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Properties of Lipid Bilayer Membranes Separating Two Aqueous Phases: The Effects of Fe^{+3} on Electrical Properties

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Summary. Ferric ion has been found to alter the electrical properties of lecithin-cholesterol-decane bilayer membranes. Within minutes after the addition of microgram quantities of $FeCl_3$ to the ambient aqueous phase, the resistance of the membrane falls by a factor of 10^5 to 10^6. No change in capacitance is observed. The resistance change is obtained with membranes made from synthetic lecithin (fully saturated fatty acids) as well as by those formed from egg lecithin. The conductance of the modified membrane exhibits both time and voltage dependent behavior; the time dependence of the current is similar to that of an inductance, and the voltage dependence of the current is exponential. Concomitant with the resistance change, the modified membrane becomes permselective, passing chloride almost to the complete exclusion of sodium. Anion selectivity can be converted to cation selectivity by the subsequent addition of certain chelating agents. Area-conductance measurements show the resistance change occurs in the thin film. The addition of a reducing agent causes the effect of the ferric ion to be reversed, and the conductance returns to that characteristic of unmodified membranes. When ferric ion is added to only one side of the membrane, the system rectifies with current ratios of up to 20:1. It is concluded that the alteration of membrane properties owes its origin to the hydrolysis of membrane-bound ferric ion. The interaction of ferric ion with aqueous dispersions of lecithin has been investigated by several techniques, and evidence is presented that the dispersions bind charged species of iron and that this charge diminishes under conditions where iron hydrolysis occurs.

It is clear that cell membranes, in spite of their apparent simplicity of appearance in the electron-microscope, are in fact, extremely complex structures. The specificity and variety of reactions which occur at membrane interfaces are far greater than would have been anticipated for structures which serve only to define the interface between two compartments. Progress in membrane research has revealed that membranes are integral parts of many diverse cellular functions.

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Our knowledge of membrane properties has been enhanced not only by analysis of biological membranes but also by the study of model membrane systems. One of the most useful models is the lipid bilayer membrane. Investigations on lipid bilayer membranes have both extended and served to emphasize the limitations of membrane models. Principal among these models is the Davson-Danielli model (*see* Davson & Danielli, 1952), which postulates that membranes consist of a lipid bilayer core, covered on both surfaces by enzymes and other proteins. The demonstration in 1962 by Mueller, Rudin, Tien and Wescott, that the lipid bilayer part of the Davson-Danielli model is, on a macroscopic scale, a stable entity is a tribute to the insight which produced the model. Studies on the physical properties by these workers and by others have revealed that many of the properties (which would not be grossly affected by a protein layer) are quite similar to natural membranes. Among these properties are water permeability (Hanai & Haydon, 1966; Hanai, Haydon & Redwood, 1966; Huang & Thompson, 1966; Cass & Finkelstein, 1967) electrical capacitance (Hanai, Haydon & Taylor, 1964; Mueller *et al.*, 1964), thickness (Huang & Thompson, 1965; Henn, Decker, Greenawalt & Thompson, 1967), and surface tension (Huang, Wheeldon & Thompson, 1964; Tien & Diana, 1968).

The principal discrepancy between the passive properties of model bilayers and natural membranes has been electrical resistance. In some cases, bilayer resistances may exceed those of natural membranes by a factor of more than 10^6. Although for this and other unrelated reasons, it has been suggested that the Davson-Danielli model is incorrect (Korn, 1966), it has been found that bilayer resistances can be varied over a wide range by addition of simple components to the system. Mueller *et al.* (1964) have found that a protein obtained from a variety of sources can lower bilayer resistances by a factor of 10^4 or more and at the same time confer upon the membrane certain properties of excitable natural membranes. Similar results were obtained with the cyclic polypeptide, alamethicin (Mueller & Rudin, 1968). In addition, these and other workers have found that several antibiotics can cause drastic lowering of bilayer membrane resistances (Van Zutphen, Van Deenen & Kinsky, 1966; Lev & Bushinsky, 1967; Mueller & Rudin, 1967). It has also been demonstrated that bilayer resistances are lowered by detergents (Seufert, 1965), oxidative phosphorylation uncouplers (Hopfer, Lehninger & Thompson, 1968; Lieberman & Topaly, 1968) and antigen-antibody complexes (Del Castillo, Rodriquez, Romero & Sanchez, 1966; Barfort, Arguilla & Vogelhut, 1968). The present investigation is based on the finding by Miyamoto and Thompson (1967) that ferric ion produces a large decrease in bilayer resistance. Since the modifying agent in this case is a cation, the system appeared to be simple enough that further study was warranted. In addition, there is reason to believe that iron, a common and ubiquitous cellular constituent, is also a constituent of some membranes (Colburn & Maas, 1965). Finally, it appeared that a study of this nature would reveal something of the mechanism of the interaction of ferric ion with mitochondrial membranes (Cash & Grady, 1965).

Materials and Methods

Unless otherwise noted, inorganic chemicals were reagent grade. Water was twice distilled, the second time from glass. Decane was supplied by Matheson, Coleman and Bell (No. 5845) and was used both without treatment and after passage over activated alumina.

Egg lecithin was prepared by a method slightly modified from standard procedures. Fresh egg yolks were extracted according to the method of Bligh and Dwyer (1957). Lecithin was recovered from the extract as its Cadmium Chloride salt, according to Pangborn (1951). Final purification was achieved by column chromatography on silica gel (Unisil, supplied by Clarkson Chemical Co.). Purity of the final product was established by thin-layer chromatography. The fatty acid composition of the lecithin was very similar to that previously reported (Huang *et al.*, 1964). Commercially prepared synthetic dipalmitoyl lecithin was purified by column chromatography.

$FeCl_3$ stock solutions were 0.1 M, prepared fresh weekly to avoid extensive hydrolysis.

Solution

All membranes were made from a solution containing ($\pm 20\%$), 10 mg lecithin, 1.7 mg cholesterol, and 0.05 ml methanol per ml decane. These amounts could be varied over rather wide ranges without obvious changes in membrane properties, except that some loss in membrane stability was observed when the cholesterol concentration was lowered.

The aqueous phase was normally 0.1 M NaCl. The salt was usually biological grade; however, in some experiments Merck Suprapur (distributed by the Brinckmann Co.) was used. Membranes formed in solutions of the latter grade had higher and more constant resistances.

Cells

The cell in which membranes were formed was similar to that described by Miyamoto and Thompson (1967). The membrane support was 1/32 inch plexiglas with a 1.3 mm diameter orifice. The seal between the two halves of the chamber was cerasin wax-impregnated sheet rubber. The external surfaces of the cell were coated with cerasin wax to minimize surface conductivity. Magnetically driven fleas provided for stirring the aqueous solution. Membranes were formed by the brush technique. Membranes with resistances lower than about 50 MΩ were rejected.

The cell used for studies at elevated temperatures was similar to the standard cell, except that it was surrounded by a close-fitting metal water jacket. Water from a temperature regulated bath was circulated through the jacket and the temperature read from a thermometer placed directly in the cell.

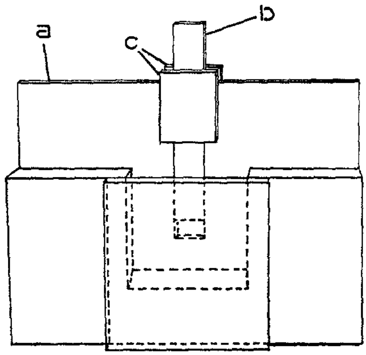

Fig. 1. Variable area cell. (*a*) Partition; (*b*) Sliding section; (*c*) Guides for sliding section

The cell used to change the area of the membrane is depicted in Fig. 1. The movable partition attached to a micromanipulator permitted the area of the membrane to be smoothly and closely controlled. Contact areas between the sliding and stationary parts above the area of the opening were lubricated and made electrically insulating with a liberal coating of hexadecane. Membranes were formed with the slide up, allowed to thin, and then compressed by lowering the slide. Membrane area could also be increased by raising the slide; however, this method had the disadvantage that with each increment of area increase, one must wait for newly formed membranes to thin. Membrane areas were measured with a calibrated reticle in the eye-piece of the observation microscope.

Electrical Measurements

The electrodes for most experiments were calomel with agar-saturated KCl bridges. The resistance of a pair was about 500 Ω and they usually exhibited asymmetry potentials of less than 2 mV.

Membrane resistances were determined with a circuit similar to that described by Miyamoto and Thompson (1967). Unless otherwise noted, the potential across the membrane was held at 20 mV. Current monitored throughout the lifetime of the membrane. Resistances were calculated from Ohm's law.

Capacitance was determined by a d–c transient method similar to that employed by Hanai *et al.* (1965*a*). These investigators charged the membrane through a large series resistor and monitored the voltage change with an electrometer voltmeter. In the present method a lower value series resistor (10 KΩ) was used and the voltage change monitored on an oscilloscope. A square-wave generator was used to provide repetitive pulses. The time constant is the same for the two methods, namely, $\tau = \dfrac{C_m}{\dfrac{1}{R_m} + \dfrac{1}{R_s}}$, when R_m is the membrane resistance, C_m its capacitance and R_s the series resistor. The advantage of using a small series resistor is that unless R_m is less than about 1/1,000 that of its unmodified value, the expression for τ reduces to $C_m R_s$ and is independent of the membrane resistance. Whenever membrane resistances dropped so low that they became comparable to 10 KΩ, the complete expression for τ was used. The accuracy of the method was checked using an appropriate parallel combination of precision capacitors and resistors in place of the bilayer membrane.

Transference numbers were calculated from the magnitude of the diffusion potential created when a salt gradient was established across the membrane. The equation appropriate to measurements using salt bridges is

$$E = \frac{RT}{F}(2t^+ - 1)\ln\frac{a_1}{a_2}, \tag{1}$$

where E is the measured diffusion potential, RT/F is 59.2 mV at 25 °C, t^+ is the positive ion transference number, and a_1 and a_2 are the activities of the salt solutions on the two sides of the membrane (MacInnes, 1961). The potentials were measured in a single-ended circuit with one electrode connected directly to the input terminal of a Cary model 31 electrometer (input impedance $> 10^{14}\ \Omega$). This method and the potentiometric method of Miyamoto and Thompson (1967) gave identical results.

Isotope Fluxes

Flux measurements were made in cells similar to those used for electrical measurements. Membranes were modified in the usual manner and when the resistance had decreased by the expected amount, Cl^{36} labeled NaCl solution was added to one compartment. The current through the membrane was set at the desired value by applying the appropriate potential. The solutions were continuously stirred and samples removed from the unlabeled compartment at various times. The volume removed was replaced by an equal volume of unlabeled salt solution. Samples were counted in 15 ml of a scintillation solution consisting of 4.0 g 2.5-diphenyloxazole, 0.1 g *p*-bis-2(5-phenyloxazolyl)-benzene, 750 ml dioxane, 125 ml anisol, and 125 ml dimethoxybenzene. Counting was done in a Nuclear-Chicago model 723 with windows set to provide maximum efficiency for Cl^{36}.

Electrophoresis

Free boundary electrophoresis was carried out in a Perkin-Elmer model 38 apparatus using a 2.5 ml micro cell. Buffer compartments were filled with 0.1 M KCl. The current was 15 ma and the field strength 7.0 V/cm. Mobilities were determined from photographs of the descending boundary. In a test run with a sucrose solution, no appreciable electro-osmosis was observed.

Lecithin dispersions were prepared by sonication with a Bronson Sonifier in distilled water under a stream of water-pumped nitrogen. Sonication was carried out for 3 hr at 0.3 maximum power. The sample was kept cool by immersion in an ice bath.

To avoid undue hydrolysis of the ferric ion, solutions were not dialyzed against the solution with which the boundary was formed. The lecithin dispersion was diluted with KCl solution so that the final concentration of KCl was 0.1 M. $FeCl_3$ solution was added and the pH adjusted. These operations were done in the cold and as rapidly as possible.

Spectral Studies

UV spectra were obtained on a Cary model 14 spectrophotometer with matched 1-cm path length cells. Difference spectra were obtained using double sector cells having 1-cm light paths in each section.

Results

Membrane Studies

In summary, the principal results obtained in the study are as follows: Addition of small amounts of $FeCl_3$ to the bilayer membrane causes the system resistance to decrease by 5 to 6 decades. This effect occurs only between pH 4 and pH 6.5 in the absence of chelating or reducing agents. Below pH 4, membrane resistance can be lowered if the ferric ion is hydrolyzed. Ferric hydroxide itself has no effect on membrane resistance. The current-voltage characteristics of the modified membrane are logarithmic. The capacitance of the membrane does not change during the resistance drop. The resistance modification occurs with fully saturated lecithin. The resistance modification can be reversed by reducing the ferric to ferrous ion with dithionite. When ferric ion is added to only one side of the membrane, time-dependent rectification is developed, the polarity being that which would be expected if chloride were encountering a lower barrier when penetrating the membrane from the ferric ion-containing solution than from the other side. The magnitude of NaCl diffusion potentials across the modified membrane have established its anionic permselectivity. Chelating agents are unable to remove the iron from the membrane. Instead, they bind to it and confer upon it cation permselectivity. The low resistance of the modified membrane has been shown to be proportional to black membrane area, excluding the torus of bulk membrane solution as a conduction pathway.

Effect of $FeCl_3$ on the Resistance of Bilayer Membranes

Fig. 2 shows the time course of the resistance change of a bilayer membrane formed in 0.1 M NaCl following the addition of $FeCl_3$. The result shown is typical. Although the change in resistance is quite reproducible, the absolute values of the initial and final resistances as well as the rate of change of the resistance shows considerable variation. As the NaCl concentration in the ambient aqueous phases is lowered, the effect of $FeCl_3$ on membrane resistance is diminished and disappears entirely at concentrations of NaCl $\leq 10^3$ M.

The magnitude of the resistance drop is not linearly dependent upon the amount of $FeCl_3$ added. Concentrations of lower than 10^{-7} M have little effect. Concentrations of 3 to 5 times higher than that shown in Fig. 3 (10^{-6} M) result in a final membrane resistance about 1/10 of that shown. Higher concentrations have not been studied.

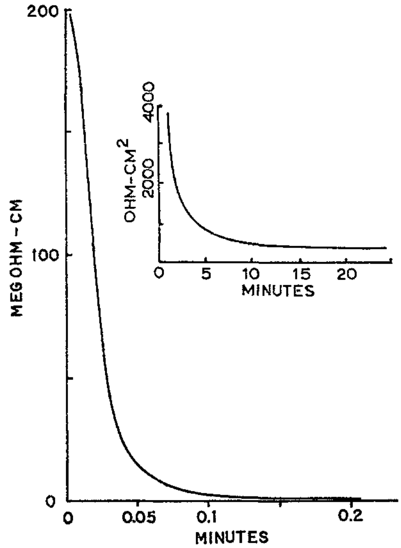

Fig. 2. Time course of resistance change after addition of $FeCl_3$. Initial membrane resistance was 200 MΩ-cm^2. At about 10 sec before time zero, the aqueous phase was made 10^{-6} M in $FeCl_3$. Zero time was when the resistance began to decrease. The insert shows resistance on a longer time scale

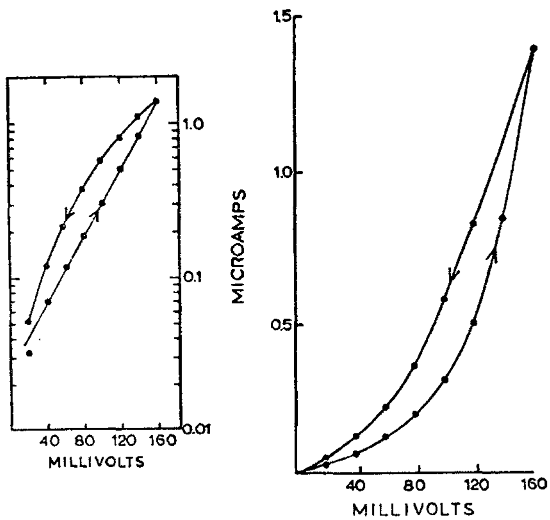

Fig. 3. Current-voltage characteristics of modified membrane. The membrane was modified by the addition of $FeCl_3$ to give a concentration of 2×10^{-5} M. The curve with the upward-directed arrow was obtained by increasing the voltage in 20 mV increments and holding the voltage for 30 sec for each increment. The curve with the downward-directed arrow was obtained immediately thereafter and in the same manner except that the voltage was decreased. The complete curve (+ and − voltage) is reproductive about zero. The inset shows the same data plotted on a logarithmic current scale

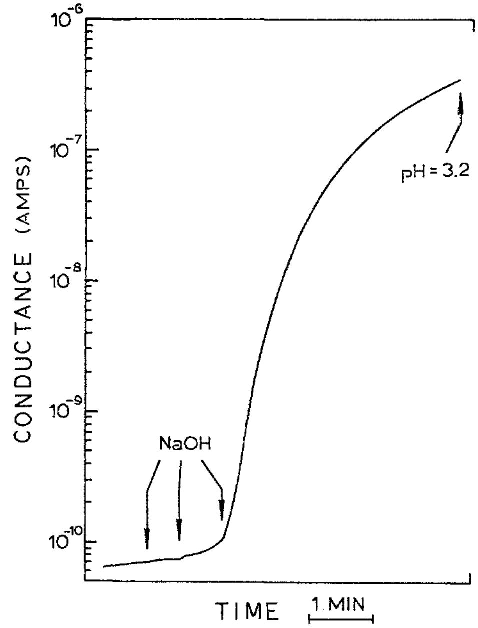

Fig. 4. Effect on membrane conductance of promoting hydrolysis of ferric ion at low pH. The cell (4 ml) was filled with 0.1 M NaCl which had been adjusted to pH 3.0 with HCl. This solution was then made 2×10^{-4} M in $FeCl_3$ and then the membrane was formed. When the membrane had thinned, NaOH (0.1 M) was added in three 10-μl portions (arrows). After the membrane resistance had decreased considerably, the solution was removed and its pH determined to be 3.2 (fourth arrow)

Typical current-voltage curves for the modified membrane are shown in Fig. 4. As indicated, when the voltage is increased and then decreased with the same polarity, considerable hysteresis is observed. Hysteresis arises because the current at any particular potential is time-dependent. It increases rapidly from an initial value to approach asymptotically a second value. The time dependency is similar to that described below for currents in the low resistance direction in the asymmetrically modified membrane (*see* Fig. 7). The magnitude of the time-dependent current increases with increasing voltage, so that when the potential is decreased from some high value, the membrane is in a lower resistance state than would have been the case had the voltage been changed to that value from some lower value. If current-voltage curves are obtained more rapidly than those shown in the figure (30 sec for each increment of 20 mV), or if the polarity of the voltage is alternated with each change in voltage, the time-dependent current makes a smaller contribution to the total current, and the curves are flatter and exhibit less hysteresis.

The magnitude of the resistance lowering is strongly dependent on the initial pH of the aqueous phase. If the pH of the bathing solution is 4 or below, $FeCl_3$ at concentrations as high as 10^{-4} M does not lower the resistance of the membrane. There is likewise no decrease of resistance if the pH of the bathing solution is above 6.5. In the latter case, a rapid and distinct coloration of the solution is seen when $FeCl_3$ is added. This probably indicates hydrolysis of the ferric ion. A suspension of ferric hydroxide[1], prepared by adjusting the pH of a solution of $FeCl_3$ to 6.5, has no effect on membrane resistance. Subsequent acidification of the bathing solution has no effect upon membrane resistance other than that which can be attributed to the pH change. Ferric hydroxide does not interfere with the subsequent reaction of the membrane with $FeCl_3$; the hydroxide suspension may be added to the membrane bathed by a solution at pH 5.5 without effect, but upon addition of $FeCl_3$ to this solution, the expected lowering of resistance is observed.

Membrane resistances at pH 3 to pH 4 are typically about 100 times lower than resistances in solutions at pH 5 to pH 6. The addition of $FeCl_3$ (10^{-6} M, final concentration) to solutions at pH 4 raises the resistance of membranes formed in such solutions to that which would have been expected in neutral solution, i.e., to approx. 100 $M\Omega\ cm^2$.

$FeCl_3$ in concentrations up to 10^{-4} M has no effect on membrane resistance if the bathing solution is initially at pH 3. If, however, the ferric ion in such solution is subsequently reacted with hydroxide ion, membrane resistances rapidly fall. The reaction consumes up to 3 equivalents of hydroxide ion, so this amount of base may be added without markedly altering the pH of the solution. Typical results of this kind of experiment are shown in Fig. 4. $FeCl_3$ was added to the bathing solution prior to the formation of the membranes because the latter were very much more stable at pH 3 if ferric ion was present. After addition of NaOH, the resistance undergoes a precipitous decrease even though the final pH is still below that at which $FeCl_3$ alone causes no decrease in membrane resistance. The predominant effect of adding hydroxide to this solution is to increase the concentration of $FeOH^{++}$ and $Fe(OH)_2^+$ at the expense of Fe^{+++} (Atkinson & McBryde, 1961).

Since considerable hydrolysis of ferric ion occurs in the first few minutes after its addition, the pH of the bathing solution is lowered. Under typical conditions, such as used for the experiment of Fig. 2, the final pH was about 4.8. Because, as will be seen below, the rate and extent of hydrolysis are

[1] Because of its common usage, the term ferric hydroxide will be retained rather than the more correct hydrous ferric oxide.

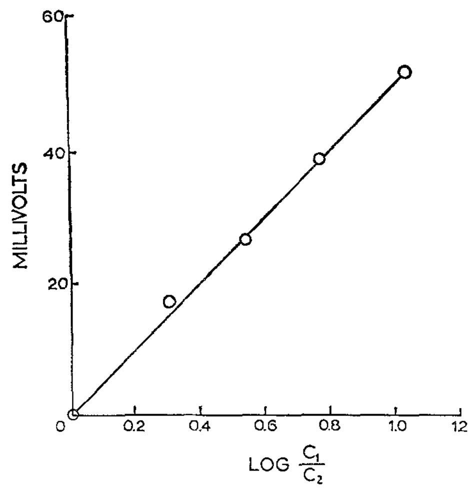

Fig. 5. Diffusion potentials generated by NaCl gradients. Membranes were formed in 0.01 M NaCl solutions. After the membranes had thinned, the aqueous phase was made 1×10^{-6} M in $FeCl_3$. Each point represents one experiment in which 5 M NaCl was added to one compartment such that the final concentration was that shown on the abscissa. The circles are experimental values, the line represents Eq. (1) using the activity ratios corresponding to the concentration ratios on the abscissa. After each experiment the membrane was broken and the resulting liquid junction potential determined. In all cases the bulk solution t^+ calculated therefrom agreed with the literature value to within experimental error

important, the conditions of pH under which the resistance change occurs are likely to be considerably different if the pH is fixed with buffers.

The effect of ferric ion on membrane resistance is inhibited by prior addition of the complexing agents EDTA, or the reducing agent, dithionite (hydrosulfite).

Permselectivity

The low resistance of membranes modified with ferric ion is accompanied by a dramatic change in transference numbers. The unmodified membrane shows slight, if any, selectivity for cations, but the modified membrane is highly selective for anions ($t^- \cong 1.0$). Evidence which will be presented below indicates that the anion selective membrane may be further modified so that it becomes cation selective.

This aspect of membrane behavior is shown in Fig. 5 where the diffusion potentials developed by several magnitudes of salt gradients are plotted. The experimental potentials closely approximate the diffusion potential calculated for an ideal anion permeable membrane ($t^+ = 0$). The theoretical

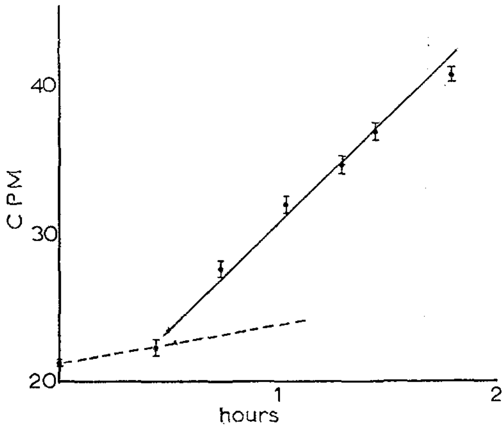

Fig. 6. Isotopic chloride flux. Membranes were made and modified (10^{-5} M $FeCl_3$) in the usual manner. When the resistance had stabilized, $NaCl^{36}$ was added to one compartment (vol, 1 ml) to a concentration of 3.5×10^9 cpm/mole. At time = 26 min, current was passed through the membrane (2 µamp, labeled compartment negative) by application of the appropriate voltage. At the times indicated, samples (250 µliter) were removed from the second compartment (vol, 5.6 ml). The current was interrupted during the time necessary for removal of the sample. The period of sampling is not included in the graph and the abscissa is correspondingly compressed (3 min for each sample). Bars signify standard deviation of counting

curve in this figure was calculated using Eq. (1), which assumes that Na^+ and Cl^- are the only ions carrying current through the membrane and that the sum of the potentials at the salt bridge junctions is zero. However, in addition to Na^+ and Cl^-, there exist in solution, OH^-, H^+, and Fe^{+3} (plus its soluble hydrolysis products, $FeOH^{+2}$, $Fe(OH)_2^{+1}$, and $Fe_2(OH)_2^{+4}$). If the product $C_i \cdot U_i$ (concentration $\times$ mobility in the membrane) for any of these ions is comparable to the product $C_{Na} \cdot U_{Na}$, the results based on Eq. (1) will be in error. Since all data in Fig. 5 were obtained at constant H^+, OH^-, and Fe^{+3} concentrations and since t^+ is constant throughout the range of variation of NaCl concentration, the potential must be a diffusion potential of NaCl alone. Moreover, the extrapolation of the experimental line through the origin shows that if potentials exist at the salt bridge junction, these must be equal and of opposite sign.

Experiments with Cl^{36} have provided additional evidence that chloride is the charge carrier in the modified membrane. Fig. 6 illustrates the results of an experiment in which the flux of Cl^{36} through the membrane was measured as a function of time and current. The current was zero until 26 min, when it was increased to 2 µamp. The isotope flux expected from a membrane permeable to Cl^- only was calculated from the current and the specific activity of the labeled compartment. This, plus the flux due only to the isotope gradient (zero current flux, represented by the dotted line) should equal the total flux of isotope. This calculated flux is shown as the solid line in the figure and is seen to be in good agreement with the measured flux.

A permeability coefficient may be calculated from the zero current flux according to $J_D = P \cdot A \cdot \Delta\text{cpm/V}$, where J_D is the flux in cpm/unit time, A is the membrane area, and Δcpm/V is the difference in isotope concentration between the two compartments. P for this membrane was 8×10^{-7}cm/sec. The diffusion flux can also be used to calculate the conductance, according to the relationship $g = F^2/RT\,J_D$ (Hodgkin, 1951) where F is the Faraday and R and T have their usual meanings. The conductance at 80 mV (which was used to drive the current in this experiment) is higher than that at zero mV ($\Delta i/\Delta V$ as $V \rightarrow 0$) because of the logarithmic nature of the i-V curve. Since the diffusion flux should be related to the conductance at zero voltage, a difference between the two values is to be expected. Indeed the conductance from the actual current and voltage used is 25 μmho while that calculated from the flux is 8 μmho.

Rectification

When $FeCl_3$ is added to only one side of the membrane, the resistance decrease occurs more slowly than when it is added symmetrically, and the time course of decrease becomes markedly dependent upon the direction in which current is passed through the membrane. When the sign of the potential gradient is such that the Fe^{+3} ions are driven toward the membrane, the resistance decreases much more rapidly than if the potential is in the opposite direction. As the resistance decreases, the membrane becomes a rectifier. The direction of the rectification is such that negative ions pass more readily from the $FeCl_3$-containing compartment into the opposite side, and/or positive ions pass from the latter compartment into the $FeCl_3$ compartment.

The type of rectification produced by asymmetric modification is time-dependent. This behavior is illustrated in Fig. 7 in which the conductance of the membrane in response to potentials of both polarities is shown. In the high resistence direction there is initially a large transient capacitance current which decays to a conductance which may either remain constant or increase slowly and linearly with time. The latter behavior is due to Fe^{+3} being forced into the membrane and is observed only in that period of time after the addition of $FeCl_3$ during which the iron is still capable of reacting with the membrane, i.e., as long as there remains in solution an

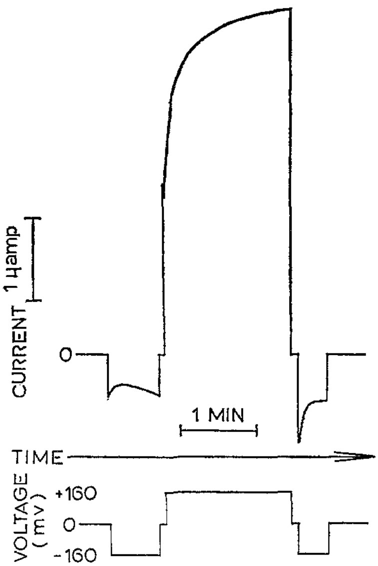

Fig. 7. Time dependence of conductance under rectifying conditions. Same membrane as in Fig. 8. Conductance response to step potentials of −160, +160, and −160 mV is plotted as a function of time. Current response to smaller negative potentials is qualitatively similar to that shown for −160 mV. In the positive direction the time-dependent part of the conductance diminishes with diminishing voltages until, at about 60 mV, the response is essentially a square wave

appreciable amount of unhydrolyzed ferric ion (about 5 min). After the membrane resistance has dropped to its lowest value, the current in this direction remains constant.

Current in the low resistance direction exhibits more pronounced time-variant behavior. In this direction there is transiently a large current which, again, is largely capacitive, but before this current has time to die away, another process occurs which produces an increase in current. This increase is initially rapid, but dies off at a rate which appears to be exponential. The magnitude of this slow conductance increase depends upon the potential. Although there is some variation from membrane to membrane, usually a potential of about 60 mV is necessary to produce the phenomenon. The magnitude of the time variant conductance increases with potential until, at about 140 to 160 mV, the increase is about 100% greater than the instantaneous value. Behavior of this nature has come to be known in the electrophysiological literature as delayed rectification. This time- and voltage-dependent increase of current in the low resistance direction is qualitatively the same as that observed in the symmetrically modified membrane.

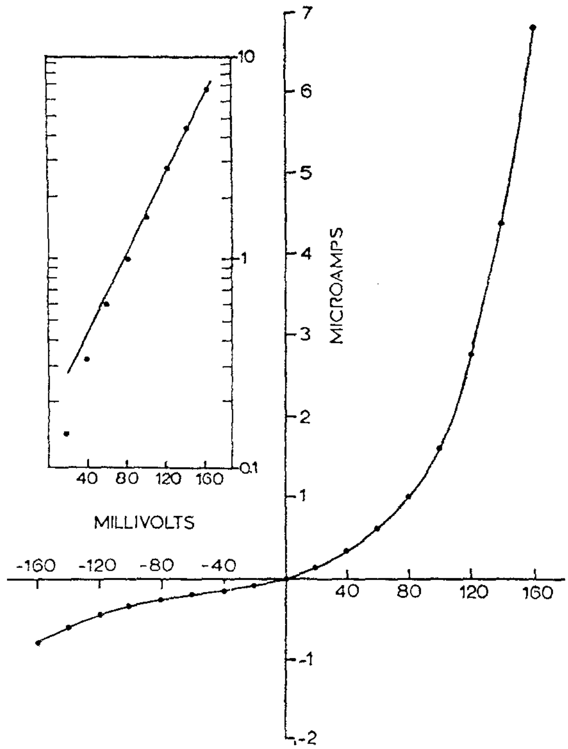

Fig. 8. Rectification of the asymmetrically modified membrane. Membrane was made in the usual manner. One compartment was then made 4×10^{-6} M in $FeCl_3$. The voltage was held at −20 mV for about 10 min and then increased (20-mV increments) to −160 mV. The voltage was then returned to zero and increased incrementally in the positive direction. Conductances were recorded about 10 sec after the voltage changes in the negative direction and at steady state (10 to 60 sec) in the positive direction (*see* text). Membrane area was approximately 2 mm². Insert shows plot of log i *vs* V. The intercept of the line drawn through the higher current values intercepts the ordinate at 0.2 μamp. Accordingly, the data fit the equation $i = 0.2$ (exp. V−1)

The rectification ratio varies from one membrane to another. In an individual membrane this ratio is strongly dependent upon the potential (or, perhaps the current density). This is shown in Fig. 8, which is the complete current-voltage curve for the membrane whose time variant behavior at one voltage was shown in Fig. 7. At voltages below about 60 mV, little rectification is observed. This is about the voltage at which the transition to a second steady state current value begins to contribute to current in the low resistance direction. As the voltage is increased, the rectification ratio increases as well. Although the voltage at which rectification becomes marked is also about the same voltage at which the time-dependent component of the current appears, oscilloscope measurements have shown that this current is only partly responsible for the rectification; much of the rectification is exhibited at very short times. As with the symmetrically modified membrane, the current (in the low resistance direction) increases with voltage in an exponential manner; the inset of Fig. 8 is a plot of log i *vs.* V. Since the current is time-dependent, the rectification ratio and the shape of the current-voltage curve depend upon the time at which the current is measured. The data in the figure were taken at the steady state value in the low resistance direction and just after the capacitance transient in the high resistance direction. Measuring the current in the high resistance direction immediately after the transient is necessary to avoid further lowering of the membrane resistance by driving into it additional ferric ion.

Since the addition of $FeCl_3$ to one side of the membrane produces a gradient of Fe^{+3} as well as H^+ and ferric ion hydrolysis products, it was necessary to show that rectification is not a consequence of diffusion potentials created by these gradients. Potential measurements were made in the presence of Fe^{+3} gradients (10^{-5} M to 0) and Fe^{+3} plus H^+ gradients (up to 2 pH units). In no case did the potential exceed a few millivolts. It may be noted that the response of the modified membrane is in sharp contrast to unmodified membranes, where pH gradients produce significant diffusion potentials. Further evidence that the pH gradient was not responsible for the rectification was obtained by determining the effect of increasing the gradient. When either the Fe^{+3}-containing compartment was made more acidic or the opposite compartment made more basic, the rectification decreased.

Capacitance

Fig. 9 shows the effect on both resistance and capacitance of adding $FeCl_3$ to the membrane. Capacitance is seen to remain constant while the resistance falls to a fraction of its original value. In a few instances, membrane capacitance was observed to decrease by a few per cent. This was probably due to incorporation into the membrane of lenses of bulk lipid from the torus. This frequently happens when the aqueous solution is vigorously stirred.

Reversal of the Effect of Ferric Ion

Complex Agents. Several iron complexing agents were assayed for their ability to reverse the ferric ion modification of the membrane. These included citrate, pyrophosphate, oxalate, thiocyanate and EDTA. When added symmetrically, none of these substances produced an appreciable increase

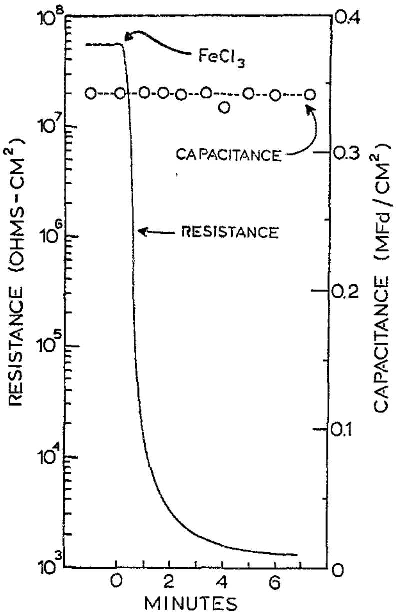

Fig. 9. Capacitance as a function of time during resistance change. Specific resistance is plotted on the left ordinate in logarithmic units. Specific capacitance is plotted on the right ordinate on a linear scale. At the time indicated by the first arrow, the aqueous phase was made 4×10^{-6} M in $FeCl_3$. Capacitance was measured as described in the text using 50 mV square waves at 800 cps. Capacitance measurement accuracy is $\pm 10\%$, precision $\pm 5\%$

in the resistance of the modified membrane, even when added in considerable excess over the ferric ion. If citrate, EDTA, oxalate, or pyrophosphate were added to only one side of the membrane, its resistance generally increased by a factor of about 10. Concurrently, a small degree of rectification (ratio $\cong 2$) was produced. The direction of the rectification corresponded to that which would have been observed if there had been no iron on the side of the membrane containing the complexing agent. Under these conditions the time dependence of the conductance following application of a step function voltage is altered. Here the current in the high resistance direction decreased with time, while current in the low resistance direction increased with time. This response is similar to what in muscle membranes is called anomalous rectification (Freygang & Adrian, 1961).

Much more dramatic effects of complexing agents were observed when changes in transference number were taken as a measure of their interaction. EDTA and citrate (the others were not tested) both caused an apparent

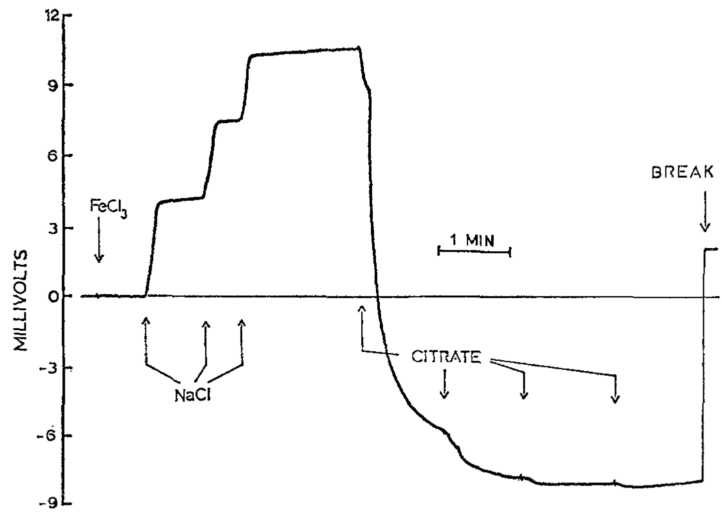

Fig. 10. Reversal of selectivity of modified membrane. Membrane potential is plotted as a function of time. Aqueous phases were 5 ml. At the time indicated, $FeCl_3$ (10^{-7} M) was added to both compartments, followed by NaCl ($3 \times 1.25 \times 10^{-4}$ M) to one compartment. A diffusion potential of 10.8 mV was developed, corresponding to a t^+ of 0.07. At the times indicated, four portions of sodium citrate (1×10^{-6} M, in solution at pH 5.5) were added to both compartments. The diffusion potential after the last addition was -8.7 mV corresponding to a t^+ of 0.88. The membrane was then intentionally broken. The resulting liquid junction potential was 2.2 mV and corresponds to a t^+ of 0.42

conversion of the modified membrane from one which was permeable only to anions to one which is permselective for cations. The effect of citrate on a ferric ion modified membrane is shown in Fig. 10. Conversion of the membrane from an anion to a cation selective membrane begins with about a 10-fold excess of citrate over Fe^{+3}, and is almost complete with a 30-fold excess. Citrate has no effect on the bulk solution value of t_{Na}^{+} although EDTA produces a small decrease, indicating some chelation.

Reducing Agents. The addition of sodium hyposulfite (dithionite, $Na_2S_2O_4$) to the modified membrane produced a rapid and sometimes complete reversal of the effect of iron and a return of the resistance to that of the unmodified membrane. This is illustrated in Fig. 11. It may be noted from the figure that a considerable excess of hyposulfite is necessary to effect a complete reversal. It is likely that such excess is required since hyposulfite is readily autoxidized and its effective concentration is rapidly decreased. In this regard, it should be pointed out that the dithionite had an effect only if its solution was strictly fresh.

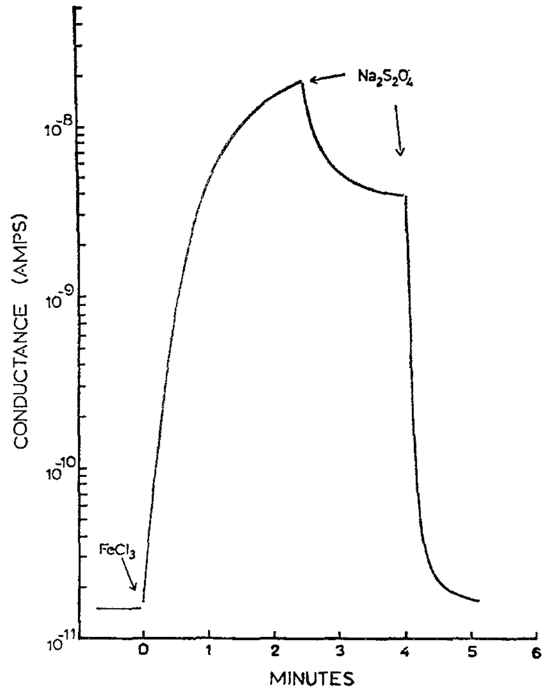

Fig. 11. Reversal of ferric ion effect by dithionite. Conductance is shown as a function of time. Membrane area was about 2 mm^2. Volumes of the aqueous compartments were 5 ml. At the first arrow, 10^{-7} M of $FeCl_3$ were added to both compartments. At the second arrow 2.5×10^{-6} moles dithionite (0.1 M, pH 5.5) were added to both compartments. At the third arrow dithionite was added again in an amount double that of the first addition

Effect of $FeCl_3$ on Membranes Prepared from Fully Saturated Lecithin

As will be seen below, ferric ion promotes the autoxidation of the unsaturated fatty acids of egg lecithin. It was consequently of interest to determine what effect, if any, ferric ion would have on a membrane which could not undergo autoxidation. It was found that membranes could be formed from dipalmitoyllecithin if the temperature was raised above 36 to 40 °C. Drainage of the membranes occurred more rapidly the higher the temperature, so these studies on saturated lecithin membranes were performed at 60 °C. Resistance of these membranes tends to be low and somewhat less reproducible at elevated temperatures. Typically, the resistance of saturated lecithin membrane at 60 °C was about 1/100 that of egg lecithin membranes at room temperature.

The resistance decrease produced in the saturated membranes by $FeCl_3$ is, provided the lower initial resistance is taken into account, similar to that observed in the egg lecithin membranes at room temperature. There

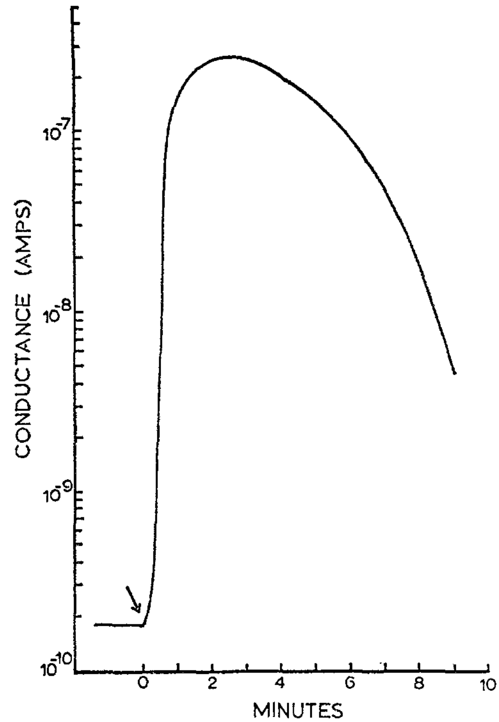

Fig. 12. Effect of ferric ion on a membrane of dipalmitolyllecithin. Membrane conductance is plotted as a function of time. Membrane area was about 0.5 mm^2. The cell and its contents were heated to 60 °C prior to membrane formation. At the arrow the aqueous phase was made 8.5×10^{-5} M in $FeCl_3$

is, however, a difference in the overall effect; in the case of the membranes prepared from the saturated lipid, the effect of the ion is transient. After about 10 min the resistance begins to return to its original value. This is shown in Fig. 12. On a few occasions when the resistance of egg lecithin membranes was recorded for long times, there was an indication that these resistances also increase. However, if complete reversal occurs in the unsaturated membranes at room temperature, it is a very slow process; the increase amounts to a factor of not more than 2 or 3 after 30 min.

Slightly larger concentrations of $FeCl_3$ are required to produce a decrease in resistance of the saturated membranes. This may be attributable to the more rapid hydrolysis of Fe^{+3} at 60 °C than at room temperature.

Conduction Pathway

To determine that the effects of iron are on the thin membrane and not on the bulk lipid in the torus or on a leak pathway, measurements of the

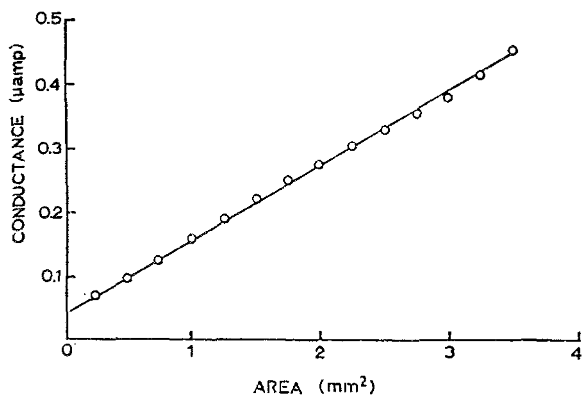

Fig. 13. Conductance as a function of area. The membrane was modified with 10^{-5} M $FeCl_3$. The area was then decreased in increments as shown on the abscissa. For details, *see* text. Specific resistance calculated from the slope of the line is 1,700 Ωcm^2

resistance of modified membranes as a function of area were carried out. The measurements were done using the variable aperture cell described in Materials and Methods. Because ferric ion drastically slowed down the thinning process, membranes were first formed on the open aperture and then the $FeCl_3$ was added. The aperture was then reduced stepwise and conductance recorded for each decrement in area. A plot of conductance *vs.* area is shown in Fig. 13. Similar data were obtain for several other membranes; however, in a few cases the correspondence to an area function was very much poorer than that shown in the figure.

Studies on the Interaction of Lecithin with Ferric Ion

In an attempt to better understand the mechanism by which the membrane is modified in the presence of ferric ion, a number of investigations were carried out on the binding of ferric ion to lecithin under various conditions.

Both $FeCl_3$ and egg lecithin are soluble in ethanol; however, when solutions of these are mixed, a yellow precipitate forms. The precipitate is soluble in 60% aqueous alcohol and in alcohol acidified with HCl. No precipitate is formed with ferrous salts.

Density gradient (H_2O-D_2O) centrifugation produced evidence that the interaction of lecithin and ferric ion (or its hydrolysis products) persists in aqueous solutions; when 0.001 M $FeCl_3$ was present in gradients with a pH between 3.0 and 3.8, the density of the lecithin dispersion was increased from 1.021–1.022 g/cc to 1.022–1.030 g/cc.

Table 1. *Electrophoretic mobilities of lecithin dispersions in the presence of ferric ion*

pH	Fe (M)	Lecithin (mg/ml)	Mobility (μ/sec/V/cm) U_1	U_2	Charge density ($100 \cdot e^-/80$ Å^2) 1	2
3.0	0	10	(−) 0.01	–	0.6	–
2.5	0.0005	5	1.31	0.67	6.2	3.1
3.2	0.001	7.5	0.91	0.58	4.3	2.8
3.3	0.001	5	1.43	0.95	6.9	4.5
3.3	0.0005	5	0.80	0.48	3.6	2.2
3.3	0.001	20	0.60	0.40	2.8	1.9
3.8	0.00025	2.5	0.95	0.56	4.5	2.6
4.1	0.001	10	0.72	0.40	6.9	3.6
4.5	0.001	5	0.06	–	0.8	–

Information on the charge of the iron species which binds to lecithin in aqueous solutions was obtained from free boundary electrophoresis. Sonic dispersions of lecithin in water were utilized. These dispersions consist of closed vesicles of 300 to 500 Å diameter (Litmann). It is likely that these vesicles possess a bilayer structure.

Free boundary electrophoresis of such dispersions was carried out in the presence of various concentrations of $FeCl_3$ at a number of different pH's. These data are presented in Table 1. At 0 $FeCl_3$ concentration the dispersion is essentially immobile, moving only slowly toward the anode. At low pH, the dispersion acquires a positive charge in the presence of iron, which increases as the ratio of iron to lecithin is increased. As the pH is raised, the charge on the dispersion due to iron binding decreases until, at pH about 5.5, it has the same mobility as the untreated dispersion.

A constant feature in the electrophoretic pattern was the appearance of two schlieren peaks. Such a system of two components run against a buffer containing neither of the components has been studied by others, who have shown both theoretically and experimentally that the presence of two bands is characteristic of an interacting system (Alberty & Marvin, 1950; Smith & Briggs, 1950). The other possibility, that one band is due to the lecithin and the second to some species of iron has been ruled out by area measurements of the two bands and of $FeCl_3$ and lecithin run separately. Not only is the amount of $FeCl_3$ in these experiments too small to contribute to the area under the schlieren peaks, but also the amount of lecithin in the dispersion cannot be accounted for unless the area of both peaks is taken into account.

The fact that positively charged species of iron bind to the lecithin vesicles suggests that the iron so bound would be rendered less susceptible to hydrolysis. This could be demonstrated by comparing the pH of $FeCl_3$ solutions in the presence and absence of lecithin dispersions. The initial pH of the 10^{-4} M $FeCl_3$ solutions is 3.75. When this concentration of $FeCl_3$ is prepared in the presence of a 0.0014 M lecithin sonic dispersion, the pH is 4.20. Higher pH's are observed when the lecithin is more concentrated. These results show that lecithin dispersions diminish either the extent or the rate of ferric ion hydrolysis. As may be expected from the solubility data, similar experiments in 60% ethanol indicate much stronger inhibition by lecithin of ferric ion hydrolysis.

Spectral Studies

Although $FeCl_3$ exhibits absorption bands in the UV region, no evidence could be gained from difference spectra for an interaction with egg lecithin dispersions. The difference spectrum obtained from egg lecithin and $FeCl_3$ and lecithin plus $FeCl_3$ shows strong differential absorption at 235 and 270 nm. These bands are characteristic of autoxidation of unsaturated fatty acids (Holman, 1954). The same two bands appear if the lecithin is merely allowed to stand in contact with air, supporting this assignment and strongly suggesting that ferric ion promotes the oxidation of the unsaturated ester constituents of egg lecithin. This is perhaps to be expected since it has long been known that iron and other substances which can exist in two different but adjacent oxidation states can promote autoxidation of many labile compounds.

On the other hand, dipalmitoyllecithin (which contains no unsaturated centers) and $FeCl_3$ does exhibit a difference spectrum in the UV region. The difference spectrum is of such small intensity that a similar difference spectrum in the case of the egg lecithin would have been obscured by the much larger differences of extinction due to the autoxidation products. At zero time the difference spectrum (Fig. 14) exhibits a maximum at about 250 nm and a minimum at about 300 to 305 nm. With time, the maximum diminishes and the difference at the long wavelength end of the spectrum increases. This change is due both to a change in the reference cell absorption ($FeCl_3$ and dispersion in separate compartments) and to a change in sample cell absorption (mixture of $FeCl_3$ and dispersion). Both exhibit strong absorption at about 240 nm and a shoulder at about 295 nm. In both cases, the absorption at the short wavelength end of the spectrum increases with time. The shoulder becomes less distinct but this is probably

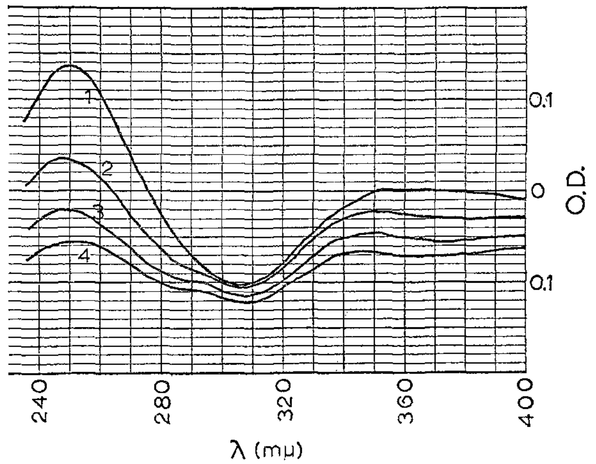

Fig. 14. Difference spectrum of lecithin dispersion and $FeCl_3$. Sample cell contained 3×10^{-4} M $FeCl_3$ and 2.5×10^{-3} M dipalmitolyllecithin (sonic disperson) in the first compartment and distilled water in the second compartment. Reference cell contained 3×10^{-4} M $FeCl_3$ in the first compartment and 2.5×10^{-3} M lecithin in the second compartment. Spectra are compensated for deviation of baseline from linearity. Curves 1, 2, 3, and 4 were run at 0, 5, 10, and 15 min after the addition of the components to the cells

because of increased background absorption and not from any increase in the intensity of the band. This general increase of absorption at shorter wavelengths may be ascribed to scattering of light produced by particles of $Fe(OH)_3$.

Discussion

Binding Studies

Understanding of the effect of ferric ion on the planar bilayer is facilitated by utilizing the information gained on the interaction between iron and lecithin dispersions. The binding studies will therefore be discussed first.

The precipitation of lecithin from alcoholic solutions by ferric but not by ferrous ion clearly reveals an affinity of the former for lecithin which, if shared by the latter, must be very considerably weaker or of a different nature. The ferric ion-lecithin interaction persists in aqueous solution, although it appears that the association is much weaker than in alcohol. That association between one or more of the species present in $FeCl_3$ solutions and lecithin occurs in aqueous solution is evident from the results of density gradient centrifugation. These experiments do not, however, distinguish between the several possible charged species and the uncharged ferric hydroxide.

The extent of ferric ion hydrolysis is appreciable in all but very acid solutions. Even at pH 3, all of the partial hydrolysis products are present in significant concentrations. At equilibrium in a 10^{-3} M solution, the approximate distribution of these species is: $Fe^{+++}=30\%$; $Fe_2(OH)_2^{++++}=30\%$; $FeOH^{++}=30\%$; $Fe(OH)_2^{+}=10\%$ (Atkinson & McBryde, 1961). The cathodic migration of lecithin dispersions in $FeCl_3$ solutions indicates that one or more of these charged species binds to the vesicle surface. The appearance of two peaks in the schlieren pattern suggests further that the interaction with at least one of the species is reversible.

Since the separation between bound charges is large, the binding in this system would be expected to follow the simple adsorption isotherm, $N=\alpha C$, where N is the number of bound ions per unit micelle surface, C is the concentration of those ions in solution, and α is the adsorption coefficient (Davies & Rideal, 1963). For our purposes we may take α to be the number of charges per surface molecule of lecithin per molar concentration of $FeCl_3$. So calculated, α falls between 110 M^{-1} at the lowest pH (2.5) and 7.5 M^{-1} at the highest pH (4.5). These values must be considered as approximations.

The fact that the charge on the lecithin dispersion is greatest at low pH when the concentration of Fe^{+3} is the highest suggests that binding of the unhydrolyzed species may dominate.

The effect of lecithin dispersions on the pH of $FeCl_3$ solutions also provides evidence that lecithin vesicles interact with one of the charged, incompletely hydrolyzed species; but, like electrophoresis, these experiments are unable to distinguish between the possible species.

Spectra

Ferric ion and ferric ion plus dipalmitoyl lecithin dispersions give a difference spectrum that has the characteristics which would be expected if the spectrum of partially hydrolyzed ferric ion were subtracted from that of an unhydrolyzed solution. Ferric ion exhibits an absorption band at 240 nm (Turner & Miles, 1957). Likewise, the difference spectrum has a peak in the same region. One of the hydrolysis products of ferric ion, $Fe(OH)^{+2}$, has an absorption band at about 300 μ (Turner & Miles, 1957). Correspondingly, the difference spectrum exhibits a trough at this wavelength.

From spectra run on $FeCl_3$ and $FeCl_3$ plus lecithin separately, it is found that the $Fe(OH)^{+2}$ band is initially about twice as large in the former as in the latter solution. Assuming all of the iron species are in equilibrium, and that inhibition of hydrolysis is due to binding, an adsorption coefficient of 200 M^{-1} is calculated. This is about 3 to 5 times larger than that found from electrophoresis at comparable pH's.

It has been observed that the difference spectrum between a mixture of lecithin plus $FeCl_3$ and lecithin undergoes the same qualitative changes with time that are exhibited by $FeCl_3$ alone. These time-dependent changes are indicative of ferric ion hydrolysis, and it is apparent that part, at least, of the hydrolysis inhibition is due to a retardation of the rate of hydrolysis. These experiments do not reveal whether the retardation is because the bound species hydrolyzes more slowly or because the concentration of free species is diminished. This suggests that electrophoresis, which requires at least 30 min per run, underestimates the adsorption coefficient.

The extrapolation of these results to the membrane is somewhat tenuous because of the large differences in iron concentration and surface area of lecithin. However, if it is assumed that the adsorption coefficient for the two systems is the same, a binding constant for the membrane can be approximated. The pH of the membrane bathing solution in the presence of 1×10^5 M $FeCl_3$ is about 4.7 to 4.8. The adsorption coefficient from the electrophoresis run at pH 4.5 was 7.5 M^{-1}.

Since hydrolysis undoubtedly occurs during the experiment and because this hydrolysis may involve species already bound to the dispersion, this value is probably a lower limit. The upper limit could be nearer 100 M^{-1}, which was obtained at lower pH's.

Bilayer Studies

Resistance Change

Since the capacitance remains constant over a resistance change of over four orders of magnitude, it is unlikely that the change in membrane structure caused by $FeCl_3$ is the result of either a homogeneous change in either the membrane thickness or in the membrane dielectric constant or both.

The alternative to a homogenous change in properties is the formation of a heterogenous membrane. The effect of iron might be to produce areas of lowered dielectric constant or very thin regions. If the area of high permeability was small compared to the total area of the membrane, no change in the capacitance would result. A lower limit on the area which need be involved in ion penetration can be obtained by assuming that pores, having the same conductivity as the bulk aqueous phase, exist. Since the conductance of a 100 Å thick sheet of 0.1 M NaCl is about 60×60^{-6} $\Omega\mathrm{cm}^2$, a 200 $\mathrm{M}\Omega\,\mathrm{cm}^2$ membrane would need only about 1×10^{-7}% pore area to have a resistance of as low as 600 Ωcm^2. Such a small fraction of conduction area would have no measurable effect on capacitance.

Indeed, the data on binding of ionic species of iron to the surface of lecithin dispersions suggests that only a small fraction of the vesicle surface is involved in the interaction. From those studies, an adsorption coefficient for binding to the bilayer was estimated to be less than 100 M^{-1}. Since $N_{\text{iron}}/n_{\text{lecithin}} = \alpha\, \text{FeCl}_3$, this means that a few tenths of a per cent or less of the lecithin molecules of the bilayer would be involved in binding. The interaction of iron with this fraction of lecithin molecules might cause fairly large local structural perturbations, but the fractional area modified would still be considerably below the few per cent demanded by the invariance of the capacitance.

It is evident that the simple binding of ferric ion to a fraction of the molecules at the surface of the membrane does not in itself produce a conductance increase. This is shown by the fact that at low pH, $FeCl_3$ binds very well to the lecithin surface (electrophoresis) but does not increase bilayer conductance. Nor does ferric hydroxide affect the membrane. The requirement appears to be that the iron first bind to the membrane and then be (at least partially) hydrolyzed. This sequence is strongly suggested by both the pH dependence of the $FeCl_3$ effect and the observation of a conductance change at low pH only following hydrolysis of the ferric ion by alkali. That iron hydrolysis occurs in the presence of lecithin vesicles (albeit more slowly than in their absence) is in accord with this inference.

Additional support for the hypothesis that the resistance change is due to the hydrolysis of a lecithin-ferric ion complex is found in the observation that $FeCl_3$ actually raises the resistance of membranes made in aqueous solutions of pH 4. This clearly indicates that ferric ion can interact with the bilayer surface and provides an additional point of comparison between the studies on dispersions and complex formation and those on the bilayer. It is interesting to note that both lanthanum and cadmium have a similar effect (Harris, 1967; Miyamoto & Thompson, 1967). These ions also form alcohol-insoluble complexes as does ferric ion. The reason that ferric ion raises bilayer resistance at pH 4 and not at pH 3 may be because of proton competition; as noted above, the lecithin-ferric ion complex is soluble in acid solution.

Although the resistance decrease is probably related to a change caused by the hydrolysis of a ferric ion-lecithin complex, occupying a relatively small fraction of the membrane area, it should be noted that gross observation of the membrane reveals a marked change in its optical properties. Shortly after the addition of $FeCl_3$, the reflectance of the membrane increases.

To be easily observed, this change must involve a sizable fraction of the membrane area. In view of the considerations above, it is unlikely that the thickness of the low dielectric portion of the membrane increases. Since the change of reflectance lags by many minutes the drop in resistance it seems reasonable that the reflectance increase is due to precipitation of ferric hydroxide on the membrane surface. Thus, the effect on resistance may be due to the first layer(s) of iron, and the change in optical properties to subsequent addition of ferric hydroxide. Since the resistance of this sheet of hydroxide gel is low, it should not affect the membrane capacitance.

A decrease in membrane resistance means that the activation energy for the penetration of an ion is substantially lowered. Although the bilayer structure consists of a hydrocarbon core sandwiched between ionic lattices, it is unlikely that these layers constitute separate barriers for ion penetration. Since the polar end and hydrocarbon tail are part of the same molecule, if the polar group lattice is disturbed, the packing of the hydrocarbon part of the molecule must be disturbed also. Thus, a distortion of the ionic lattice such as might be produced by separating the polar parts of two adjacent lecithin molecules (in the plane of the membrane) requires that the hydrocarbon tails of these molecules also be separated to a degree.

Based on such simple considerations, it seems reasonable to propose that the resistance lowering is due to bound Fe^{+3} hydrolysis. Because of its small size it is likely that ferric ion could enter the ionic lattice without grossly perturbing its structure. Indeed, cross linking by Fe^{+3} to the phosphate group on adjacent lecithins may tighten the structure and be the cause of the increased bilayer resistance observed at pH 4. Hydrolysis of the iron species after it is in the lattice would increase its size considerably, and it is reasonable to propose that this change would affect the balance of forces at the interface sufficiently that a perturbation involving the non-polar region occurs.

It is clear that any disruption of the ionic lattice and concomitant creation of hydrocarbon-water interfaces require energy. In the present case, it appears likely that this energy is provided by the hydrolysis energy of the ferric ion. This energy is clearly not available if the ion is hydrolyzed prior to its entering the lattice, and may account for the lack of effect of ferric hydroxide.

A number of other mechanisms for resistance lowering are possible, but each is inconsistent with one or more pieces of evidence. Electronic conduction is inconsistent with isotope flux data and diffusion potential reversal. A lowered dielectric constant of the membrane by catalyzed autoxidation is inconsistent with dithionite reversal, the response of the saturated lecithin membrane and the capacitance invariance. Accumulation of ions by a charged surface layer may contribute to the conductance increase, but it, in itself, is quantitatively inadequate. A carrier mechanism is unlikely since, in order to account for the isotope flux data, it would have to be uncharged and therefore consist of a complex involving ferric hydroxide. This substance has no affinity for chloride and has no effect on membrane resistance.

That the current is an exponential function of voltage in these membranes is to be expected from a conduction process which involves an activation energy. This is because the number of ions energetic enough to cross the barrier is proportional to a Boltzmann factor containing the difference between the activation energy (E) and the energy due to the potential imposed (eV for monovalent ions). A simple derivation reveals that the current across a symmetrical barrier should be given by $i=p\, e^{-E/kT}(e^{eV/kT}-e^{-eV/kT})$, where p is a proportionality factor. Aside from the time-dependency of the current, this equation describes the current-voltage curve obtained for the symmetrically modified membrane (Fig. 3). At positive or negative voltages as low as 60 mV, the term on the right closely approximates a single exponential and predicts the logarithmic dependence of the current on voltage which is shown in the insert of Fig. 3. The principles involved in the derivation may be found in standard texts of solid state physics or physical electronics. It should be noted, however, that these works are concerned with asymmetric barriers as exist in devices such as diodes. Indeed, the equation above also describes the current-voltage curve for a pair of rectifiers in an anti-parallel configuration.

It should be pointed out that an exponential i-V relationship rules out the possibility that ions traverse an area of the membrane which has the same resistance as bulk solution and in which the ionic transference members are the same as in bulk solution. Were such large, uncharged water filled channels present, the membrane would behave as an ohmic conductor.

The reason for the time-dependencies of the i-V curves is not clear. One possible explanation is that because of the equilibrium between Fe^{+3} and its hydrolysis products, there exists some ferric ion in the membrane. When the potential is such that Cl^- is driven toward the membrane, OH^- is also driven in the same direction. The pH on that side is raised slightly and some of the residual Fe^{+3} combines with the OH^- and provides additional permeable sites. A second possibility is that the permeable areas are small enough and the current density consequently high enough, so that changes in chloride concentration occur at the membrane surface, or perhaps in the permeable area of the membrane itself. The latter explanation has the additional merit of predicting the rectification which is observed when the membrane is asymmetrically modified. Ion depletion has indeed already been proposed to explain time-dependent resistances in bilayers which have been modified to be proton permeable (Lieberman & Topaly, 1968). It is of interest that the time-dependence of the conductance is similar to that of an inductance. A similar phenomenon has been observed in nerve (Cole, 1955).

Rectification

Rectification seems to be a result of the anion permselectivity conferred upon the membrane as a result of interaction with iron. If it is assumed that iron penetrates the membrane only slowly, as appears to be the case, then formation of an asymmetric membrane can explain the rectification observed. Although it is true that the solutions bathing the membrane are dissimilar, there is no evidence that suggests that this and not membrane asymmetry is the cause of rectification. Diffusion potentials are not observed when either pH or ferric ion gradients are established across the membrane. The magnitude and direction of the diffusion potential in the presence of NaCl gradients as well as the isotope flux data show that current through the membrane is carried by chloride ion.

Any membrane which is asymmetrically charged may be expected to rectify, provided only that its transverse resistance at zero current is relatively uniform. The reason for this qualification is explained at the end of the section on permselectivity. As pointed out by Kedem and Katchalski (1961), a composite membrane consisting of an uncharged layer in series with an ion-exchange layer will accumulate electrolyte in the intermembrane space when the current is in the opposite direction. In the steady state, the current-voltage curve for a composite membrane is identical to that for a diode rectifier, namely, i is proportional to $(e^{eV/kT}-1)$ (Katchalski, 1968). Aside from the increase in current at high negative values, this equation describes the curve shown in Fig. 8.

In view of such considerations, it is reasonable to propose that in the asymmetrically modified membrane, the surface facing the $FeCl_3$ solution behaves as an anion exchanger and the remainder of the membrane contains permeable regions which are either non-selective or weakly selective. The latter areas would correspond to the perturbation which was inferred to be produced in the non-polar layer when bound ferric ion becomes hydrolyzed.

Rectification is frequently observed in biological membranes. Katz (1949) and Freygang and Adrian (1961) have observed rectification in muscle when the inside and outside concentrations of potassium are equal and the resting potential close to zero. The *i*-V curves obtained by these authors are similar to those exhibited by the asymmetrically modified bilayer.

Permselectivity

The permselectivity of the membrane becomes explicable if colloidal ferric hydroxide is taken to be representative of the substance bound to the membrane. For an extensive discussion of the properties of hydrous oxide sols, *see* Weiser (1935). Unless the pH of a sol is quite high, it remains as a dispersion because it contains positively charged, incompletely hydrolyzed species of iron which provide repulsion energy to prevent aggregation. If either the pH is raised, or the concentration of electrolyte is increased, this charge is diminished and at some critical point, the sol will flocculate. When the electrolyte is a chloride salt, the diminution of charge is due to chloride binding. Chloride in such gels may be displaced by other anions and, in fact, these properties have been utilized for the preparation of ion exchangers (Kraus, 1958). Thus electrophoresis may underestimate the charge on lecithin dispersions in ferric-potassium chloride solution both because of ferric ion hydrolysis and because of chloride binding.

Whether the selectivity of the membrane is caused by a thick layer of ferric hydroxide containing a relatively small proportion of positive centers or by individual ionic species which by virtue of their effect on the membrane surface produce both the low resistance and permselectivity is not known. Although the high selectivity of the membrane would suggest that the charge is located at the local region of high permeability, the answer to this important question must await further data.

Not only are positively charged ferric hydroxide gels with adsorbed anions known, there also exist negatively charged sols which contain positive counterions. Although the exchange properties of such sols appear to have been less extensively investigated than those of positively charged gels, there is no reason to suspect that their behavior would not be analogous. The negatively charged gel can be prepared by adsorbing to it a multiply charged oxy-anion such as citrate (Weiser, 1935). That this can be done without dissolving the gel accounts for the fact that citrate reverses the ionic selectivity of the modified membrane.

From simple considerations of series resistances, it is evident that the semipermeability in bilayer membranes will not be seen as long as the membrane resistance is in the 100 MΩcm region. If the membrane resistance is so high, an ion exchanger at the membrane surface would either have to be a perfect exchanger, or have a comparably high resistance. If the conductivity of the ion exchange layer to co-ions (those with the same charge as the exchanger) is not considerably smaller than that of the non-selective part of the membrane (the lipid core), semipermeability will not be exhibited by the membrane as a whole. Since this is such a stringent requirement for an exchange membrane of the dimensions of the bilayer, it is evident that semipermeability will probably be seen in bilayers only when they are modified both with respect to ion exchange capacity *and* resistance.

Occurrence of Iron in Biological Membranes

It has been shown that bilayer membranes which are modified by reaction with ferric ion exhibit several of the passive properties of cell membranes. Although iron is one of the most abundant cellular cations, there is little information about its occurrence in membranes. Synaptosomes and myelin contain several μg of iron per g tissue from which they are isolated (Colburn & Maas, 1965). These quantities are significant and led the authors to propose a role for Fe (and Cu) in membrane function. If iron is bound to these membranes in the same way it binds to the bilayer, then it appears to be present in more than sufficient quantities to have an effect on the permeabilities of those biological membranes.

It is well known that mitochondria contain non-heme iron and in view of the present findings, it would not be implausible that some of it could be membrane associated (Cash & Brady, 1965). Investigation of the swelling of mitochondria revealed that ferric ion does interact with the mitochondrial membrane. On the basis of the present studies it is likely that the result is to cause the mitochondria to become Cl^- permeable. Ferric ion is also thought to be involved in the binding of succinic dehydrogenase to the mitochondrial membrane (Cerletti, Giovenco, Giordano, Giovenco & Strom, 1968). Finally, it may be noted that the ferric ion chelate of ADP penetrates the mitochondrion membrane faster than the magnesium chelate (Strickland & Goucher, 1964).

The fact that part of the non-heme iron in mitochondria undergoes oxidation and reduction and the present observation that modified bilayer resistances are dependent upon the redox state of the iron make it tempting to speculate that by a similar mechanism, the anion permeability of the mitochondrion could be controlled by its redox state.

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