# Discrete Conductance Fluctuations in Lipid Bilayer Protein Membranes

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ABSTRACT Discrete fluctuations in conductance of lipid bilayer membranes may be observed during the initial stages of membrane interaction with EIM ("excitability inducing material"), during destruction of the EIM conductance by proteolysis, and during the potential-dependent transitions between low and high conductance states in the "excitable" membranes. The discrete conductance steps observed during the initial reaction of EIM with the lipid membranes are remarkably uniform, even in membranes of widely varying lipid composition. They range only from 2 to 6 $\times$ $10^{-10}$ $ohm^{-1}$ and average 4 $\times$ $10^{-10}$ $ohm^{-1}$. Steps found during destruction of the EIM conductance by proteolysis are somewhat smaller. The transition between high conductance and low conductance states may involve steps as small as 0.5 $\times$ $10^{-10}$ $ohm^{-1}$. These phenomena are consistent with the formation of a stable protein bridge across the lipid membrane to provide a polar channel for the transport of cations. The uniform conductance fluctuations observed during the formation of these macromolecular channels may indicate that the ions in a conductive channel, in its open state, are largely protected from the influence of the polar groups of the membrane lipids. Potential-dependent changes in conductance may be due to configurational or positional changes in the protein channel. Differences in lipid-lipid and lipid-macromolecule interactions may account for the variations in switching kinetics in various membrane systems.

## INTRODUCTION

Many of the electrical characteristics of excitable cells can be reproduced in experimental lipid bilayer membranes when modified by a bacterial substance, called "excitability inducing material," or EIM (Mueller and Rudin, 1963, 1967 *a*, 1968 *a,b*) or the cyclic peptide antibiotic, alamethicin[1] (Mueller and Rudin, 1968 *a*). A number of substances, such as organic ions (Hopfer, Lehninger, and Thompson, 1967; Skulachev, Sharaf, and Liberman, 1967), cationic dyes (R. C. Bean, unpublished data) and other organic cations (Bean, Shepherd, and Chan, 1968), and iodide ions (Läuger, Lesslauer,

[1] Upjohn Compound No. 22,234.

Marti, and Richter, 1967) may alter the membrane conductance in a manner suggesting a carrier mechanism. In other instances, as for the cyclic peptide antibiotics (Mueller and Rudin, 1967 *b*; Andreoli, Tieffenberg, and Tosteson, 1967) the formation of stable channels (or pores) has been proposed but disputed by other investigators in favor of a carrier mechanism (Stein, 1968; Pressman et al., 1967).

Several behavioral and kinetic characteristics of the EIM-membrane system suggest consideration of a long-lived polar channel rather than a carrier mechanism. A major factor is the demonstration that the EIM conductance may be destroyed by proteolysis from either side of the membrane, even though EIM is added only on one side (Mueller and Rudin, 1968 *b*; Bean, Chan, and Eichner, 1968). Confirmation of the existence of a stable protein channel in the experimental lipid bilayers may have a significant bearing upon concepts of the structure and action of biological membranes. The evidence offered in this report, showing the existence of long-lived, incremental changes in membrane conductance during the interaction of EIM with the membrane as well as during the destruction of the EIM conductance by proteolysis, provides further support for the concept of a stable channel. In addition, the demonstration of smaller conductance steps in the potential-dependent transition between high and low conductance states of the EIM membrane suggests the existence of quantized conduction states within these channels which may be due to progressive conformational changes in the protein channel.

## EXPERIMENTAL

### *Methods and Materials*

MEMBRANE FORMATION The methods for membrane formation and the configurations of the supporting assemblies were similar to those used by Mueller, Rudin, Tien, and Wescott (1962, 1963, 1964) in their original studies. A polyethylene cup, with a small aperture (about 1 mm diameter) in a thinned area of the wall was supported inside a low glass or polymethacrylate cup. Medium was placed in both the inner and outer compartments, equilibrated at the experimental temperature, and a lipid solution was brushed across the aperture with a small, trimmed brush or thin, tapered Teflon spatula. Conductance measurements were carried out after spontaneous thinning of the films to the secondary black stage. EIM was generally added on only one side of the membrane, while monitoring current, at constant potential, as indicated below.

During the experiments both inner and outer compartments were continuously stirred with stainless steel wire, magnetic stirring bars. The plastic cup (inner compartment) was supported slightly above the floor of the outer compartment so that a single magnetic stirring motor could drive both stirring fleas, one above the other, in the two compartments.

The experimental temperature was maintained either by circulating water from a

constant temperature bath through a jacketed outer compartment or by heating with a small DC heater under the outer compartment of the assembly. Temperature was monitored with a chromel-alumel thermocouple through a Hewlett-Packard microvoltammeter (Hewlett-Packard Co., Palo Alto, Calif.)

The medium used in the membrane assembly was 0.1 M NaCl, buffered at pH 7.0 with 5 mM histidine, unless otherwise noted.

CONDUCTANCE MEASUREMENTS Electrical contact with the membrane was maintained through agar-salt bridges to calomel electrodes. Measurements were all carried out under constant potential conditions (low resistance in series with the membranes) rather than at constant current. Potentials were generated from a 1.5 v cell through a potential divider circuit. Potentials across the membrane were monitored with a Keithley electrometer (Model 610 B) with a $10^{14}$ ohm input impedance. Currents were measured with a Hewlett-Packard microvoltammeter (Model 610 B). Electrometer and ammeter outputs were recorded on an X-Y recorder, enabling direct recording of either current-voltage curves or current-time curves at constant potential. Noise factors generally limited reliable measurement of conductance or resistance changes to about $\pm$ 0.2 $\times$ $10^{-10}$ ohm (5 $\times$ $10^{10}$ ohm).

EIM PREPARATION The EIM was prepared by procedures adapted from Mueller and Rudin (personal communication) and Kushnir (1968). *Enterobacter cloacae* (ATCC 961) was grown initially on a standard thioglycollate medium. An inoculum from 16 to 24 hr growth in this medium was transferred to a glucose-salt medium, containing 0.2% glucose, 0.1% ammonium sulfate, 0.3% $KH_2PO_4$, 0.7% $K_2HPO_4$, 0.5% sodium citrate·$3H_2O$, and 0.01% magnesium sulfate·$7H_2O$. Glucose and salts were autoclaved separately and mixed before inoculation. After 16 hr of aerobic growth at 37°C, the cells were harvested by centrifugation. The supernatant medium, under these growth conditions, contained little or no EIM activity, in contrast with other procedures (Kushnir, 1968) in which the supernatant medium contained most of the EIM activity. EIM was extracted from the harvested cells by resuspending them in distilled water to one-tenth the volume of the growing medium, adjusting to pH 9.5–10.5 with NaOH, and incubating with occasional shaking for 30 min. The cells were sedimented and treated once more by the same procedure, with similar volumes.

During the incubations the pH usually fell to a point near neutrality once more. The second extract normally contained the most activity and was retained. After completing adjustment to pH 7 it was stored, frozen, to be used in the experiments presented here. Ultraviolet spectra of undialyzed extracts showed a 260 m$\mu$ absorption peak corresponding to 1–3 mM nucleoside material which could be removed by dialysis without altering activity. After dialysis less than 20–50 $\mu$g/ml of macromolecular substances normally remained. An easily detectable change in membrane resistance could be induced in sensitive membranes by as little as 0.1 $\mu$l of extract per ml of buffer. Thus, the crude material may be active at concentrations as low as 2 $\times$ $10^{-9}$ g per ml. The specific activity of the preparations could be doubled or tripled by passage through gel filtration columns having an exclusion volume of 155,000 (BioGel 150, Bio-Rad Laboratories, Richmond, Calif.) in 0.2 M NaCl solution, retaining the frontal protein peak. However, a major part of the activity was lost during such fractionation and the product was normally unstable upon storage.

Therefore, the original extracts, which were stable for at least a year of storage at freezing temperatures and several weeks at room temperature, were used experimentally. The extracts appear to have some antibiotic activity.

LIPID SOLUTIONS Specific compositions of membrane solutions are indicated, as required, in the text, figure legends, and tables. Membranes were usually formed from solutions containing a phospholipid component and a plasticizing solvent, such as $\alpha$-tocopherol, decane, or a fatty acid ester, in chloroform-methanol (2:1). Cholesterol, cholesterol butyrate, or cholestane was frequently added as a stabilizing factor. Hydrocarbon plasticizing solvents for phospholipids, such as decane, inhibited the reaction of EIM with the membranes greatly so that a modified membrane-forming procedure was required, where such systems were used, in order to obtain any significant reaction with the EIM. In these cases, a chloroform-methanol solution of the phospholipid, or phospholipid plus cholesterol, was brushed across the aperture. After waiting a suitable interval for loss of solvent to the medium, pure decane or decane in chloroform-methanol was brushed across the aperture. This resulted in the formation of membranes which reacted readily with the EIM. In contrast, no special procedures were required to obtain EIM reaction with membranes formed from oxidized cholesterol (Tien, Carbone, and Dawidowicz, 1966) in decane.

The mixed brain lipids were extracted from fresh beef brain white matter and partitioned with water for 2 days according to the procedure of Folch and Lees (1951) to remove proteolipids. Where formulations specify "brain lipids" this crude mixture was used. Brain phospholipid fractions were prepared from these extracts by adsorption on activated silicic acid, washing with 2% methanol in chloroform, and retaining fractions eluted by 80% methanol in chloroform. The lipids were stored at $-10°C$ in chloroform.

Sphingomyelin (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as received. Phosphatidylethanolamine (Mann Research Laboratories, Inc., N.Y.) was reprocessed to remove colored contaminants by precipitating from chloroform solution with acetone and fractionating on silicic acid columns, retaining the fractions eluted by 20% methanol in chloroform. Soy bean lecithin (A. E. Staley Manufacturing Co., Decatur, Ill.) was similarly fractionated, retaining a fraction eluted at 50% methanol in chloroform.

Cholesterol, from various commercial sources, was recrystallized from ethanol to remove oxidation products. Other steroids, cholesterol butyrate and cholestane (Mann Research Laboratories), were used as received.

$\alpha$-Tocopherol was a gift of the Roche Division of Hoffman-LaRoche Laboratories (Nutley, N.J.). We are indebted to Dr. J. W. Mason and Mr. R. J. Salisbury for synthesis of the cyclohexyltetradecanoate (CHDT).

## EXPERIMENTAL RESULTS

The black membranes, as formed initially, have a high specific resistance, $10^6$–$10^8$ ohm cm$^2$, which decreases significantly with increasing polarizing potentials across the membranes, as illustrated in Fig. 1 A. Upon addition of a very small quantity of EIM to the medium on one side of the membrane, the

membrane conductance (at low potentials) increases rapidly after a short induction period, as indicated in Fig. 1 B. The conductance may increase by a factor of $10^3$–$10^5$, bringing the specific resistances as low as 100–1000 ohm $cm^2$, well within ranges of resistance for biological membranes. In addition, the membrane develops a variable, potential-dependent conductance, frequently displaying a steep negative differential resistance region (Fig. 1 C) similar to that found for membranes of excitable cells.

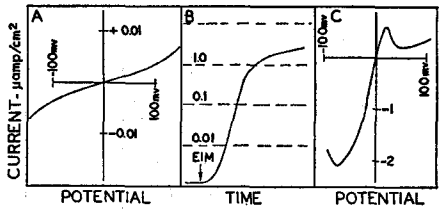


FIGURE 1. General conductance characteristics of the lipid bilayer membrane and the EIM-lipid bilayer membrane. A, current-voltage characteristics of the simple lipid membrane. Membrane composition, phosphatidylethanolamine-cholesterol-tocopherol. B, change in membrane conductance after addition of EIM. Crude EIM, $10 \times 10^{-9}$ g/ml, was added to the inner (reference) compartment of the membrane cell at the point indicated while maintaining a $-10$ mv polarizing potential (current is negative with cations flowing out of EIM compartment). Following a short induction period, which varies with EIM concentration, lipid composition, and compartment geometry factors affecting EIM diffusion, the conductance initially increases proportionally to $t^x$ where $x$ may vary from 2 to 4. Subsequently a linear relation develops. C, current-voltage characteristics of the EIM-lipid membrane. A positive potential and current indicate flow of cations into the compartment containing EIM. The EIM membrane is entirely selective for cations under these conditions. The asymmetry is characteristic of most phospholipid membranes.

However, the conductance of the membrane does not change as smoothly as represented by the logarithmically compressed curve of Fig. 1 B. Instead, as shown by the direct tracing of the current-time recording in Fig. 2, as soon as the EIM reaction starts, the current, at constant 10 mv polarizing potential, may fluctuate greatly. Recorder attenuation in this figure tends to mask the increasing fluctuation in the intermediate stages of reaction but the amplitude of the oscillations may continue to increase until a statistical blanking occurs at the higher levels of reaction.

If the EIM concentration is reduced, or temperature lowered, or a more refractory membrane is used to lower the rate of reaction, the current-time curves may take on a different character, as shown in Fig. 3. In this case, the initial reaction is characterized by several distinct conductance steps of a fairly uniform character. The step-like character may still be observed in the later portions of this curve, although steps are irregular in size and numerous reversals appear.

Under selected conditions of EIM concentration, temperature, and lipid composition, remarkably uniform step recordings, represented by the tracing in Fig. 4, may be obtained. In this case, it appears that all conductance changes occur in discrete jumps with each conductance change in an integral multiple of a common conductance increment. In other cases, as shown in Fig. 5, a large number of individual steps may be distinguished, almost all conforming to an integral multiple of a common increment. The basis for the few exceptions will be discussed in a later section of this report.

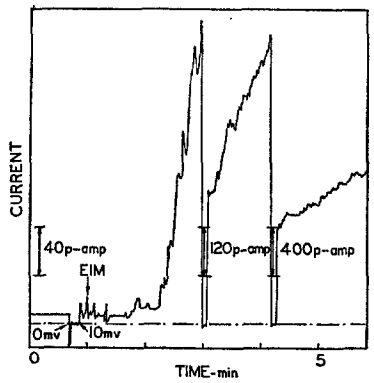


FIGURE 2. Recorder tracing of current, at constant potential, during membrane interaction with EIM. Membrane composition, phosphatidylethanolamine, 2%; cholesterol, 2%; tocopherol, 20%; in chloroform-methanol. Polarizing potential, −10 mv. Current is negative, although represented as positive to indicate increasing conductance. Temperature, 45°C. Chart speed, 50 sec per inch. After constant conductance was attained, 5 $\mu$l of crude EIM (about $20 \times 10^{-9}$ g protein) added, at the point indicated, to 2 ml of medium in the inner (reference) compartment. Before addition of EIM only a low noise fluctuation appears in the current. Just prior to and during EIM addition, large fluctuations occur due to pipet contact and manual stirring. Current spikes also occur at each change of polarizing potential due to capacitive currents.

As indicated above, the observation of steps may be greatly affected by membrane composition, temperature, and EIM concentrations. Certain systems, such as sphingomyelin-tocopherol membranes, may show a much greater tendency than others toward rapid fluctuations, which mask the incremental jumps. However, it has been possible to observe and measure conductance steps in all membrane systems which show any tendency to react with EIM. A partial summary of these observations is given in Table I. It is immediately apparent that there is only a very slight difference in the conductance increments observed in various membranes, regardless of lipid composition. Whether the amphiphilic agent is mainly sphingomyelin, phosphatidylethanolamine, phosphatidylcholine, a complex mixture of brain lipids, or oxidized cholesterol; or whether the plasticizing solvent is a straight

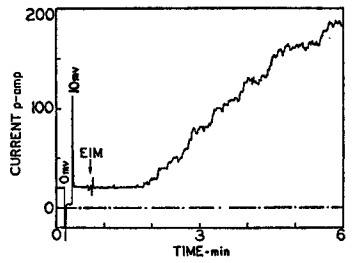


FIGURE 3. Current fluctuations during membrane reaction with limited EIM. Membrane composition, brain phospholipid, 2%; cholesterol, 2%; tocopherol, 15%. Polarizing potential, −10 mv. Temperature, 37°C. Chart speed, 50 sec per inch. At the point indicated, EIM, about $20 \times 10^{-9}$ g per ml, was added to the inner (reference) compartment. Although the concentration of EIM used here was greater than that for the membrane in Fig. 2, the lipids in this membrane were more refractory toward interaction with the EIM and a much greater concentration was required to obtain equivalent rates of interaction.

chain hydrocarbon (decane), or a bulky-headed molecule (cyclohexyltetradecanoate), or an amphiphile itself (tocopherol); or whether the steroid component is cholesterol, cholesteryl butyrate, or cholestane, or absent entirely; all conductance steps observed during the reaction appear to average close to $4 \times 10^{-10}$ mhos. For any single membrane, all the steps are extremely consistent, within the limits of the experimental system. Variability may be as great between two membranes formed from identical solutions as between membranes of entirely different composition. Thus, the size of the conductance increment appears to depend more upon a common factor, the EIM, than it does upon the nature of the lipid in the membrane.

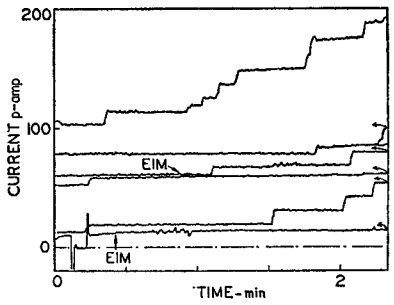


FIGURE 4. Long-lived conductance steps during membrane interaction with EIM. Membrane composition, brain phospholipid, 1.5%; tocopherol, 15%. Polarizing potential, −15 mv. Temperature, 36°C. Chart speed, 3 inches per min. EIM, to about $20 \times 10^{-9}$ g per ml, was added to the reference compartment at the two points indicated. The recording was made on an X-Y recorder, necessitating the overlapping, sequential scans. This particular batch of brain lipids consistently gave remarkably uniform and distinct step reactions similar to those shown here.

The steps cannot be considered as an artifact of the observational system since the same instrumentation applied to other reactions consistently shows different types of conductance-time relations. An increase in membrane conductance caused by the cationic triphenylmethane dye, brilliant green, proceeds perfectly smoothly, with no evidence of fluctuations or steps above the normal background noise level (Fig. 6). Similar curves are found for a number of other dyes (e.g., methylene blue, acridine orange) and iodine. On the other hand, the cyclic peptide antibiotic, tyrocidin, causes rapid current fluctua-

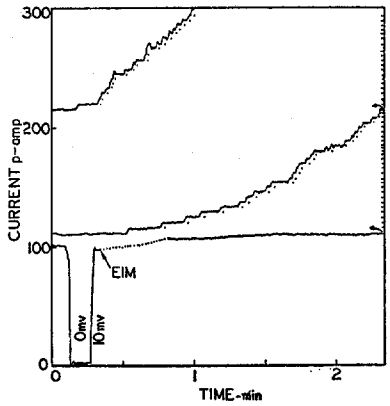


FIGURE 5. Extended conductance step sequence during the EIM interaction. Membrane composition, brain phospholipid, 2%; cholesterol, 3%; tocopherol, 15%. Polarizing potential, −10 mv. Temperature, 36°C. Chart speed, 3 inches per min. Manual stirring immediately following addition of EIM caused violent current fluctuations so the trace is omitted at that point. More than 40 uniform steps may be distinguished, as indicated by the markings on the right side of the chart. A few steps appear to be considerably smaller than normal for this system. Such variations may be accounted for in the transitional mechanism as outlined in the text.

tions (Fig. 7), which increase in magnitude with increasing conductance, but no steps may be distinguished under a variety of conditions of temperature, tyrocidin concentration, or electrolyte or membrane composition.

Therefore, it appears that the discrete, uniform conductance steps occurring during the reaction of EIM with the membranes may be uniquely attributed to the changes which EIM induces in the membrane structure. It is further apparent that the structural alterations have a very long life, in molecular terms, although some portion of the changes may be rapidly reversible.

*Proteolytic Destruction of EIM Conductance* Mueller and Rudin (1968 *b*) state, without further experimental amplification, that various endopeptidases may destroy the EIM conductance, including the variable resistance

characteristics, and that the proteolytic action is effective from either side of the membrane, except in the case of chymotrypsin. Experiments in our laboratory with proteolytic enzymes supported similar conclusions with the exception that chymotrypsin was effective from both sides of the membranes under appropriate conditions. In addition, proteolysis always appeared to

TABLE I

INCREMENTAL CONDUCTANCE CHANGES IN LIPID BILAYER MEMBRANES DURING REACTION WITH EIM

| Membrane | Composition‡ | No. of steps observed | Conductance increment* | | |
|---|---|---|---|---|---|
| | | | Average | Minimum | Maximum |
| | | | $ohm^{-1} \times 10^{10}$ | | |
| BL-TOCO | 2:20 | 73 | 3.2 | 2.7 | 5.0 |
| BL-TOCO-CHOL | 1.5:20:2 | 251 | 3.8 | 3.0 | 6.0 |
| BPL-TOCO-CHOL | 2:15:2 | 120 | 4.1 | 3.0 | 6.0 |
| SM-TOCO | 6:40 | 113 | 3.4 | 2.2 | 5.2 |
| SM-TOCO-CB | 3:20:3 | 71 | 3.5 | 2.2 | 4.2 |
| SM-TOCO-CHOL | 3:20:3 | 10 | 4.0 | (4.0) | (4.0) |
| PE-TOCO-CHOL | 1:20:2 | 55 | 4.1 | 3.4 | 4.4 |
| PC-TOCO-CB | 1:20:2 | 8 | 4.0 | (4.0) | (4.0) |
| BPL-CHTD-CHOL | 1:10:2 | 35 | 3.4 | 2.8 | 4.2 |
| BPL-CHTD-CHOLESTANE | 1:10:3 | 31 | 4.3 | 2.8 | |
| BPL-DECANE | | 15 | 3.6 | 3.4 | 3.8 |
| BPL-DECANE-CHOL | | 10 | 3.9 | 3.6 | 4.2 |
| OXIDIZED CHOL-DECANE | 1:99 | 25 | 3.6 | 2.2 | 5.0 |

* Measurements carried out at 10 mv, constant potential. Conductances were calculated by dividing total current change for a series of single steps by the number of steps. Average is calculated from total of all observations made. Maximum and minimum values represent largest and smallest average values for a series of steps in a single membrane. Individual steps could only be measured with an accuracy of $\pm$ 0.2 p-amp at 10 mv or a conductance change of $\pm 2 \times 10^{-11}$ $ohm^{-1}$.

‡ Abbreviations: BL, brain lipid (including phospholipid plus neutral lipids); BPL, brain phospholipid; SM, sphingomyelin; PE, phosphatidylethanolamine; PC, phosphatidyl choline; TOCO, tocopherol; CHTD, cyclohexyl tetradecanoate; CHOL, cholesterol; CB, cholesteryl butyrate.

leave a residual membrane conductance, somewhat higher than that of the original, protein-free membrane, after destruction of the variable conductance properties. These observations suggested that the EIM conductance was due to the formation of polar channels through the lipids in which the conductance was dependent upon certain configurations of the protein which could be destroyed by endopeptidases. The residual conductance, without the variable resistance behavior, was taken to indicate that some portion of the protein might remain associated with, or trapped in the lipid membrane after destruction of the high conductance configuration, still creating a path of less resistance than the surrounding lipids.

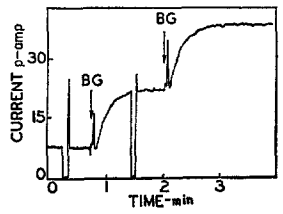


FIGURE 6. Conductance change in lipid bilayer membranes due to interaction with brilliant green. Membrane composition, sphingomyelin, 3%; cholesteryl butyrate, 3%; tocopherol, 15%. Polarizing potential, −10 mv. Temperature, 34°C. Chart speed, 50 sec per inch. Brilliant green dye was initially added, as indicated, to give $5 \times 10^{-7}$ M concentration in the reference compartment. Conductance changed rapidly, but smoothly (except for normal noise) in contrast with the fluctuating behavior with EIM. A second addition caused a similar change indicating that conductance is directly proportional to brilliant green concentration. A salt gradient did not produce a diffusion potential suggesting that conductance is probably due to migration of the cationic form of the brilliant green. I(V) curves for the brilliant green system show a transition between two conductance states, as for the EIM system, but not a negative resistance.

A further examination of the proteolysis phenomenon was made under conditions similar to those used for detection of conductance steps during the EIM reaction. EIM, at a relatively low concentration, was allowed to react with the membrane, to a limited degree, and then chymotrypsin was added to the medium (together with sufficient magnesium ion for enzyme activation). As shown in Fig. 8, the increase in EIM conductance is soon reversed after the addition of the enzyme. Under the conditions of this experiment, steps

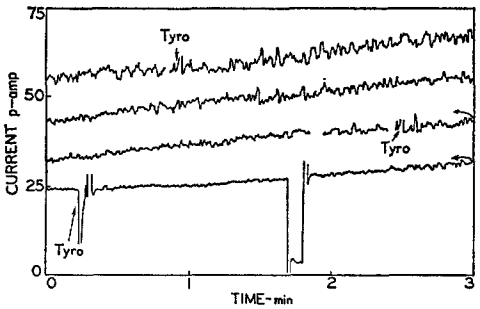


FIGURE 7. Conductance fluctuations during membrane interaction with tyrocidin. Membrane composition as in Fig. 6. Tyrocidin (Nutritional Biochemicals Corp.) was added at the points indicated to give $10^{-8}$ M tyrocidin in the reference compartment. This may include some gramicidin, normally present in commercial tyrocidin. Current fluctuations increased above normal noise level almost immediately after the addition and continued to increase as total conductance increased. Membranes were highly specific for cations and showed about a 10-fold discrimination for potassium over sodium ion. I(V) characteristics are similar to those illustrated for the bare lipid membrane (Fig. 1) even though conductance may approach that of the medium.

may be observed during the reversal as well as in the forward reaction. The reversal increments, however, are smaller than those of the forward reaction. In some cases, as for that illustrated, the degree of reversal ultimately attained appears to be roughly proportional to the size ration of reversal to forward steps. This would be expected if the proteolysis destroys the structure of the EIM channel sufficiently to reduce the maximum conductance per channel, but leaves sufficient protein penetrating the lipid to maintain a significantly greater conductance than that of the simple lipid membrane.

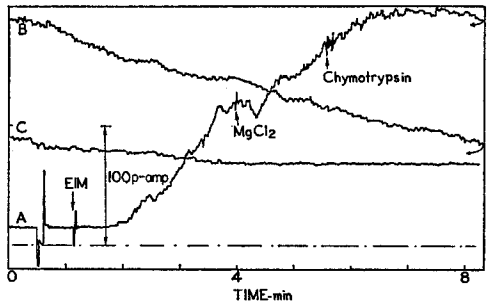


FIGURE 8. Discrete conductance fluctuations during proteolytic destruction of EIM conductance. Membrane composition, phosphatidylethanolamine, 1%; cholesterol, 1%; tocopherol, 20%. Polarizing potential, −10 mv. Temperature, 34°C. Chart speed, 50 sec per inch. At the indicated point, EIM, to $2 \times 10^{-9}$ g/ml was added to the reference compartment. After a partial reaction had occurred, magnesium chloride, 1 mM, was added and then chymotrypsin, 50 μg/ml (two times crystallized) was added to the same compartment as the EIM. The enzyme-activating magnesium ion generally caused a slight, but temporary inhibition of the EIM interaction. Shortly after addition of the enzyme, a reversal was initiated. Conductance increments during reversal appear to average about $2 \times 10^{-12}$ amp while the forward steps average about $3 \times 10^{-12}$ amp. After 30 min of incubation, conductance was constant again with about 75% of the EIM conductance destroyed. No variable conductance characteristics remained.

The possibility that the reversal was actually due to causes other than proteolytic destruction of the EIM channel (e.g., interference and blocking of the channel by proteins or displacement of the EIM by other proteins) was investigated by examining the action of a number of other nonproteolytic enzymes and proteins on the EIM membrane. Although several caused specific alterations in the EIM conductances, none, aside from the endopeptidases, caused a significant reversal.

*Discrete Conductance Fluctuations in Potential-Dependent Conductance Transitions* The most striking property of the EIM conductance is its response to changes in polarizing potentials across the membrane, switching reversibly between a low conductance and a high conductance. In their theoretical discussion of this conductance transition, Mueller and Rudin (1963) treated it

initially as a two-state system, with a low and high conductance, having specific transition energies associated with the forward and backward transitions between these two states. It would matter little to the theoretical model whether the transition occurred by this simple two-state mechanism or through a series of intermediate, smaller steps but a structural interpretation of the transition may be greatly affected by the two possibilities. Mueller and

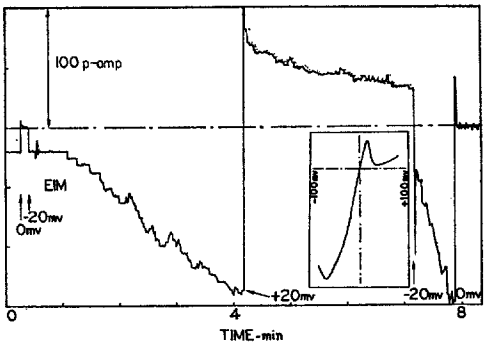


FIGURE 9. Discrete conductance fluctuations in potential-dependent resistance transitions of the EIM-lipid membrane. Membrane composition, brain phospholipid, 2%; cholesterol, 2%; tocopherol, 15%. Temperature, 40°C. Chart speed, 50 sec per inch. Polarizing potential during initial reaction with $2 \times 10^{-9}$ g EIM per ml was held at −20 mv (cation flow out of EIM compartment). This was switched to +20 mv to develop the high resistance state. Inset indicates I(V) characteristics of the membrane as determined after observation of conductance fluctuations. Maximum conductance occurred at −25 mv. The minimum conductance increment average during the EIM reaction sequence is $3.0 \times 10^{-10}$ $ohm^{-1}$. Increments and their frequencies, observed during the transition (including both forward and reverse steps), were: $0.5 \times 10^{-10}$, 13 times; 1.0, 14 times; 1.5, 3 times; 2.0, 6 times; 3.0, once; 6.0, once. In other experiments similar increment values were observed but relative frequencies depended upon the potential applied.

Rudin (1968 *b*) observed some potential fluctuations in the EIM membranes which suggested the existence of quantized conductance states in the EIM channels. We reinvestigated the transitional problem to determine whether these quantized states did exist, in fact, and to determine whether the transitions would be best described by a two-state or multistate mechanism.

A membrane having a large number of channels will show a relatively smooth, exponential transition between conductance states in response to a potential change, as described by Mueller and Rudin. However, when switching is induced in a membrane containing only a few channels, as in Fig. 9, a more random behavior and a number of incremental fluctuations may be observed. In this instance, EIM was allowed to react until a conductance corresponding to the formation of approximately 20 channels was attained. During the EIM interaction the membrane was polarized at −20 mv, the approxi-

mate potential giving maximum conductance in this membrane system. To initiate the transition to the high resistance state, a +20 mv was applied, a potential which might be expected to cause 60–90 % decrease in EIM conductance according to the behavior of similar membranes. As the recorder tracing shows, the positive potential causes an initial drop in conductance, completed within the overshoot and recovery time of the recorder, of about half of the original conductance. This is then followed by a series of step transitions as conductance continues to decrease slowly. Similar step transitions were obtained several times in this membrane and in a number of others. In addition, maintenance of an intermediate potential (+10 mv) created a continuing current fluctuation in which positive and negative steps of similar nature were observed. The latter procedure, corresponding roughly to the conditions under which Mueller and Rudin observed the step changes, was somewhat complicated by the continuing reaction of EIM and development of additional channels during the holding periods.

The individual steps, in all cases, tended to be much smaller than those found during the insertion of EIM. In the case illustrated, the transitional steps appear to be about one-fifth the formation step, or about one-tenth the normal average channel conductance. This suggests then, either that the transition between conductance states in a single channel may take place through a multistep, rather than a single-step process, or that the unit channel conductance is represented by the small switching steps and the larger steps, observed during EIM reaction or switching must be due to multiunit coupling.

## DISCUSSION

Mueller and Rudin (1968 *b*) have previously suggested that EIM forms a protein bridge across the lipid membrane to provide hydrogen bonding and charged sites for the movement of ions across the membrane. It is difficult to attribute the incremental current fluctuations during the EIM interaction or during its reversal in proteolysis to anything other than the opening or destruction of a long-lived, unit EIM channel. The proteolysis studies, showing that channel destruction may be accomplished from either side of the membranes, but leaving a small, residual conductance due to EIM, also support the interpretation of a macromolecular bridge, or pore, which requires a certain configuration for maximal conductance. The uniformity of the incremental fluctuations observed during the EIM interactions in membranes composed of a great variety of lipids indicates that the basic, open channel conductance is mainly governed by the macromolecule bridge, i.e., the mobile ions are largely shielded from the influence of the varying polar groups of the lipids in the membrane in the maximal conductance state of the channel. It might be conceivable that the long-lived conductance increments could be developed as the result of insertion of a carrier, or carrier complex

into the membrane so that it becomes active at a constant level. However, if the average current increment ($4 \times 10^{-12}$ amps) is assumed to reflect the carrying capacity of each carrier element, it would be necessary to propose a flow of about $10^8$ ions per sec via each carrier unit. This would be much too large a turnover to be accommodated by a carrier molecule with a molecular weight of 10,000–100,000 (Stein, 1968).

The transitions between the two extreme conductance states, on the other hand, are very much subject to the influence of lipids (Mueller and Rudin, 1968) with respect to kinetics and steady-state characteristics. It has been demonstrated here that the transitions also involve incremental conductance fluctuations, which may be much smaller than those observed during the EIM insertion or adsorption. Similar fluctuations previously noted by Mueller and Rudin (1968 *b*) led to their suggestion that quantized conductance states may exist in each channel. The numerous, small incremental conductance fluctuations demonstrated here during the conductance transitions provide further support for the concept of a number of discrete conductance states between fully open and fully closed states of the EIM channels. Another possibility, that the larger conductance increments simply represent coupling of a number of unit channels, with conductances corresponding to the smaller steps, should also be considered. However, the uniformity of the channel-forming steps suggests that the coupling may be so tight, under many conditions, that it would be desirable to treat the multiunit assembly as the basic structural unit even though different mechanisms would be involved in the switching transitions. In addition, the rather low cation-cation discrimination of these membranes (Bean and Shepherd, 1965), and other factors of ion interaction, suggest a channel of relatively large diameter.

The smaller steps obtained in the transitional sequences may also account for the occasional smaller than normal steps obtained during the EIM insertion process. The high conductance state of the EIM channel is the stable state at the low, negative potentials used for monitoring the conductance during the EIM-membrane interaction. However, neither the activation energy nor the free energy for the transition from one state to another appears to be very large (Bean and Shanfield, unpublished data) and there is always a finite probability that certain channels will either partly close soon after formation or will be in a partly closed configuration as they form, giving rise to a smaller conductance change than would be expected for the normal insertion process. The number of channels in closed, or partially closed states should increase with rising temperatures in addition to increasing the frequency of fluctuation between open and closed states, leading to observation of a greater number of the smaller conductance increments and a more rapid current fluctuation. Both phenomena have been observed in a number of different types of membranes. Indeed, although the temperature coefficient

for the increase in conductance of the EIM channel with temperature is generally in the range of 1.2 per 10°C in the lower temperature ranges (Mueller and Rudin, 1968 *b*), at higher temperatures (40–45° C for sphingomyelin-tocopherol membranes or 50–55° C for phosphatidylethanolamine-tocopherol-cholesterol systems) the conductance may show a negative temperature coefficient as the population of the high resistance states increases in the low potential ranges (Bean and Shanfield, unpublished data).

Although the evidence strongly suggests that EIM forms a macromolecular, conductive bridge through the lipid membrane, it may be difficult to develop a more specific model for the EIM channels because of the complex and often contradictory reaction kinetics and behavior of the system. In most cases the development of the EIM conductance may depend exponentially on the concentration of EIM, as indicated by Mueller and Rudin (1968 *b*) but, under some circumstances, a direct, linear dependence between rate of conductance increase and EIM concentration may be obtained. Similarly, the conductance may increase linearly at a given concentration of EIM with certain lipid systems while increasing as a power function of time with other systems. Such discrepancies in behavior may possibly be attributed to a complex reaction sequence which may involve macromolecule-macromolecule, macromolecule-lipid, and lipid-lipid interactions. There is frequently a very sharp cutoff temperature for the EIM interaction in various lipid systems, which occasionally appears to coincide with a transition temperature for other changes in the physical properties of the lipid membrane (Bean, unpublished data) indicating that the rearrangement of lipids to accommodate the EIM may be a significant factor in the reaction sequence. In the presence of certain proteins (e.g., cytochrome *c*) the EIM interaction may show a linear dependence upon EIM concentration (for certain lipid systems) where an exponential function was previously found.

If the average conductance increment obtained during the EIM reaction ($4 \times 10^{-10}$ ohm$^{-1}$) is used to calculate the diameter of a (hydrated) channel which would provide this conductance (assuming ionic mobilities similar to those in water), a diameter in the range of 10 to 30 A is obtained. This diameter could be accommodated in a number of proteins, macrocyclic peptides, or the helix of such structures as bacterial pili. A channel of similar size could also be formed at the junction of several protein molecules inserted into the membrane.

It is probable, however, that the simplest molecular model to account for the complex behavior of the EIM channel would be one related to that recently proposed by Onsager (1967). He suggests that variable conductance channels may consist of nonrigid chains of the polar groups of amino acids of a protein helix, with supporting helices held in place in the membrane by suitable interaction of polar and nonpolar groups of the lipid and protein.

Conductance in such a channel could be altered by any configurational change which would change distributions of fixed ion or hydrogen-bonding sites in response to chemical factors or polarizing potentials.

In the open channel state of the EIM membrane, as suggested above, the protein configuration and its situation in the lipid matrix might provide a polar channel in which the mobile ions would be largely protected from influences of the charged and polar groups of the lipids. This minimal resistance configuration may be changed either to create larger energy barriers between polar sites or opened to allow a direct influence of the cation groups of the lipids on the mobile ions. Similarly, a change of the situation of the protein with respect to the surfaces of the lipid bilayer may increase or decrease the effects of the charged lipids upon the mobile ion. Such structural, positional changes could readily account for the influence of lipids upon the kinetics of conductance transitions in spite of their lack of effect upon the open channel conductances. However, the variable conductance properties of the EIM channels are manifested even in membranes consisting completely of uncharged lipids (oxidized cholesterol and decane), although higher potentials may be required to obtain the transitions, so that it appears probable that the spacing relations of transport sites of the macromolecules are more important than those of the lipids.

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