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During the second year of our research project we continued with the investigation of the membrane behavior and of the structure of its lipid and protein or polypeptide components under the influence of transmembrane electric fields. The model membranes investigated during this period were lipid monolayers at the mercury electrode/water interface and lipid bilayers with incorporated channel forming polypeptides alamethicin and melittin. The spread lipid monolayers were transferred after their interaction with the polypeptide in the aqueous solution, from the air/water to the mercury/water interface. Their properties were then inferred from their capacitance and ionic permeability at different electrode potentials. Ionic permeability of unilamellar vesicles upon interaction with the channel forming polypeptides and proteins was determined with and without applied diffusion potential. The change in conformation of specific membrane components with applied electric field was studied by circular dichroism (CD) and Fourier transform infrared (FTIR) measurements. Transmembrane potentials induced were either diffusion potentials (K^+ gradient valinomycin) in non-leaky membranes or Donnan potential when high channel concentrations made the membranes leaky to small ions.

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Progress Report on Contract N00014-87-G-0203
Principal Investigator: Israel R. Miller
Title: **Electrical field dependence of protein conformation and channel function in lipid membranes of different compositions.**

RESEARCH OBJECTIVES

The objective is to study the effect of electric field on membrane permeability and on the conformation of membrane proteins and channel forming polypeptides to gain insight on their function as channels or as signal transducing receptors. The research has been conducted in model systems, namely lipid monolayers and lipid bilayer membranes.

PROGRESS DURING THE SECOND YEAR STARTING JULY 1988

1. Effect of cholera toxin and of tetanotoxin interacting with their ganglioside receptors on the structure and the permeability of lipid monolayers and bilayers:

This work has been completed and the results were treated in the framework of the general problem of the effect of the interactions in the head groups on the structure and on the permeability of lipid monolayers and bilayers. It has been shown that the surface complexes formed by specific polar interaction between the proteins and the respective ganglioside head groups can be incorporated into the lipid layers perturbing their uniform layer structure lowering their sensitivity. One paper based on this research has been published, another has been submitted for publication (1,2).

2. Effect of alamethicin and of melittin on the capacitances and on the ability of lipid monolayers on Hg electrode surface and on the permeability of vesicular lipid membranes:

The differential capacitance of condensed monolayers of phosphatidylcholine (PC) or of mixtures of PC with phosphatidylserine at the mercury/water interface was measured in the presence of different concentrations of the antibiotic alamethicin or of the bee venom melittin. The degree of perturbance of the structure of the phospholipid monolayers and their penetration by the oligopeptides was inferred from the increase in differential capacity and from their suppressed impedance to polarographic currents carried by ionic depolarizer. The augmentation of the capacitance and of the ionic permeability depends not only on the degree of penetration or of displacement of the lipid monolayer by the oligopeptides but also by the surface properties of the displaced domains. Alamethicin is much more hydrophobic than melittin. Alamethicin adsorbs on the mercury surface giving a condensed monolayer of a minimal

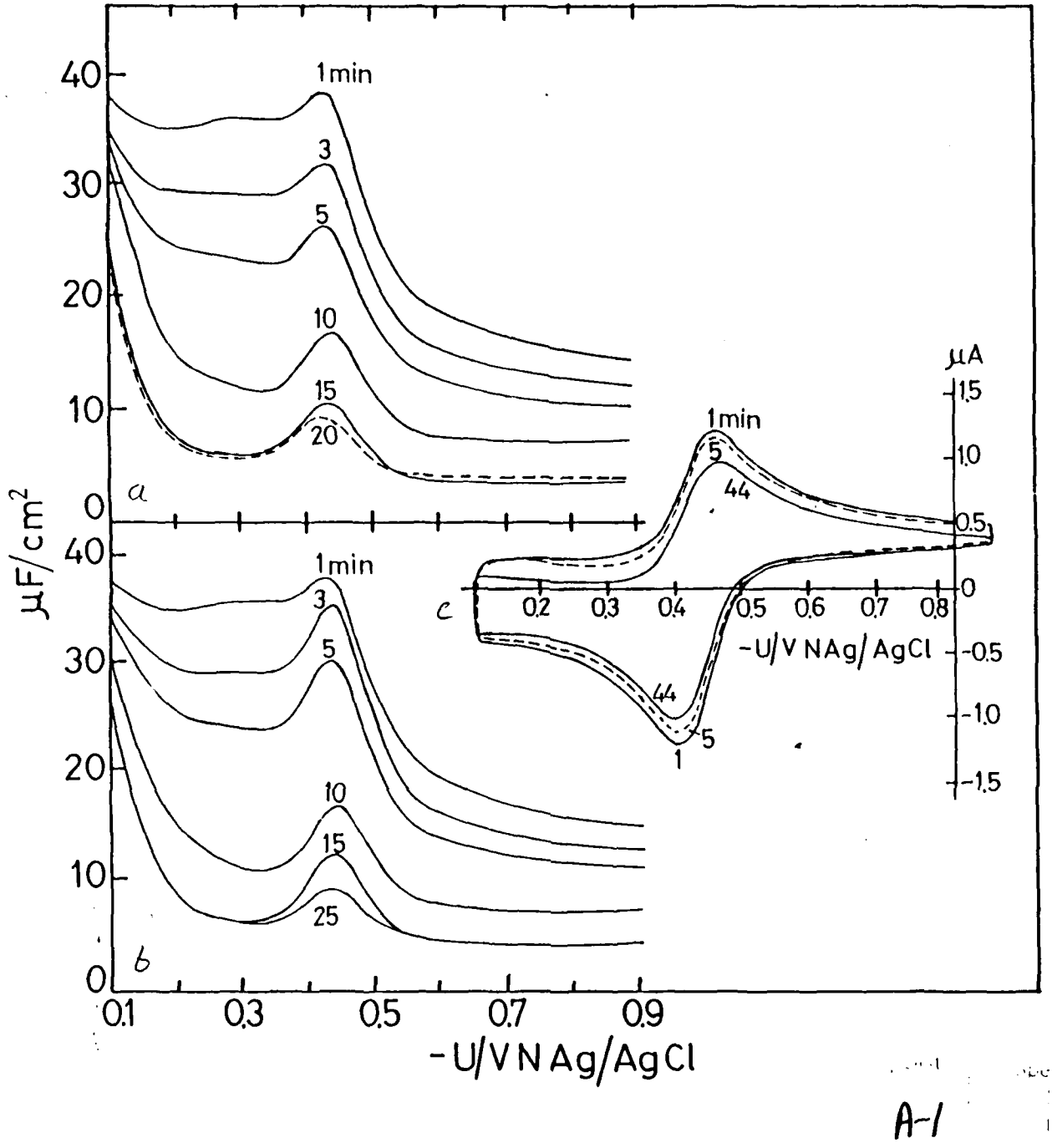

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Fig. 1: Capacitance and transport of Tl^+ across adsorbed monolayers of alamethicin.

a&b - Differential capacitance as a function of Hg drop electrode potential at different times of exposures of the mercury drop surface to adsorption from the aqueous solution containing 1μg/m alamethicin $4 \cdot 10^{-5}$M Tl^+ in 0.1M NaCl.

a - Each curve recorded on a new drop exposed to the solution for the number of minutes indicated at a constant potential of -0.1V relative to NAg/AgCl electrode.

b - The capacitance curves are recorded on the same mercury drop. (Here the pseudocapacitance peaks at the consecutive times (e.g. 3', 5') are larger because of Tl accumulation near the surface because of the previous potential sweeps).

c - Cyclic voltamograms after different exposure times of the Hg drop electrode to a solution of 1μg/ml alamethicin. Exposure times indicated on the curves.

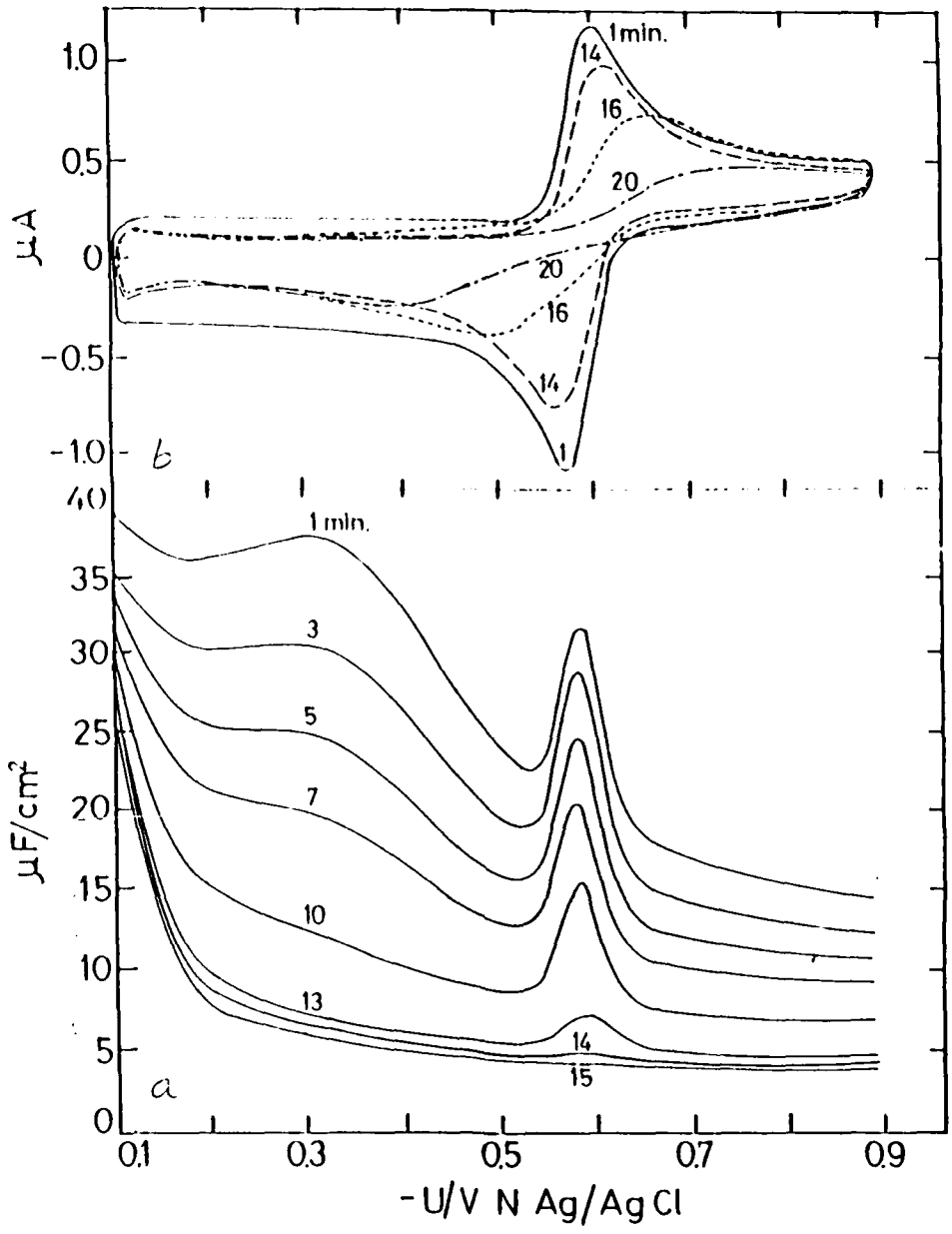

Fig. 2: **Capacitance and transport of Cd^{++} across adsorbed monolayers of alamethicin.**

a - Differential capacitance curves at different times of exposure of the mercury surface to adsorption from an aqueous solution containing 1μg/ml alamethicin $4 \cdot 10^{-5}$M Cd^{++} and 10^{-1}M NaCl.

b - Cyclic voltamograms under similar conditions. Exposure time indicated, sweep rate 0.2V/sec.

specific capacitance < 4μf/cm² from concentrations < 1μg/ml (Fig. 1a, b). It hinders the access of ions to the electrode surface suppressing the acpseudocapacitance peak of Tl^+ to less than half of its value without the monolayer and it practically eliminates the ac pseudocapacitance of Cd^{++} (Fig. 2a). Cyclic voltamograms show scan rate dependent shifts in peak potentials from which the ionic permeability of the surface layer can be calculated (Figs. 1c, 2b). Thus, even high degrees of penetration or displacement of lipid monolayers by alamethicin has only a moderate effect on the monolayer capacitance and permeability.

In Fig. 3 the effect of alamethicin on the capacitance and on the permeability to Tl^+ of a phosphatidylcholine monolayer is shown. The effect increases at relatively short times of exposure of the monolayer covered electrode to the alamethicin containing solution. It decreases at longer times until the monolayer capacitance and the Tl^+ pseudocapacitance peak (indicative of monolayer permeability to Tl^+) reach to values obtained in the absence of alamethicin. Cyclic voltametry shows (Fig. 3b) that it is the reduction current depending on the transport of Tl^+ ions across the monolayer to the electrode, which is predominantly impeded. Another reduction peak around 0.1V appears besides the one around -0.45V. The function reduced at -0.1V increases with the monolayer impedance on top of the slight negative shift of the -0.45V peak. The reoxidation rate of metallic Tl to Tl^+ (anodic peak) is only slightly affected by the monolayer and by the change of its impedance.

Melittin on the other hand is a relatively hydrophilic molecule with a substantial positive net charge. The minimal capacitance of its condensed adsorbed monolayer is around 9 to 10μf/cm² and inspite of its positive net charge it is almost freely permeable to Tl^+ without lowering its diffusion current or its pseudocapacitance peak when reduced on the electrode surface. It perturbes only little the structure of lipid monolayers since it cannot properly penetrate the lipid monolayer without exposing polar groups to its hydrophobic domain.

Inspite of the difference in hydrophobicity the effect of the two channel formers on the permeability of vesicular lipid membranes is quite similar. As seen in Fig. 4a and b, at concentrations of about 0.5μg/ml of either polypeptide, when the lipid to polypeptide molar ratios are around 100/1, complete release of Tl^+ or of any other embedded ion is attained. Below these concentrations only partial release is attained even after relatively long time. At these long times the rate of release induced by melittin is somewhat larger than that induced by alamethicin. There are two ways to explain this behavior: 1. Some of the vesicles adsorbed enough polypeptides for one or more channels, other remain without any channels. 2. All the vesicles have an adequate number of polypeptide for channel formation but the channels become inactivated before the release has been completed.

The first alternative is improbable since the vesicle diameters as by inferred from the entrapped volume were larger than 100nm which correspond to . $4x10^4$ lipid molecules per vesicle. At lipid/polypeptide molar ratio of 1000 when less than half of the release is attained, the average number of polypeptides per vesicle is about 40. This should assure more than one channel per all the

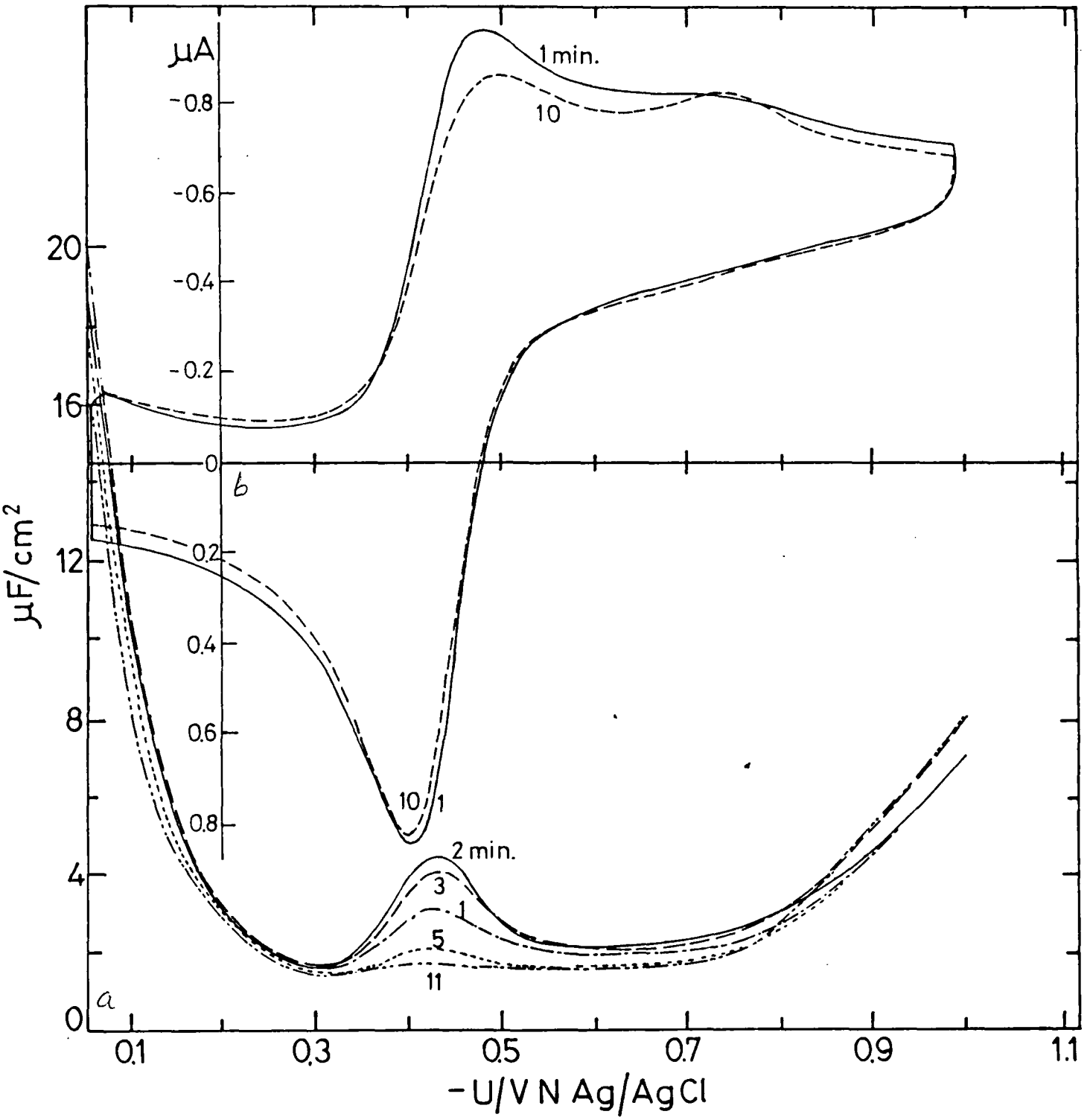

Fig. 3: **Effect of alamethicin on the capacitance and the Tl^+ permeability of a condensed PC monolayer.**

a - Differential capacitance/potential curves after exposure of the PC monolayer covered electrode to the alamethicin containing solution (1.9μg/ml) for different length of time (indicated). Tl^+ concentration $4 \cdot 10^{-5}$M.

b - Inset-cyclic voltamograms of the same after 1 minute and 11 minutes exposures.

vesicles. The time dependent channel inactivation is therefore the most likely possibility. One of the possible mechanisms for channel inactivation is the flip ones of the polypeptide to point their helical dipoles in the antiparallel direction with respect to the other helices of the channel. The small differences between the behavior of alamethicin and of melittin may be accounted for by a higher equilibrium concentration of the latter in the aqueous solution. there is also a difference in the pH dependence of ionic specificity. Melittin below pH 7 induces preferentially ion permeability while above pH 8 it forms preferentially cationic channel inspite of its still positive net charge.

3. Effect of transmembrane potential on the conformation of membrane components:

This line of investigation has been started with measuring the effect diffusion potentials brought about by K^+ gradients across vesicular membrane and the specific K^+ carrier - valinomycin, on the circular dichroism (CD) of the embedded bacteriorhodopsin. The results published in a short communication in the *Biophysical Journal* [3] show that transmembrane potentials irrespective of their direction lower the helicity of the bacteriorhodopsin in the membrane.

We have concentrated during the last year on the conformation of alamethicin embedded in vesicular bilayer lipid membranes under the influence of electric fields across these membranes. The embedded alamethicin molecules produce non selective channels and it is impossible to establish and to maintain a diffusion potential in its presence at a high concentration. We made use of Donnan equilibrium to establish and to maintain potential differences across alamethicin containing membranes. The electric field in either direction across the vesicular membrane was established by different sodium polyacrylate (NaPA) concentration inside or outside the vesicles with different salt concentrations on the opposite side. Isosmotic conditions were maintained by balancing the osmotic pressure with glucose. The vesicles were loaded with NaPA when formed by lipid solution injection [4] into NaPA containing aqueous solution. The NaPA was removed from the exterior phase by equilibration with a 10-20 fold excess of Cl^- from anion exchanger (Bio-Rad AG 1-84), e.g. 0.5g (3.5meq/g) of the dry ion exchanger = 1.75meq was washed and swollen with water. The excess water was removed with filter paper and the hydrated ion exchanger added to 1ml 0.1N NaPA (0.1meq). After equilibration during 3-4 h practically all of the PA in the external phase are exchanged for Cl^-. At the same time up to 50% of the vesicles are lost by adsorption on the in exchanger. PA containing vesicles are negatively charged and tend to adsorb on the anion exchanger even if the lipids are electroneutral. From an anion exchanger column they cannot be recovered at all. The phospholipid concentrations left after the anion exchange was determined by phosphor analysis. The NaPA left in the external phase was determined by light scattering after adding Ca^{++}. The vesicular suspensions were then dialyzed against different concentrations of NaCl and glucose, and alamethicin was added to each sample half an hour before the CD measurements.

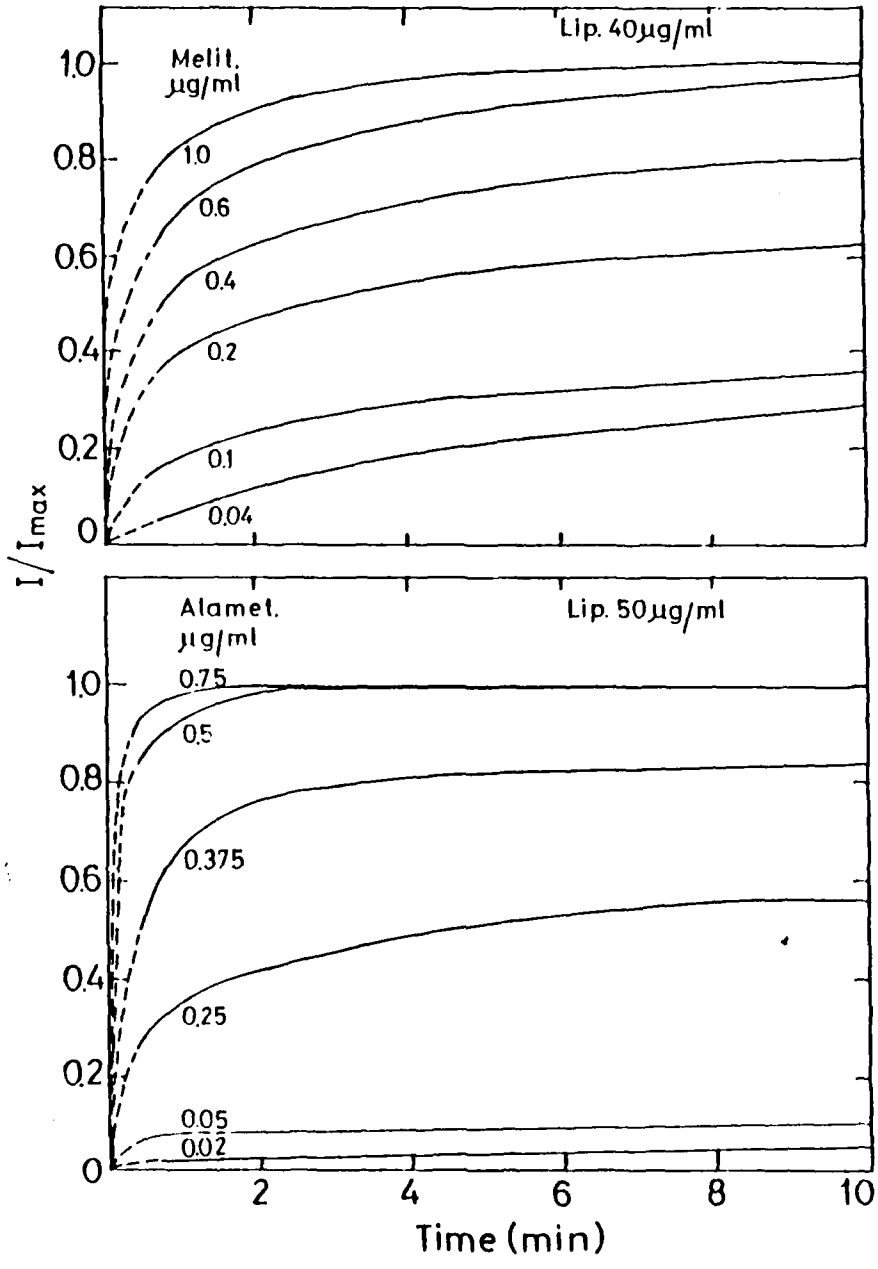

Fig. 4: **Reduced polarographic currents (I/I_{max}) of Tl^+ in the outer solution released from the vesicles by different concentrations of melittin (a) and of alamethicin (b) as a function of time.**

As shown in Fig. 5, the ellipticity at 222nm is a monotonous function of the Donnan potential. The ellipticity decreases with the NaPA concentration in the outer phase increasing the negativity of the potential on the outer side of the vesicle. The ellipticity increases, corresponding to α helicity increase, when NaPA is entrapped in the vesicles and the potential gradient across the membrane is in the opposite direction. This change in CD spectrum corresponds to decrease in helicity and to an increase in β structure. Here the behavior differs from that of bacteriorhodopsin incorporated into vesicular membrane which showed a decrease in helicity with electric file irrespective of its direction. A behavior similar to that of bacteriorhodopsin has been observed by us recently in preliminary experiments with Na^+-K^+-ATPase reconstituted in lipid vesicles. The behavior of alamethicin is in accord with the concept of electric field dependent incorporation of the polypeptide into the membrane. Compatible results are obtained by FTIR measurements, started a few months ago. The first manuscript treating these results is underway.

4. Lateral mobility of membrane components in tangential electric fields:

A paper resulting from these investigations is now in press in *Biophysical Journal* [5]. Its title is: "Electric field induced lateral mobility of photosystem I in the photosynthetic membrane: A study by photoluminescence". In course of these studies we encountered some changes in the investigated systems when exposed for larger times to electric fields. The most pronounced changes were continuous increase in electrophotoluminescence and in light scattering indicating that the electric field induces aggregation of the thylakoid vesicles. Since field dependent enter cellular interactions and the mechanisms are of biological importance, we are contemplating microscopical investigation of these phenomena. We are trying different designs for observation of vesicular cell populations in electric fields under the microscope.

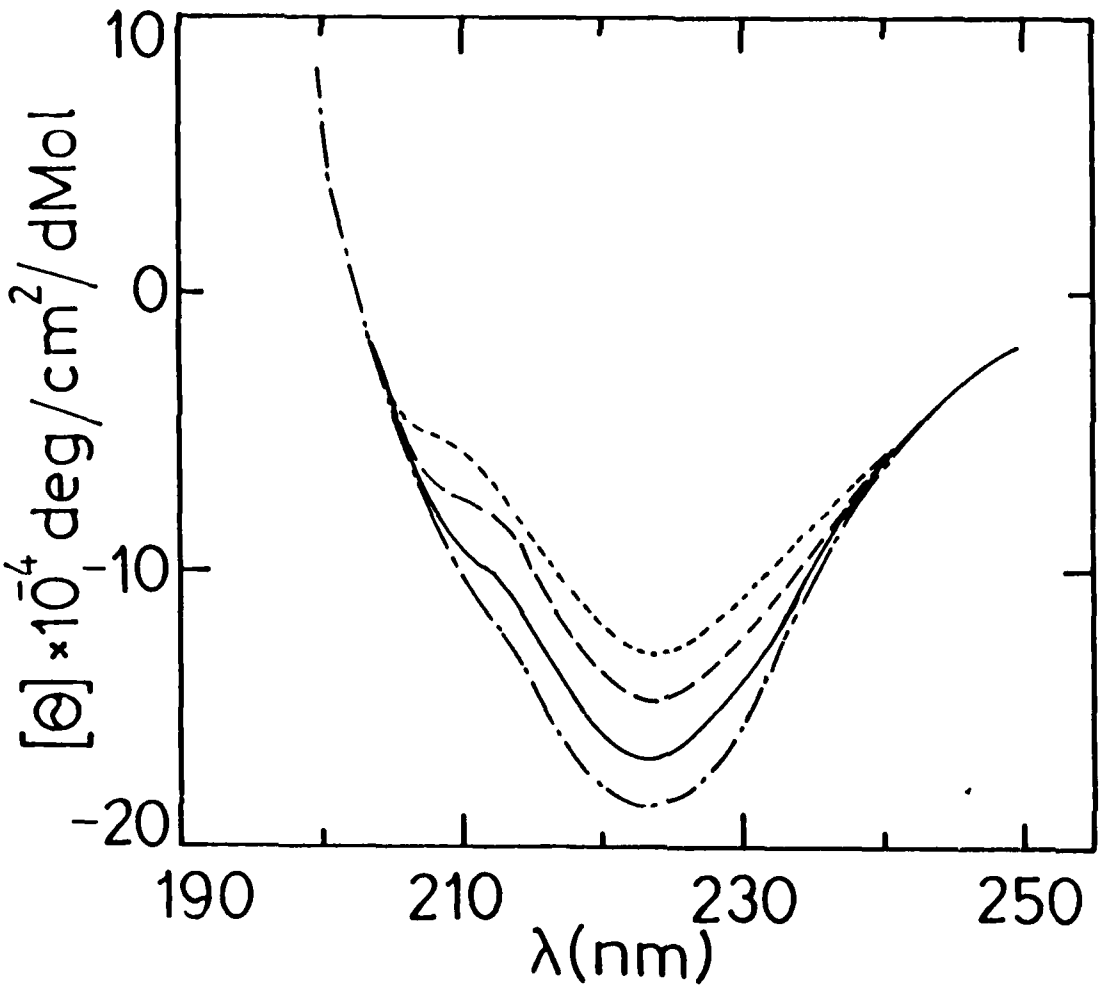

Fig. 5: **CD spectra of alamethicin adsorbed on or incorporated into vesicular egg phosphatidyl choline membranes as a function of applied Donnan potential $\Delta\psi$ across the membrane.**

—— no NaPA; $\Delta\psi = 0$

– . – 0.1 N NaPA inside; $2 \cdot 10^{-5}$M NaCl outisde the vesicles, $\Delta\psi$ = 173mV - positive outside. This value may be considerably lower if the NaPA has not been completely removed from the outer side, e.g., 2% NaPA left ($2 \cdot 10^{-3}$N) would lower the potential to 97mV.

– – *0.03N NaPa otuside, $2 \cdot 10^{-5}$M NaCl inside, $\Delta\psi$ = -70mV*

---- 0.1N NaPa otuside, $2 \cdot 10^{-5}$M NaCl inside, $\Delta\psi$ = -79mV

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