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Spontaneous Conductance Changes, Multilevel Conductance States and Negative Differential Resistance in Oxidized Cholesterol Black Lipid Membranes

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Summary. Oxidized cholesterol lipid films were studied under voltage clamp conditions. Certain kinds of conductance phenomena closely resembling effects described in the literature and attributed to various additives were observed with unmodified lipid films. These include spontaneous conductance changes, multilevel conductance states and negative resistance phenomena in films formed from aged oxidized cholesterol-in-hydrocarbon solutions. The multilevel conductance state manifests itself in the form of step changes in current under voltage clamp, the magnitudes of which correspond to conductance intervals of $5 \pm 2 \times 10^{-10}$ mho and its multiples in 0.1 N sodium chloride. These steps are attributed to channels that form in the film. Under certain conditions it has been observed that the action of the electric field was to close channels that were open at lower voltages. Under these conditions the current-voltage plot shows a distinct region of "negative differential" resistance. Films generated from freshly prepared oxidized cholesterol solutions did not exhibit multistable phenomena but under a prolonged voltage clamp the membrane conductance went into a transition from a stable, relatively noise-free-current state to a gradually increasing current with a large noise component. This sequence generally signalled imminent breakdown but the membrane could spontaneously revert to the original high-resistance, low-noise state. These kinds of intrinsic behavior sufficiently resemble certain effects produced by additives to merit special consideration during membrane reconstitution experiments.

The lipid bilayer is believed to be the basic structure of biological membranes. It is a relatively simple structure serving mainly as a permeability barrier and a matrix for other membrane components (Singer & Nicolson, 1972). These components, mainly proteins, glycoproteins and proteolipids are believed to be responsible for most of the complex functions that are carried out by the biological membrane.

A planar lipid bilayer can be formed readily *in vitro* (Goldup, Ohki & Danielli, 1970). It is generally prepared by allowing a dilute solution of

lipid in a hydrocarbon solvent to thin across a hole in a Teflon barrier (Mueller & Rudin, 1969). The composition of the final film is difficult to determine. Capacitance measurements indicate that the film thickness decreases as the hydrocarbon solvent chain length is increased by using decane, tetradecane and hexadecane (Fettiplace, Andrews & Haydon, 1971). Based on some evidence gathered by surface chemistry techniques, these authors concluded that the limiting area of a lipid molecule was independent of solvent chain length. They were then able to calculate film volume fractions which showed that the lipid volume fraction increased to 0.83 and 1.00 for glyceral mono-oleate and lecithin, respectively, in hexadecane. These results differed from direct measurements of film composition by radioactive assay which gave a value of 37% solvent composition for glyceral mono-oleate films prepared from decane or hexadecane solutions (Pagano, Ruyschaert & Miller, 1972). However, these last values may have been elevated by the presence of solvent lenses that are known to exist in these films. Recently, planar bilayers without any solvent were prepared and they were found to have capacitances within the range of values found for biological membranes, 0.9 $\mu F/cm^2$ (Montal & Mueller, 1972). These investigators suggested the possibility that the solvent in the bilayer may affect bilayer-protein interaction which may account for the difficulty encountered in incorporating large proteins into lipid films.

While capacitances of lipid films can be measured with good accuracy (White, 1970; Montal & Mueller 1972), the resistance of the lipid film, with or without solvent, is one of the least reproducible of its properties. Its value is distinctively high but it can vary over several orders of magnitude (Hanai, Haydon & Taylor, 1965; Taylor & Haydon, 1966; Läuger, Lesselauer, Marti & Richter, 1967; White, 1970; Kalkwarf, Frasco & Brattein, 1972; Montal & Mueller, 1972). Correlation of resistance with the area of the lipid film can be made only with the highest resistance values (Hanai *et al.*, 1965).

Nevertheless, the measurement of conductance (reciprocal resistance) change is an important method of monitoring biomembrane reconstitution efforts. Evidence for the incorporation of cholinergic (Parisi, Reader & DeRobertis, 1972) and adrenergic (Ochoa, Fiszer de Plazas & DeRobertis, 1972) receptors into lipid bilayers was obtained by the increase in conductance caused by the addition of the appropriate agonist. The conductance increases were blocked by the corresponding antagonist. A conductance increase was also the result of antigen-antibody and enzyme-substrate interactions at the bilayer (DelCastillo, Rodriguez, Romero & Sanchez, 1966). Bilayers treated with eel electroplax acetylcholinesterase have been re-

ported to respond to cholinergic agents with an increase in conductance that can be blocked or reversed by the action of inhibitors of acetylcholinesterase (Leuzinger & Schneider, 1972; Jain, Mehl & Cordes, 1973) and by an antagonist of the acetylcholine action at the acetylcholine receptor (Jain *et al.*, 1973).

Another kind of conductance change has been frequently observed. It has been clearly demonstrated that the proteinaceous bacterial extract, excitability inducing material (EIM), forms voltage-dependent cation-selective channels which close at high electric fields (Mueller & Rudin, 1968; Bean, Shephard, Chan & Eichner, 1969; Ehrenstein, Lecar & Nossal, 1970; Bean, 1972; Latorre, Ehrenstein & Lecar, 1972). This behavior is responsible for the "negative resistance" induced in the film by this substance (Mueller & Rudin, 1968; Ehrenstein *et al.*, 1970). Multilevel conductance phenomena indicative of voltage-sensitive channel formation have also been observed with bilayers treated with the extracts of the rat brain and eel electroplax (Goodall & Sachs, 1972), a cholinergic proteolipid from the eel electroplax (Parisi *et al.*, 1972), and a synthetic polypeptide (Goodall & Urry, 1973). Multilevel conductance phenomena have been observed also with alamethicin and gramicidin A. The alamethicin transitions were about 1,000 times faster and 10 times larger than the previously mentioned cases (Gordon & Haydon, 1972). The gramicidin channel is reported to have a unit conductance of only 5.8×10^{-12} mho and its lifetime is very sensitive to bilayer thickness (Hladky & Haydon, 1972).

In trying to repeat some of these very interesting experiments and in executing some variations of our own, we encountered some dramatic "successes" that were traced eventually to the lipid film itself. Our observations included spontaneous conductance changes, multilevel conductance phenomena and "negative differential resistance" which we believed at that time to be outside the realm of capabilities of the unmodified lipid bilayer film. These observations should serve as a note of caution to investigators initiating BLM studies with oxidized cholesterol.

Materials and Methods

Oxidized cholesterol solution were prepared by a method reported in the literature (Jain *et al.*, 1973). Twice recrystallized (from ethanol) cholesterol (590 mg) was refluxed in a solution of 10 ml tetradecane and 10 ml decane (practical grades, J. T. Baker Chemical Company, Phillipsburg, New Jersey) for 20 hr. The solution was then stored in 1- to 2-ml portions in stoppered 1-dram vials. Membranes prepared from this solution exhibited multistable behavior only after standing under ambient conditions for about a month. Storage at -2 °C retarded but did not prevent this aging process. EIM was a

generous gift from Dr. P. V. Mueller. Eel electroplax acetylcholinesterase VI, Lot No. 102C−8030 and Lot No. 11C−8080 was obtained from Sigma Chemical Company. Thin-layer chromatography plates were prepared with Silica Gel G, E. Merck AG, Darmstadt, Germany. Columns for column chromatography were filled with a 1:1 mixture of silica acid, Baker analyzed reagent and diatomaceous earth from J. T. Baker.

The bilayers were formed by blowing a bubble through a pipette coated with the lipid mixture against an opening in a Teflon septum separating two compartments of a cell. The septum was cut out of a 0.4-mm-thick Teflon sheet into which a 1.4 mm diameter hole was made with a #55 steel drill by carefully cutting through to half the thickness from either side. The hole was then gently polished with a #1 surgical silk to remove burrs along the edges.

The measurement cell consisted of two rectangular Lucite chambers of 5 ml capacity each with 1-cm diameter apertures on matching sides. Grooves were put on the periphery of the apertures into which "O" rings were fitted to prevent leakage. The Teflon sheet with the hole was placed between the "O" rings and clamped with two brass bolts that passed through the outer edges of the cell. An airtight cap was made for one of the chambers to allow the solution to be changed in the opposite chamber after film formation. Temperature control was maintained by circulating a lightweight oil through two sets of glass or brass tubing that were immersed in the aqueous compartments. The brass tubing was protected with a thin film of epoxy paint. Stirring was accomplished by two magnetic fleas in recessed wells in the bottoms of the chambers which reacted to electrically driven magnetic bars arranged in tandem below the cell.

Separate silver-silver chloride electrodes were used for passing current and for measuring voltage. The electrodes were connected to the respective chambers through potassium chloride-agar bridges. Voltage was supplied by a 1.35 volt mercury cell through voltage dividers and the output was regulated with a potentiometer. A polarity switch allowed changes in polarity. Membrane voltage was monitored with a Bioelectronic Instruments amplifier Model NFI with an input impedance of 10^{12} ohms. Current was measured with an Analog Devices 301 amplifier (open-loop gain 5×10^5), used in a simple current-voltage conversion configuration. Feedback resistors to the inverting input allowed current measurements of milliamps to picoamps. Initially the time constant of the current monitor was fixed at 50 msec. Later on the output was fed into a variable active RC lowpass filter that allowed a continuous but uncalibrated variation in time constant to a maximum of 0.2 sec. This allowed d-c measurements of very noisy membrane currents. The voltage and current outputs were fed into a dual channel Beckman Type RS Dynagraph for simultaneous recording.

The cell was cleaned between experiments by a sequence of rinses of 0.1 M aqueous KOH, 0.1 M HCl in ethanol, hexane, methanol, then distilled water. Rinses with pure solvents were repeated several times before proceeding to the next solvent. The Teflon septum was replaced whenever it was suspected to be contaminated irreversibly or when it became too heavily scored.

Results and Discussion

Three characteristic behaviors of oxidized cholesterol black lipid films were observed under voltage clamp.

In the normal state obtainable with freshly prepared lipid mixtures a very stable high resistance film could be formed. The resistance of this film was of the order of $10^8\ \Omega\ \text{cm}^2$ with a film lifetime of at least 4 hr. Another

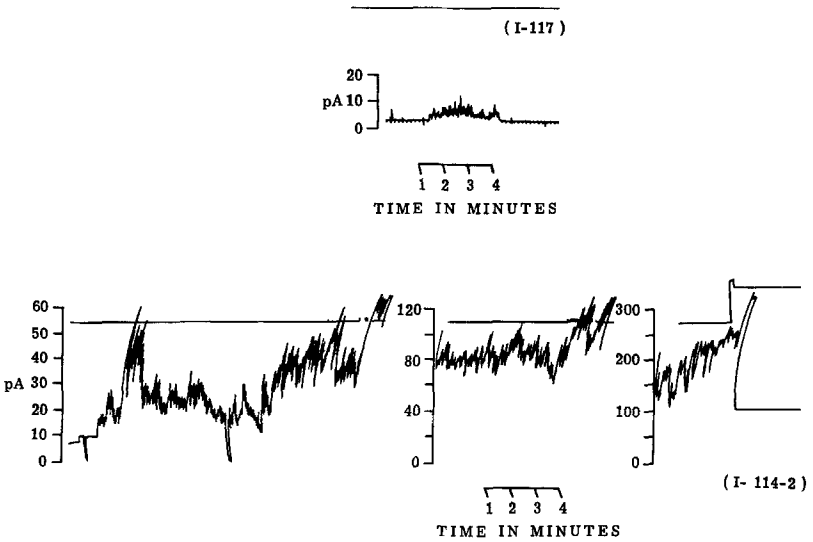

Fig. 1. Two current records of a high resistance membrane under a prolonged voltage clamp of 70 mV. 1.0 M KCl, 10 mM Tris HCl, pH 7.0 (adjusted at 25 °C), 34 °C. The membrane made a transition from a high resistance, relatively noise-free state to a noisy state. The record in the top trace is of a membrane that entered the noisy state, then returned to the low noise state. The lower trace is another current record of a membrane under the same conditions. In this case the transition to the noisy state was followed by a progressive deterioration of the permeability barrier that culminated in the rupture of the film. Note the change in the recorder gain setting as the current increased. The continuous current record was separated during reproduction so that the scale changes could be inserted. The regularly spaced markers in the top trace are artifacts caused by the pickup of pulses from a wave-form generator that was on at that time. Two zero checks in the early part of the bottom trace can be seen as dips to zero in the current trace. The lower current trace is a record of an experiment in which acetylcholinesterase (6 μg/ml) was added to the aqueous phase 9 min before the onset of the noisy state. This record is similar to other records of experiments without additives. This particular trace was selected for publication because the record of the entire noisy state was of a convenient size for reproduction

characteristic of this state was a low level of current noise under voltage clamp. The second type of behavior occurred when this membrane was clamped at 70 mV for a prolonged period of time. A transition occurs characterized by: (1) large random fluctuations (noise) in the current, and (2) a gradual decrease in the membrane resistance. Occasionally the membrane entered the noisy state then returned to the noiseless state. More often the onset of the noisy state signalled the beginning of a gradual deterioration in the membrane resistance until the membrane broke. This process generally lasted a few minutes, although it has lasted for as long as an hour. A representative current trace is reproduced in Fig. 1. An

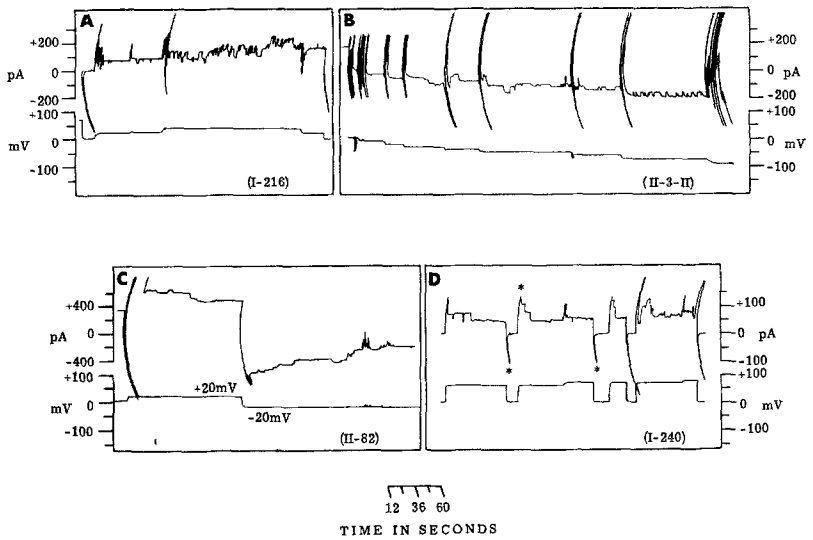

Fig. 2. (*A*) Multilevel conductance phenomena in 0.1 M NaCl, 10^{-3} M PO_4^{3-}, pH 7.0 at 24.4 °C. Multilevel conductance phenomena appeared when the clamping voltage was 25 mV and higher. The time scale for *A* is 5 times slower than the indicated time scale which applies to *B*, *C* and *D*. (*B*) Multilevel conductance phenomena in artificial seawater (465 mM Na^+, 5 mM K^+, 25 mM Ca^{++}, 10 mM Tris HCl, pH 7.4 at 25 °C). Multilevel conductance phenomena appears at −30 mV. (*C*) Effect of voltage on a membrane exhibiting multistable phenomena. 0.4 M NaCl, 4×10^{-3} M PO_4^{3-}, pH 7.0, 30.1 °C. The current can be seen dropping in steps from a high to a low value. The trend started at +20 mM and continued when the sign of the voltage was reversed to −20 mV. (*D*) Effect of a voltage step on a membrane in a multilevel conductance state. 0.1 M NaCl, 10^{-3} M PO_4^{3-}, pH 7, 28.5 °C. The conductance dropped in random steps with the application of a voltage step. The process was repeated several times. Asterisks mark capacitative currents

example of a short-lived excursion into the noisy state has been included. The relationship of these changes to the time and voltage dependent transition of an "initial state" membrane capacitance to a "final state" membrane capacitance described by White (1970) remains to be determined.

A third type of behavior was observed with aged oxidized cholesterol mixtures and was characterized by a wide range of membrane resistance of at least an order of magnitude lower than a normal film. Film lifetime was unchanged and a noisy state again signalled imminent breakdown. The most interesting feature of this state was the occurrence of sharp "step-function" transitions in current between apparent multilevel conductance states. Fig. 2 (*A*) and (*B*) are representative observations. It should be noted that these transitions are very similar in form and magnitude to the observa-

tions reported for EIM channels (Bean *et al.*, 1969; Ehrenstein *et al.*, 1970; Bean, 1972; Latorre *et al.*, 1972), the potassium selective channels from rat brain and eel electroplax (Goodall & Sachs, 1972), the bistable conductance induced by a cholinergic eel electroplax proteolipid (Parisi *et al.*, 1972), and a synthetic voltage dependent channel (Goodall & Urry, 1972). A significant difference is that unlike the situation with EIM, the number of transitions occurring in the unmodified oxidized cholesterol film could not be controlled. The lipid film occasionally went into this multilevel conductance state from a high resistance state. After thinning, the film, under a voltage clamp, may be in a high resistance state for a variable period, then make a transition in one step into a higher conductance state after which the multilevel conductance phenomena can be observed. The lifetime of the high resistance state that preceded the multilevel conductance state diminished with progressive aging of the lipid mixture. The magnitude of the conductance jumps varied from $5 \pm 2 \times 10^{-10}$mho to about 2×10^{-9} mho with 0.1 N NaCl (buffered to pH 7 with 10^{-3}M phosphate). The data collected from over 80 different films formed from three different batches of oxidized cholesterol, each batch prepared by the same procedure, indicated that the conductance steps were multiples of a single unit of about $5 \pm 2 \times 10^{-10}$ mhos. This was true for membranes with total conductances that varied over an order of magnitude. Observations on very leaky membranes ($10^5\ \Omega\ cm^2$) showed that transitions of similar magnitude occurred but measurements of current increments became inaccurate with increasing net current because of the necessity of reducing the current sensitivity on the recorder.

One possible explanation for the increase in conductivity and the stepwise change in the conductivity is the spontaneous formation of channels in the membrane formed from an aged lipid solution. The wide variation in membrane resistance observed in this state can be explained in terms of a variability in the number and size of the channels. The smallest conductance step of $5 \pm 2 \times 10^{-10}$ mho probably represents a minimum radius or a maximum curvature that can be maintained by a channel in the lipid film.

The membrane in the multilevel conductance state showed no permselectivity. This was determined by the absence of membrane potentials with sodium chloride and potassium chloride concentration gradients. A small bi-ionic potential of 21 ± 7 mV was observed when 100 mM tetraethyl ammonium chloride was placed opposite 100 mM NaCl, the TEA compartment being positive. A potential of this magnitude can be explained by a diffusivity ratio of 2:1 between the salts. Apparently the channels are large

enough such that no additional restrictions outside of normal solvent drag are felt by the diffusing ions.

The most interesting feature of the multilevel conductance state was the voltage dependence of the conductance. In many cases, when the voltage was applied the current started from a high value then dropped stepwise in fairly random manner to a lower value. This can be seen in Fig. 2 (*C*). This effect was more convincingly demonstrated by repetitively applying a voltage step on the membrane after a brief rest at zero voltage. The result was a cascading stepwise drop of current from a high to a low value. This is reproduced in Fig. 2 (*D*). A threshold voltage was required to activate the multilevel conductance phenomena (Figs. 2*A* and 2*B*). This threshold voltage decreased with decreasing membrane resistance. When the membrane resistance was in the range of 10^6 to 10^7 Ω cm^2, the multistable phenomena was not observed until a voltage of 20 to 30 mV was applied. The membrane voltage was obviously sealing the conductive channels. For this kind of voltage effect a negative differential resistance would be expected and was observed. This is shown in the voltage-current plot in Fig. 3 which has a conspicuous "negative resistance" region. However, in contrast to observations with EIM and alamethicin, the negative resistance curve was not reversible.

As stated earlier, a membrane exhibiting multistable behavior could have a widely varying specific resistance with older lipid mixtures tending towards lower resistances However, membranes with resistances lower than 10^5 Ω cm^2 were generally not studied for reasons stated earlier. Often a membrane became progressively and irreversibly "leakier" with time and increasing voltage. In such situations the effect of the membrane voltage seemed to be to create channels rather than to seal them. The film lifetimes were also short and the state of noisy deterioration observed with high resistance films were encountered more frequently. These factors plus the lack of reversibility of the I-V curve indicates the presence of other random forces acting on the channel formation and elimination process.

A cursory analysis of the oxidized cholesterol mixtures by TLC (acetic acid, chloroform, hexane; 5:20:75 v/v) showed the presence of five major components including cholesterol. A difference between fresh (inactive in terms of multilevel phenomena) and aged (active) cholesterol was not observed by this method. Three attempts at the isolation of a possible "active ingredient" by column chromatography were without success. All fractions came out as mixtures and those that were tested were active.

The possibility that the increased conductivity was caused by leakage between the lipid torus border and the Teflon septum was considered. This

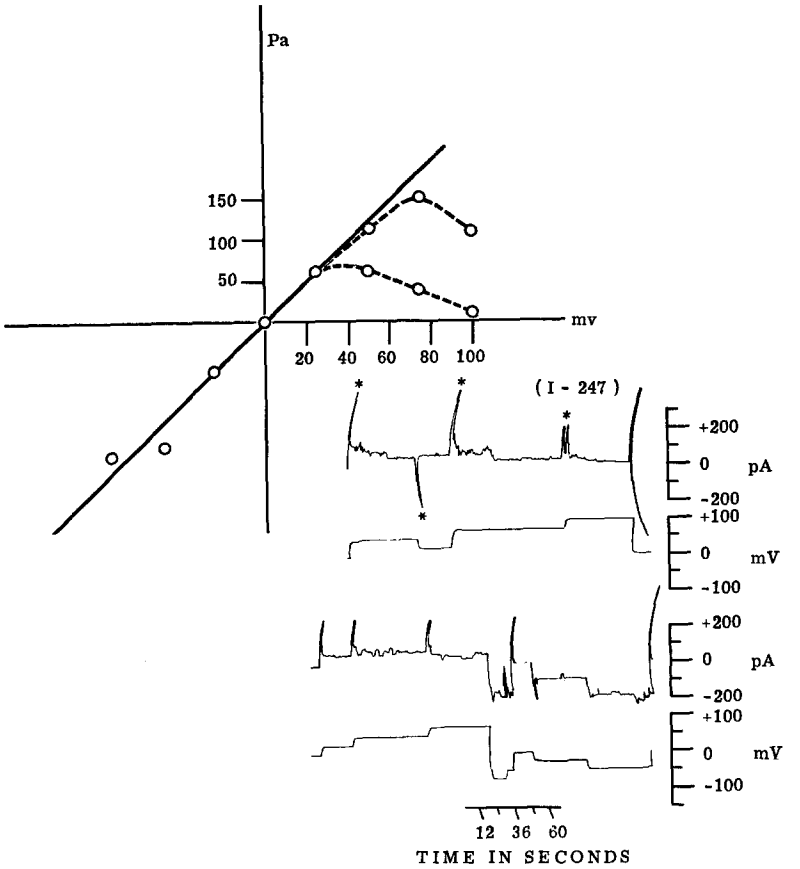

Fig. 3. Negative resistance exhibited by a membrane in a multilevel conductance state. 0.1 M NaCl, 10^{-3} M PO_4^{3-}, pH 7.0, 27 °C. The "negative resistance" points are connected by broken lines. The two curves follow the high and low conductance observed in the positive quadrant. Only the lower current values are shown in the negative quadrant. Asterisks refer to capacitative currents

problem was difficult to resolve with adequate certainty but it was found that the bilayer could be made to thicken by tapping the cell. When the film thickened, the multistable phenomena was completely extinguished and returned only when the film became black again. The time of reappearance of the multistable phenomena corresponded closely with the thinning process. This observation, together with the fact that aging was always required for this phenomena, comprised the evidence against leakage at the border.

The membrane voltage appeared to have two kinds of effects on the membrane. High voltages on stable films or moderate voltages on unstable

films induced a conductance turbulence which resulted in a noisy current trace and a deterioration of the permeability barrier. This behavior may be related to the mechanism for the dielectric breakdown. The membrane voltage could also partially restore the permeability barrier. This was done apparently by forcing the channels that exist to close. The source of this force may arise from the charges ($Q = VCm$) that accumulate on either side of the bilayer. This force is believed to act on the bilayer to cause a 10 to 15% reduction in the bilayer thickness (White, 1970). It is possible that channels would be squeezed out under these conditions. The variation in membrane area introduced by these channels appearing and disappearing would be infinitesimal.

The similarity of these intrinsic phenomena to effects elicited by certain additives can be troublesome in reconstitution experiments. We have repeated the EIM experiments and found that channels were induced very readily in lipid films as reported (Ehrenstein *et al.*, 1970). These EIM channels were qualitatively similar to the intrinsic variety except for the fact that the number of EIM channels induced could be controlled. This brings forth the possibility of the simultaneous appearance of EIM and native channels. Such superpositions may account for the fractional steps observed by Bean (1972).

The interaction of incorporated acetylcholinesterase with acetylcholine (Jain *et al.*, 1973) results in conductance changes that resemble the current trace in Fig. 1 in the following respects: (1) the magnitude of the conductance increase, (2) the time course of the increase, and (3) the increase in current noise. We attempted to generate the acetylcholinesterase effect without success but found the spontaneous conductance changes particularly vexing because of their similarity to the esterase effect. The key difference was the absence of manipulability of our system. Perhaps the increase in noise accompanying the acethylcholinesterase effect is more pertinent to basic bilayer phenomena than to the esterase-substrate reaction. The possibility also comes to mind that a particular conformation of the esterase promotes the noisy state of imminent breakdown, and that the role of the substrate is to induce this particular conformation or arrangement in the protein.

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