Biochimica et Biophysica Acta, 465 (1977) 429—442

BBA 77654

PROPERTIES OF BILAYER MEMBRANES IN THE PRESENCE OF DIPICRYLAMINE

A COMPARATIVE STUDY BY OPTICAL ABSORPTION AND ELECTRICAL RELAXATION MEASUREMENTS

J. WULF *, R. BENZ and W.G. POHL

Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz (G.F.R.)

(Received August 24th, 1976)

Summary

In order to test the question if a pool of lipophilic ions may exist in black lipid membranes which cannot be detected by electrical relaxation measurements we have performed simultaneously measurements of the optical absorption of a lipophilic ion. The absorbance of membrane-bound dipicrylamine at 410 nm was measured with a sensitive spectrophotometer which can detect absorbance changes $\geqslant 4 \cdot 10^{-5}$. A minimal concentration of about $6 \cdot 10^{11}$ dipicrylamine ions per cm^2 of the membrane could be detected with this instrument. The dipicrylamine concentration in the membrane obtained with the optical method N_t^{opt} is compared with the concentrations N_t^{el} obtained from simultaneous electrical relaxation measurements. N_t^{opt} and N_t^{el} agreed at low dipicrylamine concentrations (10^{-8}—10^{-7} M in the aqueous phase) and showed saturation at higher concentrations (up to $5 \cdot 10^{-6}$ M). In the saturation range N_t^{opt} was maximally four times higher than N_t^{el}. The significance of this difference is discussed together with general aspects of the saturation phenomenon.

Introduction

Black lipid membranes first described by Müller et al. [1], have been used in the past as tools for physicochemical studies as well as models for biological membranes. Transport phenomena across these membranes have been studied by many investigators [2—6]. It has been found that lipid bilayer membranes

* Present address: University of Ulm, Abteilung Biologie IV, D-7900 Ulm, G.F.R.
Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

show a high specific resistance ($\approx 10^8\ \Omega \cdot cm^2$) in the presence of common electrolytes like NaCl or KCl in contrast to biological membranes [7]. This high specific resistance is lowered by many orders of magnitude if certain carrier antibiotica like valinomycin or nonactin are present. Similar effects have been found if weak organic acids like dinitrophenol or carbonylcyanide-*n*-chlorophenylhydrazone are added to the aqueous phase [8].

Large organic ions, such as tetraphenylborate or dipicrylamine, added to the aqueous phase do not change the steady-state conductivity of bilayer membranes considerably [3,4,9], but cause current transients under voltage clamp conditions. These transients have been studied in detail by different authors [9—12] and it has been found that this is caused by redistribution of charges across the membranes as well as by diffusion polarization associated with concentration changes of the permeable ion in the unstirred layers adjacent to the membrane surface, which result from current flow through the membrane [9,13].

A detailed mechanism for the transport of hydrophobic ions across lipid bilayer membranes has been proposed earlier [9]. From the data of relaxation measurements it has been shown that the transport of lipophilic ions occurs in three different steps, namely, adsorption from the aqueous phase to the interface of the membrane, translocation to the opposite interface and desorption into the aqueous phase. The rate constant k_i for the exchange of lipophilic ions between the deep potential-energy minima at the membrane solution interface was derived from the current transient [9—11]. A value for the rate constant k_{ma} of the transport from the membrane into the aqueous phase cannot be given because of the slow aqueous diffusion [14]. The interpretation of the fast current transient in terms of a redistribution of ions across the central energy barrier has been confirmed in recent voltage-jump and charge-pulse relaxation experiments [12,15].

For small concentrations of lipophilic ions the concentration in the membrane was found to be proportional to the concentration in the aqueous phase [9—11,15]. For larger concentrations ($c \geqslant 10^{-7}$ M for dipicrylamine) saturation phenomena for the hydrophobic ions in the membrane were observed [9,11,15]. The effect has been discussed on the basis of binding sites at the membrane surface for the lipophilic ions [16]. Recently, McLaughlin [17] gave an estimation of the surface potential on the basis of a refined adsorption model and received higher values than Ketterer et al. [9]. Surface potentials of the magnitude estimated by McLaughlin [17] are sufficient to explain the observed saturation in the binding of lipophilic ions to the bilayer membrane. In the saturation range, a large decrease of the translocation rate constant k_i has been observed. It is not clear, whether this effect is caused by electrostatic interactions mentioned above and/or rather by a change in the structural properties of the membrane.

So far it was not clear whether the concentration of lipophilic ions in the membrane derived by electrical measurement agrees with the real total concentration. It could not be excluded that a special pool of lipophilic ions exists in the membrane which did not participate in the charge transport. To decide this question and to get more insight into the saturation phenomena occurring at high concentrations of lipophilic ions more information about the real concen-

tration in the membrane is needed. This information may be obtained by optical measurements. Recently, Pohl et al. [18] measured the concentration of dansyl-valinomycin in the membrane by electrical as well as by fluorescence measurements and found with the optical method a higher concentration than derived from the conductance measurements. These measurements were not performed simultaneously because of the high time resolution needed for electrical measurements which required a small membrane area, whereas a large area was necessary for the fluorescence measurements.

In this publication we describe experiments with lipophilic ions performed at the same membrane with optical absorption and electrical relaxation measurements. For the optical absorption experiments with dipicrylamine as lipophilic ion, a spectrometer with a resolution of about $4 \cdot 10^{-5}$ absorbance units was constructed. With an extinction coefficient of dipicrylamine of $\epsilon \approx 2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ a sensitivity of $4 \cdot 10^{-5}$ allows to detect about $6 \cdot 10^{11}$ dipicrylamine ions per cm^2 of the membrane.

Description of the transport model

Lipid bilayer membranes have a non-polar interior and therefore form thin barriers ($\approx$ 5 nm) in aqueous solutions. For common electrolytes such as KCl or NaCl the membrane has a high specific resistance ($10^8\ \Omega \cdot cm^2$). This is caused by the high energy needed to introduce a small ion from the aqueous phase into the membrane which makes the solubility of these ions in the membrane extremely small. In contrast, for large organic ions such as dipicrylamine or tetraphenylborate the energy of transfer is substantially lowered so that an increased permeability is observed.

The transport of lipophilic ions through a lipid bilayer membrane is assumed to occur in three different steps [9]. (i) Adsorption from the aqueous phase (concentration c') to the left side of the membrane (surface concentration N' in mol/cm^2, rate constant k_{am}); (ii) translocation for the left side energy minimum across the central barrier to the minimum at the right side (surface concentration N'', rate constant k_i); (iii) desorption from the right side energy minimum to the aqueous phase (concentration c'', rate constant k_{ma}). The rate of change of N' and N'' per unit time is given by the following equations:

$$\frac{dN'}{dt} = k'_{am}c' - k'_{ma}N' + k''_i N'' - k'_i N' \tag{1}$$

$$\frac{dN''}{dt} = k''_{am}c'' - k''_{ma}N'' - k''_i N'' + k'_i N' \tag{2}$$

At equilibrium (voltage $V_m = 0$) the interfacial concentrations for identical aqueous solutions ($c = c' = c''$) are equal and given by the partition coefficient γ:

$$N' = N'' = N_t/2 \tag{3}$$

$$\frac{N_t}{2c} = \frac{k_{am}}{k_{ma}} = \gamma\frac{d}{2} \tag{4}$$

N_t is the total concentration of the lipophilic ion in the membrane and d is the membrane thickness. The rate constants k'_{am}, k''_{am}, k'_{ma}, k''_{ma}, k'_i and k''_i are, in

principle, functions of the applied voltage V_m. In the limit of small voltage, however, the voltage dependence of k_{ma} and k_{am} can be neglected [12]. For k_i' and k_i'' the following voltage dependence according to an Eyring treatment may be assumed for monovalent lipophilic ions:

$$k_i' = k_i \exp(+u/2) \tag{5}$$

$$k_i'' = k_i \exp(-u/2) \tag{6}$$

where u is the reduced voltage:

$$u = \frac{V_m \cdot F}{RT} \tag{7}$$

R is the gas constant, T the absolute temperature and F the Faraday constant.

The solution of the Eqns. 1 and 2 under non-stationary conditions (voltage jump) was given previously [9]. For the membrane current $I(t)$ the following expression has been found:

$$I(t) = I_\infty + (I_0 - I_\infty) \exp(-t/\tau) \tag{8}$$

with

$$\tau = \frac{1}{k_{ma} + 2k_i \cosh(u/2)} \tag{9}$$

I_0 is the initial current and I_∞ the current at long times. In the limit of small voltages ($|u| << 1; |V_m| << 25$ mV), Eqn. 9 reduces to:

$$\tau = \frac{1}{k_{ma} + 2k_i} \tag{10}$$

In addition the following expressions hold for the initial conductivity $(\lambda_0)_{t=0}$ and the conductivity $(\lambda_0)_{t=\infty}$ at long times:

$$(\lambda_0)_{t=0} \equiv \lambda_{00} = \frac{F^2}{2RT} N_t k_i \tag{11}$$

$$(\lambda_0)_{t=\infty} \equiv \lambda_{0\infty} = \frac{F^2}{2RT} N_t \frac{k_{ma} k_i}{2k_i + k_{ma}} \tag{12}$$

Under the assumption $k_i >> k_{ma}$ [9,10] or in the case of slow aqueous diffustion [14] Eqn. 10 reduces to:

$$\tau_0 = \frac{1}{2k_i} \tag{13}$$

Materials and Methods

Design of the spectrophotometer

In Figs. 1 and 2 the optical set-up and a block diagram of the electronic system are shown. The light source was a xenon-mercury arc lamp (Hanovia 901B1, 200 W, Newark, N.J., U.S.A.) and was supplied by a Hewlett-Packard Model 6274A dc power supply (stability better than 0.03% during 8 h). Monochromatic light was obtained by using a grating monochromator (model 33-86-48

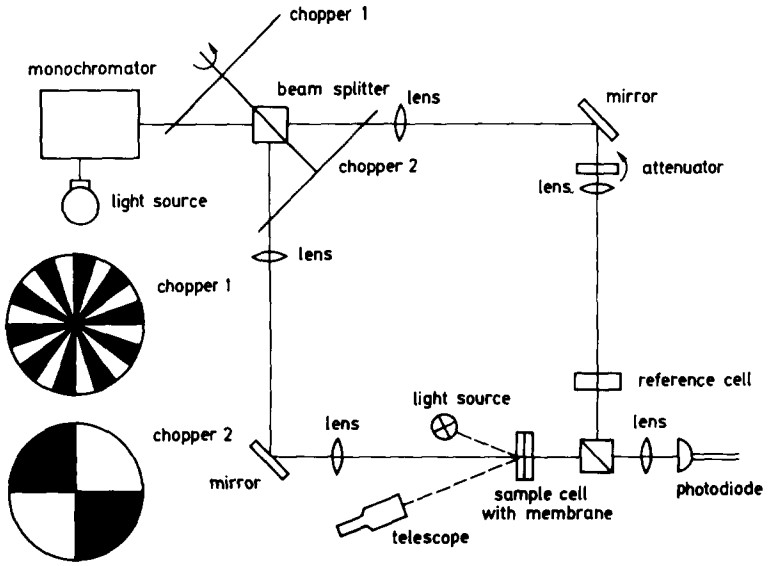

Fig. 1. Principle of the spectrometer (chopper 1 has in reality 20 black sectors).

from Bausch and Lomb, Rochester, N.Y., U.S.A.) with a bandwidth of 6.6 nm combined with an achromatic condensor. The light was modulated by means of a rotating chopper (1) and was split into two beams. A second chopper (2) synchronized to the first by mounting it on the same axis chopped the light with a low frequency alternatively into the two light paths [19]. The sample beam was focussed on the membrane and after passing the cell the beam was recombined with the reference beam in a second beam splitter. For balancing the light intensities of sample and reference beam a light attenuator was installed in the reference

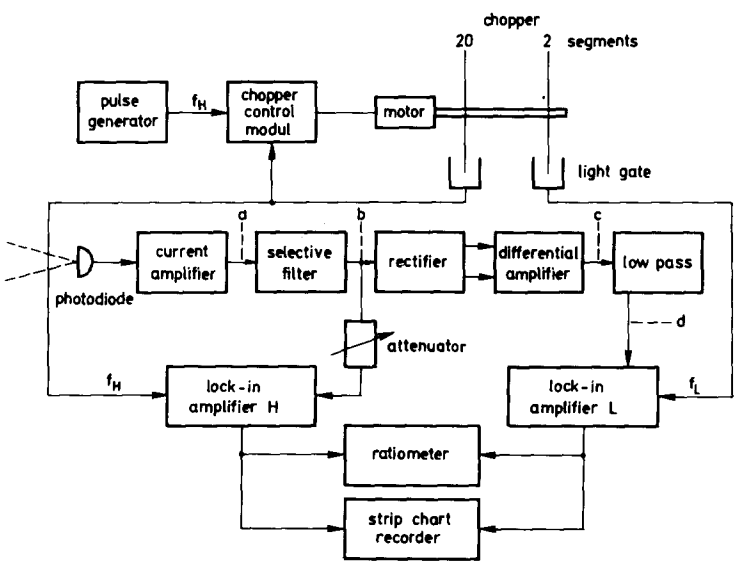

Fig. 2. Block diagram of the electronic circuit of the spectrometer.

channel. A soot-blackened platelet was mounted on a worm reduction gear and allowed smallest light attenuation by altering the angle of the platelet. The recombined light signal was detected by a photodiode (PIN 10 UV United Detector Technology, Santa Monica, Calif., U.S.A.). In Fig. 3a the signal is represented which is generated by the two choppers. The incident light was pulsed with the high frequency $f_H \approx 115$ Hz so that the amplitude of this frequency was proportional to the sum of the light intensities of both reference and sample beams. The second chopper had only one-tenth of the segment number of the first and therefore modulated the light with a frequency $f_L = 11.5$ Hz. The amplitude of the group frequency f_L was proportional to the difference of the reference and sample beam intensities. The signal amplitudes at both frequencies were detected by two lock-in-amplifiers which were synchronized by light gates at the chopper wheels. The amplitude of the f_H signal was measured by a lock-in amplifier model 220 from Princeton Applied Research, N.J., U.S.A. The total signal was fed into a selective amplifier tuned to f_H which rejects signals of interfering frequencies and forms all pulses to a unique shape (Fig. 3b). A precision rectifier (output signal given in Fig. 3c) combined with a low-pass filter (model 4213 Ithaco, Ithaca, N.Y., U.S.A.) demodulated the low frequency signal (Fig. 3d) which was thereafter coupled to the second lock-in amplifier (model 391A Ithaco). The prefiltering with the selective filter improved the phase stability of this lock-in. From the sum and difference signals of both lock-in amplifiers the negative absorbance was obtained using a

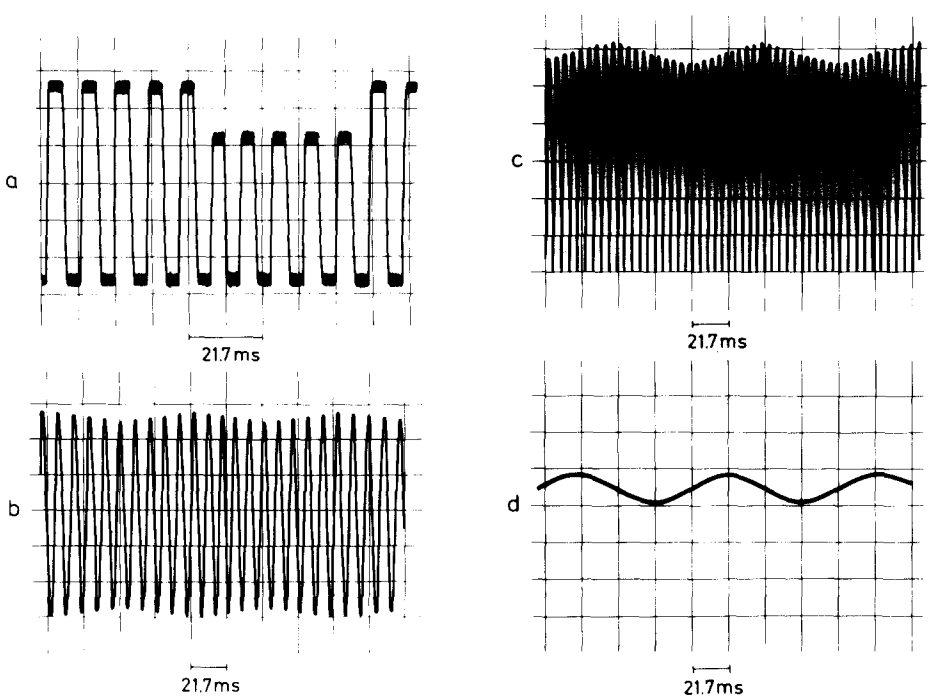

Fig. 3. Electronic signals at different points a, b, c and d of the electronic circuit as indicated in Fig. 2. For further explanations see text.

ratiometer (model 3512, Ithaco) according to the equation $-A = \lg [(|y| - |x|)/(|y| + |x|)]$ (y and x being the high frequency and low frequency lock-in signals, respectively). For absorbances smaller than 10^{-3} a strip chart recorder was used for direct recording of the signals x and y. The absorbance was calculated from the equation given above. Before measuring, the gain of the lock-in amplifiers was calibrated. This was done by occluding the sample beam. In order to balance both signals prior to the formation of a membrane, a variable electronic differential amplifier (model AM502, Tektronix) was used to attenuate the x-amplitude to exactly the same value as y. The linearity of the instrument was tested with a series of dye solutions of varying concentration and with grey filters of known transmission. With dye solutions absorbance changes of $4 \cdot 10^{-5}$ could be detected at wavelengths between 260 and 700 nm.

Membrane experiments

Black lipid membranes were formed in the usual way [20] from a 1% (w/v) solution of 1,2-dierucoyl-*sn*-glycerol-3-phosphorylcholine in *n*-decane. The lipid was synthesized and purified in our laboratory by Janko [21]; it gave a single spot in a thin-layer chromatogram. Dipicrylamine (Fluka, Buchs, Switzerland, purissimum) was used as a concentrated stock solution in ethanol. Small aliquots of the stock solution were added to a 0.1 M NaCl (Merck, Darmstadt, G.F.R., analytical grade) solution in twice-distilled water to get a final concentration between 10^{-8} and $5 \cdot 10^{-6}$ M. The aqueous phase was buffered with 10^{-2} M HEPES (Serva, Heidelberg, G.F.R., analytical grade) and had a pH of 6. Some experiments were performed in aqueous solutions without HEPES and some with KCl instead of NaCl. The results of these experiments agreed with those obtained unter normal conditions (0.1 M NaCl, 10^{-2} M HEPES) within the scatter of the experimental data. All experiments were performed at 25°C. The cell used for bilayer formation was made from black Teflon. Each compartment on both sides of the wall had a volume of 5 ml. The circular hole in the Teflon wall had a diameter of 4 mm (area 12.5 mm^2). Because of the large membrane area a long time for blackening of the membranes was needed (20 min). All measurements were performed 30 min after the membrane had turned completely black. During this time a substantial increase of the concentration N_t of dipicrylamine in the membrane was observed. After this period N_t increased very slowly.

Voltage-jump measurements were performed as described previously [9]. A battery-operated pulse generator with a rise time of about 200 ns (built in our electronic workshop) was used together with a storage oscilloscope (Tektronix 5115/5A22). The current was measured as a voltage drop across a series resistor. Depending on the required time resolution of the set-up the resistance was chosen between 100 Ω and 1 kΩ. Small voltages of about 10 mV were applied through Ag/AgCl electrodes to the membrane.

For the analysis of the optical measurements the absorbance of dipicrylamine dissolved in different solvents has been measured with a double beam spectrometer (Zeiss, DMR 10). It was found that for more polar solvents like *n*-hexanol, ethanol and water the wavelength of the absorption maximum was larger than for non-polar solvents like *n*-hexane or chloroform. The molar extinction coefficients for several solvents at the wavelength of maximal

TABLE I

Wavelength of maximal absorption λ_{max} and molar extinction coefficient $\epsilon_{\lambda max}$ obtained at λ_{max} for dipicrylamine dissolved in different solvents (at a concentration of $7 \cdot 10^{-6}$ M). The aqueous solutions contained 0.1 M NaCl. $T = 25°C$.

Solvent or membrane system	λ_{max} (nm)	$\epsilon_{\lambda max}$ (10^4 $M^{-1} \cdot cm^{-1}$)
n-Hexane	370	1.0
Chloroform	376	1.7
1,4-Dioxane	380	1.7
1-Hexanol	412	2.9
Ethanol	412	2.6
0.1 M NaCl, pH 1.5	379	1.6
0.1 M NaCl, pH 6	420	2.2
2% (w/v) egg phosphatidylcholine in 0.1 M NaCl, 10^{-2} M HEPES, pH 6	420	2.5
Dierucoyl phosphatidylcholine/*n*-decane membranes + 10^{-2} M dipicrylamine in the membrane-forming solution aqueous phase: 0.1 M NaCl, 10^{-2} M HEPES, pH 6	≈410	—

absorption are given in Table I. The molar extinction coefficient of dipicrylamine added to a 2% (w/v) dispersion of egg phosphatidylcholine in 0.1 M NaCl is also given in Table I. This measurement was performed in order to study the dipicrylamine molecule in a similar environment as in a planar bilayer membrane.

It is seen from Table I that the shift in the wavelength of the absorption maximum may be caused, in principle, by different protonation states of dipicrylamine. This is suggested from the results at low pH where most of dipicrylamine is protonated (pK = 2.66 [22]), whereas at high pH nearly all is protonated. The wavelenght of maximal absorption of dipicrylamine in membranes made from dierucoyl phosphatidylcoline in *n*-decane was measured in the following way. Dipicrylamine was dissolved to a concentration of 10^{-2} M in the membrane-forming solution. The absorption was measured at wavelengths between 370 and 430 nm, for at least three membranes at each wavelength, by taking the difference of the signal before and after destroying the membrane. The results indicate that most of the dipicrylamine in the membrane is deprotonated.

The other optical measurements with planar membranes were performed at a wavelength of 410 nm. The absorbance was calculated from the difference of the signal before and after destroying of the membrane by an electrical pulse. The absorbance for undoped membranes ($\approx 5 \cdot 10^{-5}$) was subtracted in each case. For the evaluation of the dipicrylamine concentration in the membrane the extinction coefficient for dipicrylamine dissolved in the suspension of egg phosphatidylcholine vesicles ($\epsilon = 2.5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) was used.

Results

Electrical and optical measurements were performed at the same membrane. The results of the electrical measurements are given in Fig. 4 and 5. The initial specific conductivity λ_{00} at low voltages as well as the time constant τ_0 of the

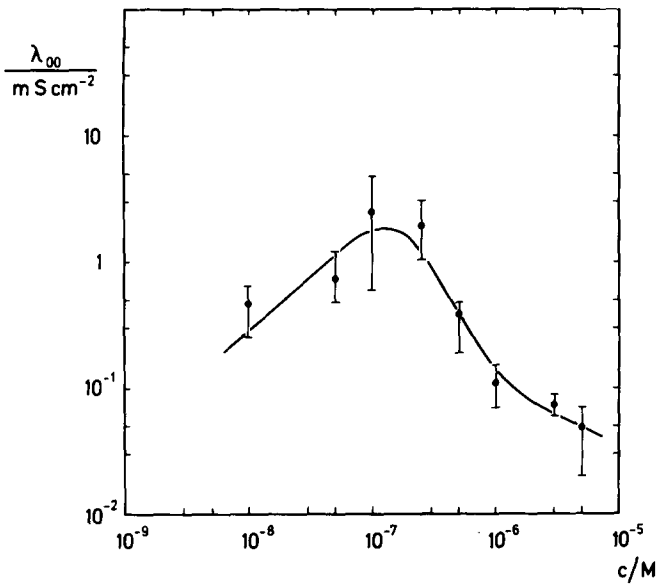

Fig. 4. Initial conductance λ_{00} (V_m = 10 mV) of dierucoyl phosphatidylcholine/*n*-decane membranes in the presence of different dipicrylamine concentrations *c*. The aqueous phase contained 0.1 M NaCl and 10^{-2} M HEPES, pH 6; T = 25°C. The bars indicate the range of measured values.

relaxation were derived from the semilogarithmic plot of log current versus time as described earlier [9]. For low concentrations of dipicrylamine in the aqueous phase ($c \leqslant 5 \cdot 10^{-7}$ M) the current across the membranes decayed in a single exponential function to values which nearly corresponded to the conductance of undoped membranes. At higher concentrations a larger current at

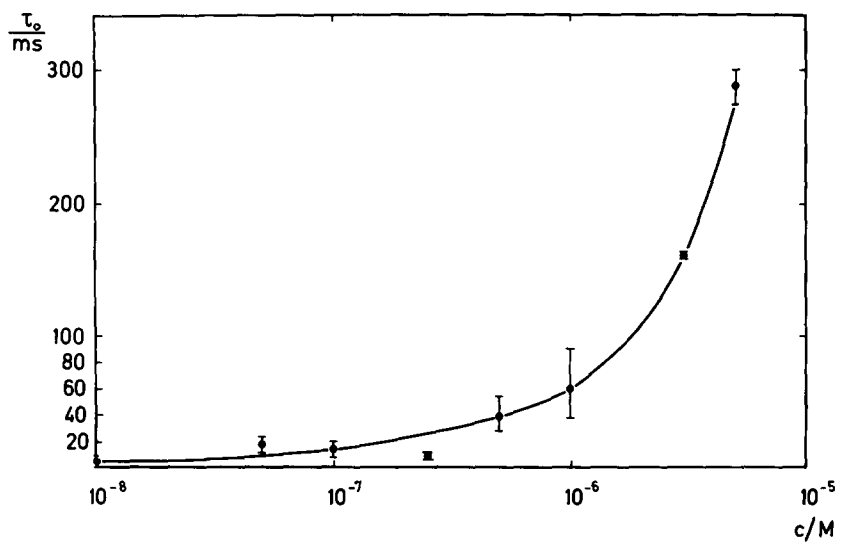

Fig. 5. Relaxation time τ_0 from voltage-jump measurements with dierucoyl phosphatidylcholine/*n*-decane membranes in the presence of different dipicrylamine concentrations *c*. The aqueous phase contained 0.1 M NaCl and 10^{-2} M HEPES, pH 6; T = 25°C. The bars indicate the range of the observed values.

long times was observed which showed a slow additional relaxation in the time range of seconds. There is a strong evidence based on theoretical arguments that this current is limited by diffusion polarization in the unstirred layers adjacent to the membrane [23]. It was therefore not possible to evaluate the value of the desorption rate k_{ma} from the experiments.

From Fig. 4 it is seen that the initial conductivity at low voltages, λ_{00}, shows a saturation behaviour at high dipicrylamine concentrations. Similar results have been obtained by other authors [9—12,15]. Below 10^{-7} M dipicrylamine in the aqueous solution λ_{00} has been shown to be a linear function of the dipicrylamine concentration [9,10]. The saturation behaviour of the initial conductivity λ_{00} is accompanied by a large increase of the relaxation time τ_0 (Fig. 5). Between 10^{-8} and $5 \cdot 10^{-6}$ M dipicrylamine τ_0 increased from 6 to 280 ms. This corresponds to a decrease of the rate constant of translocation k_i by a factor of about 45. A large increase of k_i with increasing dipicrylamine concentration has already been observed by Bruner [10]; similar results were obtained also for tetraphenylborate [11,12,15].

The total dipicrylamine concentration N_t^{el} in the membrane from electrical measurements was calculated from the data of τ_0 and λ_{00} according to Eqn. 11. The results are given in Fig. 6 together with the total dipicrylamine concentration N_t^{opt} from optical measurements. N_t^{opt} was calculated from the absorbance A according to:

$$N_t^{opt} = \frac{A}{\epsilon \cdot 10^3} \text{ mol/cm}^2 \tag{14}$$

For the molar extinction coefficient ϵ the value obtained for dipicrylamine in egg phosphatidylcholine vesicles (Table I) has been used. The dependence of

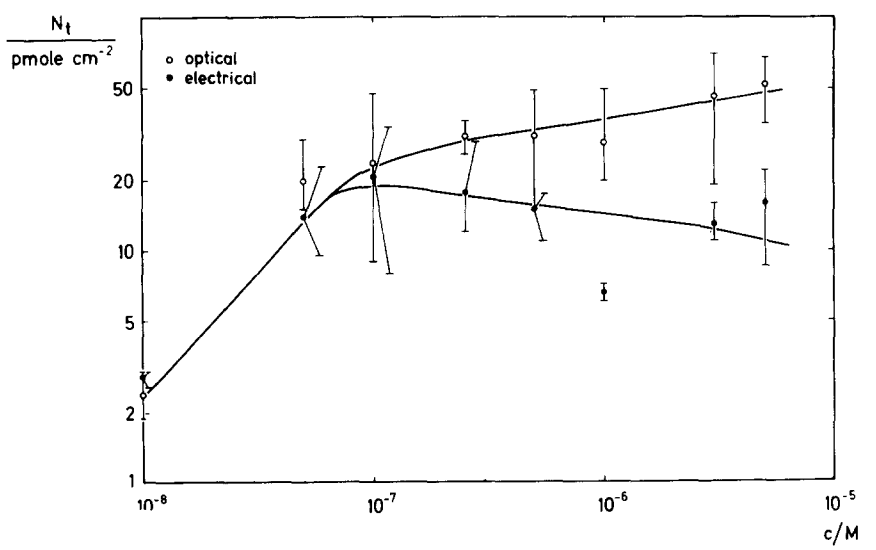

Fig. 6. Total dipicrylamine concentration N_t in the membrane as calculated from electrical and optical measurements. 0.1 M NaCl, 10^{-2} M HEPES, pH 6; $T = 25°C$. The bars indicate the range of the observed values.

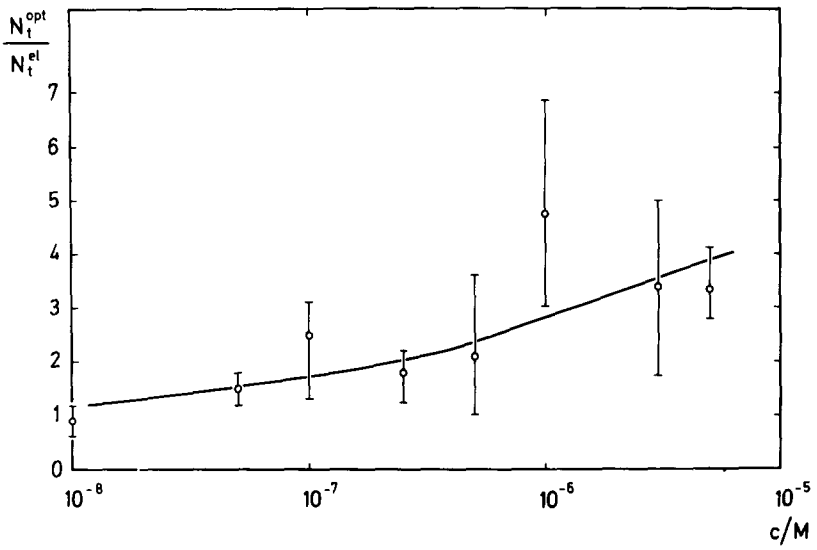

Fig. 7. Ratio N_t^{opt}/N_t^{el} calculated from simultaneous optical and electrical measurements with the same membrane. The bars indicate the range of the observed values.

both N_t^{el} and N_t^{opt} on aqueous dipicrylamine concentration c between 10^{-8} and 10^{-7} M, as shown in Fig. 6, agrees with the previously observed linear relationship between N_t and c at low concentrations [9,10]. A partition coefficient $\gamma \approx 4.5 \cdot 10^5$ for dipicrylamine may be calculated from this linear range (using $d = 5.7$ nm [21]). For higher dipicrylamine concentrations in the aqueous phase, N_t saturates, as observed in the optical as well as in the electrical measurements. Whereas the values for N_t derived for both types of measurements closely agree at low dipicrylamine concentrations, a large difference between N_t^{el} and N_t^{opt} is observed in the saturation range. This is also shown in Fig. 7 where values of the ratio N_t^{opt}/N_t^{el} taken from the same membrane are given. N_t^{opt}/N_t^{el} varies between 1 and 4 in the concentration range of 10^{-8}—$5 \cdot 10^{-6}$ M.

Discussion

It has been shown in the previous section that the values of the total concentration N_t of dipicrylamine in the membrane obtained from optical and electrical measurements are closely correlated. The quantitative evaluation of the optical measurements requires the knowledge of the absorbance of the undoped membrane and of the molar extinction coefficient of dipicrylamine in the dierucoyl phosphatidylcholine/*n*-decane membrane. The first point is only of importance at low dipicrylamine concentrations. Although the mean absorbence value $\bar{A}_0$ for bare membranes was subtracted in each case, the value of A_0 varies to some extent from membrane to membrane, thereby increasing the error in N_t^{opt}. The second point may lead to errors in N_t^{opt} in the whole concentration range. The molar extinction coefficients for dipicrylamine in different solvents, as given in Table I, refer to isotropic bulk phases. For the calculation of N_t^{opt} the extinction coefficient obtained from a suspension of egg phosphatidylcholine vesicles has been used. In this case the dipicrylamine molecule

presumably is in a similar environment as in the planar bilayer, but the value of ϵ represents an average over all possible orientations of the molecule with respect to the light beam.

A membrane is not isotropic and if the molecules are ordered in the membrane-water interface the molar extinction coefficient in a planar membrane and in a bulk phase may differ [24—26]. On the other hand, the good agreement between optical and electrical measurements at low dipicrylamine concentrations is in favour of the view that the uncertainties mentioned above do not seriously influence the interpretation of the optical measurements. We cannot exclude, however, that at higher concentrations a reorientation of the dipicrylamine molecules in the membranes takes place as well as optical saturation effects may occur which influence the value of ϵ. A smaller value for ϵ should be expected in these cases [27], which would lead to a higher N_t^{opt}, increasing the discrepancy between electrical and optical measurements.

From the results given in Table 1 it is not possible to draw clear conclusions on the environment of the dipicrylamine molecule in the membrane. The wavelength of maximal absorption seems to depend on the charge of dipicrylamine. Most of the dipicrylamine adsorbed to the membrane is likely to be in the ionized state. This is consistent with the results of measurements with dioleoyl phosphatidylcholine/*n*-decane membranes at different pH values in the presence of dipicrylamine in the aqueous phase [12]. From the observation that the dipicrylamine molecule experiences approx. 95% of the voltage drop across a dierucoyl phosphatidylcholine/*n*-decane membrane [12] it is very likely that the molecule is situated close to the polar layers of the membrane. Similar results have been obtained by G. Szabo from measurements with tetraphenylborate in glycerol monooleate membranes [15] and by Anderson and Fuchs [11] from phosphatidylethanolamine membranes.

For large aqueous concentrations of dipicrylamine a saturation behaviour of the concentration N_t in the membrane as well as for $k_i = 1/2\tau_0$ have been observed. The saturation of N_t has been observed in the optical as well as in the electrical measurements. The results obtained by the two methods differ in the extend of the saturation by a factor of maximally 3—4 (Fig. 7). This difference is too large to be explained on the basis of the inaccuracy of the measurements. A possible explanation may lie in the high surface concentration of dipicrylamine in the saturation range (1 molecule/6.7 nm^2). At such a high concentration not all adsorbed lipophilic ions may participate in the transport under the influence of an applied potential difference.

The saturation of the surface concentration of dipicrylamine in the membrane has already been discussed in the literature [9—12,15]. It is not possible to explain the saturation only on the basis of a Gouy Chapman potential caused by the surface charge of the adsorbed lipophilic ions [9,17]. For the number of adsorbed ions per cm^2 reported here from the optical measurements at $5 \cdot 10^{-6}$ M dipicrylamine in the aqueous phase, a surface potential of about −30 mV may be calculated from the Gouy Chapman equation (ionic strength $\approx$ 0.1 M) [9]. If we define the partion coefficient γ as being given by the ratio of $N_t/c_- d$, were c_- is the dipicrylamine concentration in the aqueous layer immediately adjacent to the membrane surface and if we assume γ to be independent of c_-, a potential difference $\Delta\psi'$ may be calculated which is responsible for the satu-

ration in the membrane:

$$c_- = \frac{N_t}{\gamma \cdot d} = c \exp(\Delta\Psi' \cdot F/RT) \tag{15}$$

Under the assumption that at $5 \cdot 10^{-8}$ M ($\gamma \approx 7 \cdot 10^5$, from N_t^{opt}) dipicrylamine in the aqueous phase c_- is nearly equal to c (i.e. $\Delta\psi' \approx$ o or $N_t \sim c$) a potential $\Delta\psi' = -95$ mV is calculated for a dipicrylamine concentration of $5 \cdot 10^{-6}$ M in the aqueous phase. (For other concentrations see Fig. 8). The absolute value of the surface potential is considerably higher than the potential value of −30 mV obtained by the Gouy Chapman equation. McLaughlin [17] recently pointed out that boundary potentials which are considerably larger than the Gouy Chapman potential may be observed if the adsorption plane of the lipophilic ions is shifted more towards the hydrophobic interior of the membrane. Because of the high specific capacity of the polar region a surface concentration similar to that given above may cause a boundary potential of the order of −100 mV [17]. The qualitative agreement between the potential calculated according to Eqn. 15 and the boundery potential estimated on the basis of McLaughlins treatment supports the notion that the saturation of N_t may be partly caused by electrostatic interaction. We cannot exclude, however, that also distortions of the lipid structure at high N_t contribute to the saturation behaviour.

The large increase of τ_0 with increasing dipicrylamine concentration has been discussed on the basis of a decreasing membrane fluidity [10] or, in the case of tetraphenylborate, by an effect of the surface potential [15]. We found a 45-fold increase of τ_0 between 10^{-8} and $5 \cdot 10^{-6}$ M dipicrylamine in the aqueous phase. It is difficult to explain the corresponding large decrease of the translocation rate constant on the basis of a fluidity change of the membrane interior alone. In principle, the decrease of k_i to k_i^* for high dipicrylamine concentrations may be explained assuming an additional potential $\Delta\psi''$ acting on the

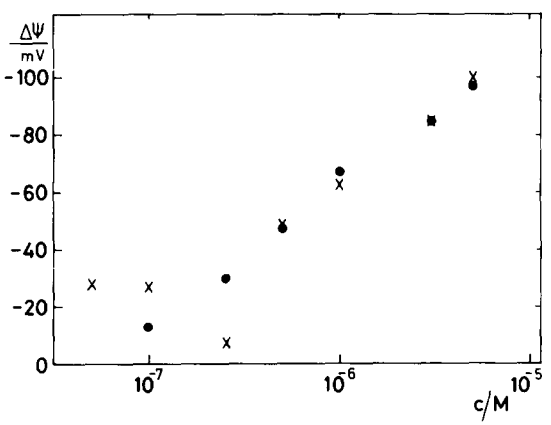

Fig. 8. Potential difference $\Delta\psi$ calculated from the saturation behaviour of N_t^{opt} (full circles, $\Delta\psi'$) and from the increase of τ_0 (crosses, $\Delta\psi''$), as a function of dipicrylamine concentration c. For further explanations see text.

single molecule:

$$k_i^* = k_i \exp(\Delta\Psi'' \cdot F/RT) \quad (16)$$

For $c = 5 \cdot 10^{-6}$ M dipicrylamine in the aqueous phase a potential $\Delta\psi'' = -100$ mV may be calculated. The results for other concentrations are given in Fig. 8.

It is interesting to note that the potential difference $\Delta\psi'$ calculated for the saturation of N_t^{opt} and the potential difference $\Delta\psi''$ which may be responsible for the decrease of k_i^* have similar values and the same dependence on the dipicrylamine concentration in the aqueous phase (Fig. 8). It seems that high concentrations of adsorbed dipicrylamine influence the membrane properties in different ways. The adsorption may lead to a boundary potential, as well as to a change of the dipole moment in the polar layers of the membrane. Further experiments with lipophilic ions and carriers may give information on the relative contributions of the mentioned effects on the behaviour of the concentration N_t in the membrane and of the translocation rate constant k_i.

Acknowledgements

The authors wish to thank Drs. P. Läuger, G. Stark and E. Bamberg for many helpful discussions. This work has been financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138).

References

1 Müller, P., Rudin, D.O., Tien, H.T. and Wescott, W.C. (1962) Nature 194, 979—980
2 Müller, P. and Rudin, D.O. (1967) Biochem. Biophys. Res. Commun. 24, 398—404
3 Le Blanc, Jr., O.H. (1969) Biochim. Biophys. Acta 193, 350—360
4 Liberman, E.A. and Topaly, V.P. (1969) Biophys. J. 14, 477—487
5 Eisenman, G., Ciani, S. and Szabo, G. (1969) J. Membrane Biol. 1, 294—345
6 Tosteson, D.C. (1968) Fed. Proc. 27, 1269—1277
7 Palti, Y. and Adelman, Jr., W.J. (1969) J. Membrane Biol. 1, 431—458
8 Hopfer, U., Lehninger, A.L. and Thompson, T.E. (1968) Proc. Natl. Acad. Sci. U.S. 59, 484—490
9 Ketterer, B., Neumcke, B. and Läuger, P. (1971) J. Membrane Biol. 5, 225—245
10 Bruner, L.J. (1975) J. Membrane Biol. 22, 125—141
11 Andersen, O.S. and Fuchs, M. (1975) Biophys. J. 15, 795—830
12 Benz, R., Läuger, P. and Janko, K. (1976) Biochim. Biophys. Acta 455, 701—720
13 Szabo, G. (1974) Nature 252, 47—49
14 Haydon, D.A. and Hladky, S.B. (1972) Q. Rev. Biophys. 5, 187—282
15 Szabo, G. (1976) in Extreme Environment; Mechanism of Microbial Adaption (Heinrich, M.R., ed.), Academic Press, New York, in the press
16 Bruner, L.J. (1970) Biophysik 6, 241—256
17 McLaughlin, S. (1976) Submitted to Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 9
18 Pohl, G.W., Knoll, W., Gisin, B.F. and Stark, G. (1976) Biophys. Struct. Mech. 2, 119—137
19 Ithaco Application Note 33, Ithaco, Ithaca, N.Y., U.S.A.
20 Benz, R., Stark, G., Janko, K. and Läuger, P. (1973) J. Membrane Biol. 14, 339—364
21 Benz, R. and Janko, K. (1976) Biochim. Biophys. Acta 455, 721—738
22 Gaboriaux, R. (1966) C.R. Acad. Sci. Paris, Sér. C, 263, 911—914
23 Läuger, P. and Neumcke, B. (1973) in Membranes, A series of Advances (Eisenman, G., ed.), Vol. 2, M. Dekker, New York
24 Steinemann, A., Alamuti, N., Brodmann, W., Marschall, O. and Läuger, P. (1971) J. Membrane Biol. 4, 284—294
25 Steinemann, A., Stark, G. and Läuger, P. (1972) J. Membrane Biol. 9, 177—194
26 Cherry, R.J., Hsu, K. and Chapman, D. (1972) Biochim. Biophys. Acta 267, 512—522
27 Kortum, G. (1962) Kalorimetrie, Photometrie und Spektroskopie, pp. 30—41, Springer Verlag