})$ versus the transmembrane potential

The above approach, which relates the effect of temperature or pressure on viscosity to flow-activation, can, in principle, be applied to the effect of membrane potential on lipid microviscosity, which was described in this study. The electrostatic effect on the lipid fluidity can be ascribed to the difference in charge distribution $\Delta Q^{\ddagger}$ in a flow unit between the activated and the resting states. Accordingly:

$$\bar{\eta}_E = \bar{\eta}_{E=0} e^{E\Delta Q^{\ddagger}/RT} \tag{6}$$

where $E\Delta Q^{\ddagger}$ is the flow activation energy due to the electrical potential. (When E is given in volts and Q in coulombs $E\Delta Q^{\ddagger}$ is given in joules and R is taken as 8.31 joules deg^{-1} mole^{-1}). A replot of the data given in Fig. 2 as $\ln \bar{\eta}_E/\bar{\eta}_{E=0}$ *vs.* E is shown in Fig. 5. As can be seen, at $E \gtrsim 50$ mV Expression (6) fits well with the experimental data. The slope of the line, $\Delta Q^{\ddagger}/RT = 1.19$ volts^{-1}, reveals that $\Delta Q = 3 \times 10^3$ coulombs mole^{-1} or $\Delta Q^{\ddagger} \dfrac{3 \times 10^3}{96{,}500} \sim 3 \times 10^{-2}$ ionic equivalents. In other words, the interpretation described in Eq. (6) suggests that during the flow activation process a transient increase of about 3% of the net charge in the flow unit takes place. In principle, this transient change in charge density can be brought about by perturbation of the equilibrium arrangment of the phospholipid headgroups or by a transient net diffusion of 3×10^{-2} equivalents of ions (either potassium or any of its counterions) per each

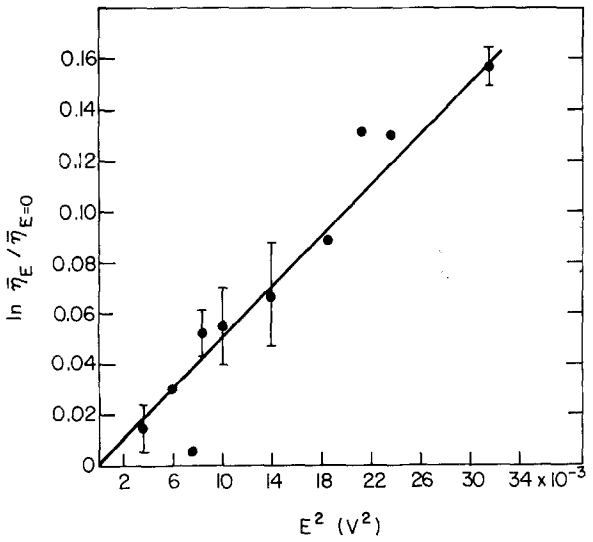

Fig. 6. Replot of the values given in Fig. 2 as $\ln(\bar{\eta}_E/\bar{\eta}_{E=0})$ versus the square of the transmembrane potential

mole of flow unit. The barrier of ~50 mV, could be ascribed to a potential breakdown point (E_o) beyond which the flow becomes dependent on membrane potential:

$$\bar{\eta}_E = \bar{\eta}_o e^{(E-E_0)\Delta Q^*/RT}. \tag{7}$$

It is interesting that a similar barrier was also observed in the change in fluorescence intensity of diS$-C_3$-(5) which could be related to the apparent change in $\bar{\eta}$ (Fig. 1).

The effect of membrane potential on flow-activation can be, alternatively ascribed to the pressure P, created by the potential which in turn affects $\bar{\eta}$ according to Eq. (2). The pressure-potential relation is given by

$$P = \frac{C \times E^2}{2d} \tag{8}$$

where C is the capacitance of the lipid layer, taken as $1\,\mu F/cm^2$ or 9×10^5 esu/cm^2 [20], d is the thickness of the hydrocarbon layer (taken as 5×10^{-7} cm) and E is taken in statvolts. The conversion of the membrane potential E (in volts) to P (in dyne $\cdot$ cm^{-2}) is therefore given by $P \approx 10^7 E^2$ which by substitution in Eq. (2) leads to

$$\bar{\eta}_E = \bar{\eta}_{E=0} e^{10^7 E^2 \Delta V^*/RT}. \tag{9}$$

A replot of the data given in Fig. 2 as $\ln \frac{\bar{\eta}_E}{\bar{\eta}_{E=0}}$ *vs.* E^2 is shown in Fig. 6. From the slope of the linear dependence a value of $\Delta V^* = 12$ liter/mole is derived. This volume is about 10 times greater than that of a mole of phospholipid. According to such an interpretation the change in molecular volume at the flow-activated state is about 10-fold which seems rather unlikely. It is noteworthy that in a recent study, where the dependence of lipid microviscosity on hydrostatic pressure was analyzed, it was found that ΔV^* for egg-PC is only 22 cm^3/mole (*in preparation*). This large difference in ΔV^* values could be partially accounted for by the fact that the pressure associated with membrane potential is vertical to the lipid-bilayer, where the compressibility is much smaller than the lateral compressibility [51]. Hydrostatic pressure, on the other hand exerts its effect mostly through lateral compression [51].

The interpretations given above are all based on simple statistical assumptions analogous to the Boltzmann distribution. Yet, from the stationary point of view, one can interpret the effect of membrane potential on lipid viscosity as exerted by changes in the average effective volume of the hydrocarbon constituents. This can be analyzed by Batschinski's equation [1] which holds exceptionally well for hydrocarbon fluids:

$$\eta = \frac{B}{V - V_\infty} = \frac{B}{\Delta V} \tag{10}$$

where V is the specific volume (i.e. the reciprocal of density), V_∞ is the limiting value of V (at infinite viscosity) and B is a constant. According to this empirical relation the viscosity is inversely proportional to ΔV which is the "vacant-volume" between the molecular backbones. It can be assumed that the observed change in $\bar{\eta}$ ensuing the change in membrane potential is mostly mediated by changes in V. Further, since interdigitation of the phospholipid chains due to electrostriction was found to be negligible [8–10], the change in V is approximately proportional to the change in the effective surface area A of the lipid molecules. Therefore, between two states (e.g. with and without diffusion potential)

$$\frac{\bar{\eta}_1}{\bar{\eta}_2} = \frac{A_2 - A_\infty}{A_1 - A_\infty} \tag{11}$$

where A_∞ is the limiting surface area, the upper value of which can be taken as ~40 Å^2, which is approximately the area of phospholipids at the collapse pressure [29]. Equation (11) indicates that the relative reduction in A ensuing the formation of membrane potential is smaller than the relative increase in $\bar{\eta}$. A possible mechanism for the decrease in the average surface area of the lipids upon the formation of membrane potential E, is a putative energy coupling between E and the surface pressure.

Changes in membrane potential of up to ~100 mV occur during most electrophysiological processes in nature. Membrane potential of such a magnitude was found to affect the structure of lipid layers in the squid axon [45, 46]. In addition, changes in membrane potential were found to affect carrier-mediated transport [30, 41], ion translocation [3, 39, 40], channel formation [43, 44] and assembly of membrane proteins [11, 52]. If these functional changes are associated with transient modulation of the membrane lipid viscosity, as those observed here in lipid vesicles, they could be related to changes in diffusion processes of the membrane proteins. It may be therefore proposed that transient increase in membrane potential may transiently decrease protein rotational and lateral diffusions, as well as modulate their degree of exposure to the external surrounding [49], with all of its physiological consequences.

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