sensitive part of hair cells. The ineffectiveness of extraluminally administered streptomycin and kanamycin might be attributable to the presence of some barrier which prevents the access of these drugs to the basal part of hair cells. However, in view of the fact that the molecular weight of kanamycin sulfate (582.6) is smaller than that of ouabain (728.77), the possibility seems remote.

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References and Notes

1. D. W. Fawcett, *Circulation* **26**, 1105 (1962).
2. V. K. Johnsen, *Acta Physiol. Scand. Suppl.* **145**, 87 (1957); J. Diamond, *J. Physiol.* **161**, 442 (1962); S. Thesleff and K. Schmidt-Nielsen, *Amer. J. Physiol.* **202**, 597 (1962); M. R. Hokin, *J. Gen. Physiol.* **50**, 2197 (1967); I. M. Glynn, *Pharmacol. Rev.* **16**, 381 (1964).
3. A. J. Duvall and J. Wersäll, *Acta Otolaryngol.* **57**, 581 (1964); J. E. Hawkins and H. Engström, *Acta Otolaryngol. Suppl.* **188**, 100 (1963); J. Farkashidy, R. G. Black, T. D. R. Briant, *Laryngoscope* **73**, 713 (1963).
4. J. Wersäll and A. Flock, *Life Sci.* **3**, 1151 (1964).
5. E. J. Furshpan and T. Furukawa, *J. Neurophysiol.* **25**, 732 (1962).
6. T. Furukawa and Y. Ishii, *ibid.* **30**, 1377 (1967); *Jap. J. Physiol.* **17**, 572 (1967).
7. I. Tasaki and C. Fernández, *J. Neurophysiol.* **15**, 497 (1952).
8. T. Konishi, E. Kelsey, G. T. Singleton, *Acta Otolaryngol.* **62**, 393 (1966).
9. C. G. Johnstone, R. S. Schmidt, B. M. Johnstone, *Comp. Biochem. Physiol.* **9**, 335 (1963); T. Naito, H. Uyematsu, T. Nakamura, K. Horii, T. Miyazaki, *Japan. J. Otol.* **68**, 1628 (1965).
10. H. Davis, *Cold Spring Harbor Symp. Quant. Biol.* **30**, 181 (1965).
11. W. Kuijpers, A. C. Van der Vleuten, S. L. Bonting, *Science* **157**, 949 (1967); Y. Tanaka and P. G. Brown, *Ann. Oto-Rhino-Laryngol.*, in press.
12. We thank Dr. H. Grundfest for reading the manuscript.

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Resistance Changes in Lipid Bilayers: Immunological Applications

Abstract. *The electrical resistance of a bimolecular lipid membrane in 0.1 molar NaCl decreases if antibody and complement are present on one side of the membrane and the homologous antigen is added to the other side. The reaction occurs within minutes and requires less than 0.1 microliter of antiserum.*

Immunological as well as enzyme-substrate reactions occurring on one surface of a lipid bilayer can lower the electrical resistance of the membrane (*1*). In addition, occasional proteins (*2*), valinomycin (*3*), and a group of related antibiotics can selectively induce an increased flux of cations, with a subsequent resistance decrease, whereas alamethicin can lower the resistance by nonselective transport of cations (*4*). We now report the effect of an antigen-antibody reaction, occurring across the membrane, upon the transmembrane electrical resistance. We have shown that when either antiserum or antigen alone is added no change in resistance is observed. When antiserum is added to one side of a membrane and the homologous antigen is added to the other side, a very significant decrease in the resistance is consistently observed.

All membranes were formed with a lipid solution containing 2.5 mg of sphingomyelin per 100 μl of a mixture of α-tocopherol, chloroform, and methyl alcohol (5:3:2). No traces of cholesterol, stearate, or palmitate could be demonstrated in the sphingomyelin which migrated as a single component on thin-layer chromatography (*5*, *6*). The α-tocopherol (*6*) had a light amber hue, an indication that some oxidation may have taken place. The lipid stock solution was kept in a nitrogen atmosphere at −80°C to reduce oxidation and evaporation.

A polyethylene cup (Beckman, 1.6 cm high and 2.0 cm in diameter) formed the septum which provided a support for the membrane and separation between the aqueous solutions. When not in use, the cup was stored in *n*-decane (*6*). The circular orifice in the wall of the cup, across which the membranes were formed, had an area of 0.0132 cm^2. Immediately before an experiment, the cup was thoroughly cleaned with Kimwipes, so that only a trace amount of decane was available for incorporation in the lipid structure (*7*). To make a membrane, a small amount of the lipid solution was applied to a thin sable brush, size 0000, which was then streaked across the orifice (Fig. 1) to deposit a sheet of lipid solution. As the chloroform and methyl alcohol diffused into the aqueous phase, bimolecular regions started to form locally (*8*); after a few minutes, a major part of the membrane area would assume the bilayer configuration. A discussion of the reproducibility and time course of the membrane resistance is given after the experimental data.

The aqueous medium was a 0.1*M* NaCl solution with 5 m*M* histidine (*6*) at *p*H 7.3. We found that concentrations of NaCl and KCl equal to or greater than 0.3 mole/liter made formation difficult and gave unstable membranes. The transmembrane electrical resistance was monitored with two fiber-junction electrodes (Beckman, No. 41239) at an applied voltage of less than 20 mv. The offset-voltage between the two electrodes was considered as part of the voltage source. The preferred temperature for membrane formation was 37° ± 1°C.

We found that the thinning procedure became unpredictable when the temperature went below 28°C, and the membranes consistently broke between 40° and 41°C. When the current density exceeded 4×10^{-8} to 10×10^{-8} amp cm^{-2}, the membranes would often break without any apparent mechanical or thermal stimulation. If only a small number of sites carry ions across the membrane, one can visualize a local increase in temperature, caused by the dissipation of (16 mv) (4×10^{-8} amp cm^{-2}) = 9.2×10^{-6} cal min^{-1} cm^{-2} in the membrane. Because lipid is a poor conductor of heat, the released energy may raise the temperature locally above the critical 41°C and hence weaken the membrane structure so that it breaks. When the difference in hydrostatic pressure between the two chambers exceeded 0.4 cm, the membranes would burst. Also, air currents in the room could give fluctuating resistance values.

In general, the resistance of a membrane increased for about 30 minutes after formation and then remained virtually constant for at least 96 hours if the temperature was held constant at 37°C. We found a transient decrease in resistance immediately after addition of a protein to either side of the membrane. The decrease was typically 10 to 20 percent of the resistance (R) value before addition of the protein, and the duration of these transients did not exceed 20 seconds. As Fig. 1 shows, deviations from a smooth curve of the relation between the logarithm of R and time were frequently encountered after addition of one of the proteins. There was no correlation between these devia-

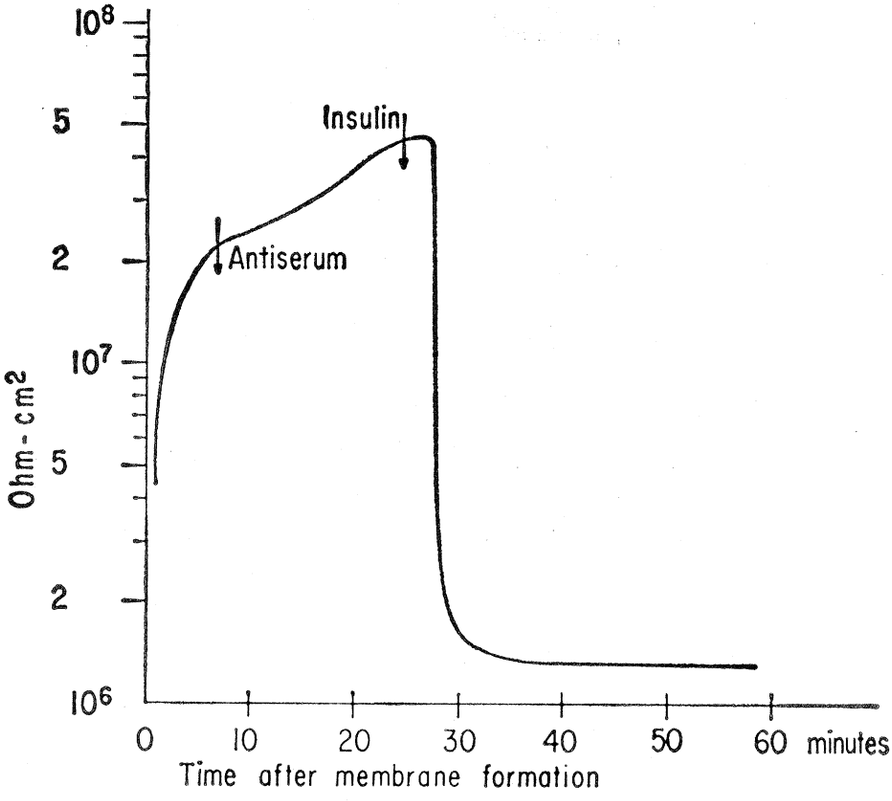

Fig. 1. Typical curve showing how the resistance of the membrane suddenly decreases when homologous antigen and antibody are added to opposite sides of the membrane.

Table 1. Side 1 contains antigen, as indicated, in 55 ml of saline solution. Side 2 contains antibody and guinea pig complement in 4.5 ml of saline solution. Pool J, guinea pig antibody to insulin; pool I, rabbit antibody to ribonuclease-A; lysozyme ab, guinea pig antibody to lysozyme.

Side 1		Side 2		Resistance decrease	Experiment (No.)
Substance	Amount (μl)	Substance	Amount (μl)		
Insulin	1000	Pool J	50	1200 ×	1
Insulin	200	Pool J	100	37 ×	3
Insulin	200	Pool J	50	70 ×	4
Insulin	200	Pool J	25	40 ×	1
Insulin	200	Pool J	22	125 ×	1
Insulin	200	Pool J	1.25	100 ×	1
Insulin	200	Pool J	0.0625	10 ×	1
Insulin	200	Heat-treated pool J and complement	50 100	10 ×	1
Insulin	200	Heat-treated pool J and complement	50 50	10 ×	1
Insulin	200	Heat-treated pool J and complement	20 50	50 ×	1
Ribonuclease-A	500	Pool I	100	100 ×	1
Ribonuclease-A	200	Pool I	20	320 ×	1
Lysozyme	200	Lysozyme ab	100	1600 ×	1
Lysozyme	200	Lysozyme ab	70	100 ×	1
Lysozyme	200	Lysozyme ab	50	200 ×	2
Lysozyme	200	Heat-treated lysozyme ab and complement	50 50	32 ×	1
		Controls			
Lysozyme	200	Heat-treated lysozyme ab	50	None	3
Lysozyme	200	Complement	100	None	1
Lysozyme	200	Complement	50	None	2
Lysozyme and complement	200 50	Heat-treated lysozyme ab	50	None	1
Insulin	200	Insulin	50	None	2
Lysozyme ab	200	Lysozyme ab	50	None	2
Pool I	200	Pool I	50	None	1

tions and the dramatic changes in resistance observed after addition of the homologous antigen or antibody to the other side of the membrane. When a protein was added to one of the saline solutions, the solution at that side was gently stirred for a few seconds. No particular precautions were necessary to guard against mechanical vibrations. The temperature was controlled with an infrared lamp outside the copper screen which provided electrostatic shielding. The current through the membrane was measured with a Keithley electrometer, type 200 B, and a decade shunt resistor (Fig. 2). The response time of our equipment was about 1 second, and $R \times C$, the time constant (*9*; Fig. 3) of the membranes, varied between a fraction of a second at low resistance values and several seconds when the resistance had reached its steady, high value. Thus, we could not have observed any fast changes (in the millisecond range) like those reported in (*1*).

Three types of antigens were used: lysozyme, insulin, and ribonuclease-A. One milligram of lysozyme (*6*) was dissolved in 0.5 ml of 0.01*N* NaOH and added to 0.5 ml of 0.22*M* phosphate buffer, *p*H 7.4. Insulin (*10*) was prepared in the same manner; 1 mg of ribonuclease was dissolved in 1 ml of 0.1*M* tris acetate buffer, *p*H 7.5. Three types of antiserums were tested: guinea pig antiserums to lysozyme and insulin, and rabbit antiserum to ribonuclease. The immunization procedure has been described previously (*11*). All complement and antiserums were stored at −80°C. In most experiments, 50 μl of antiserum was added to 4.5 ml of buffered saline in the polyethylene cup which formed the inner chamber. Antigen (200 μg in 200 μl solvent) was then added to 55 ml of buffered saline in the external chamber. A characteristic decrease in resistance was observed repeatedly when 50 μl of antiserum, diluted 800-fold, was added to the 4.5 ml of saline. In these experiments a maximum of 0.0625 μl of antiserum was available for binding at the membrane. The absolute sensitivity of this procedure can be increased by reducing the size of the inside and outside chambers.

The membrane resistance could not be changed by the addition of antiserum to one or both sides of the membrane, or by the addition of antigen to one or both sides. If, however, homologous

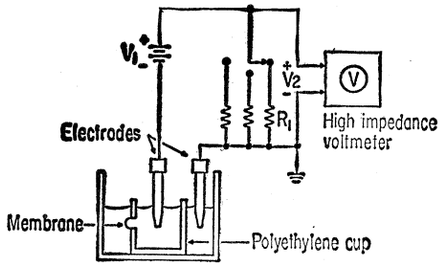

Fig. 2. Simplified diagram of the equipment. The d-c current through the membrane is V_2/R_1, and the membrane resistance $R_m = (V_1 - V_2) \times (R_1/V_2)$, where R is resistance and V is voltage. The input impedance of the voltmeter is considered infinite compared to R_m.

antigen and antibody were added to opposite sides of the lipid bilayer, a characteristic decrease in resistance was observed between a few seconds and, at most, 13 minutes after the last protein was added (Fig. 1). The large variation in the delay indicates that a mechanism other than diffusion through the saline solution and unstirred layers on the membrane is involved.

If the antiserum was kept at 0°C or room temperature for 6 to 48 hours, the ability to cause a resistance decrease was reduced. This could indicate that complement was a necessary ingredient for the reaction to be observed (*12*). We therefore performed the following experiments: Guinea pig antiserum to lysozyme was heated at 56°C for 30 minutes to inactivate complement (*12*). All the lysozyme antiserum activity remained, however, as shown by passive immune hemolysis (*11, 12*). No decrease in the membrane resistance was noted when the heat-treated lysozyme antiserum was applied to one side of the membrane, and lysozyme to the other side. If, however, 50 μl of heat-treated antiserum and 50 μl of freshly thawed complement were added to the same side of the membrane, and 200 μl of lysozyme was added to the other side, a resistance decrease took place similar to that noted with fresh antiserum. Complement and lysozyme alone, or heat-treated antiserum and complement alone, resulted in no appreciable resistance drop across the lipid membrane. When complement was added to the antigen (lysozyme), and heat-treated antiserum was added to the opposite side, no resistance change was noted. Therefore, in order to obtain the decrease in membrane resistance, it was necessary to have antibody with complement on one side of the membrane and antigen at the other side (Table 1).

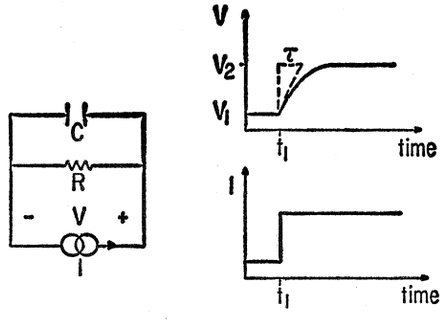

Fig. 3. A sudden change in the membrane resistance will give a current step as shown here, but after the time t_1 the membrane voltage will follow the curve $V = V_2 + (V_1 - V_2)\ \exp\ [-(t - t_1)/\tau]$, where $\tau = R \times C$.

Little is known about the exact mechanism for charge transport, especially the molecular interactions responsible for changes in ionic flux. The type of monovalent cations transferred from one side of the membrane to the other seems to be of little importance, whereas the relationship of structure and function between the bound water and the protein molecules attached to the lipid surfaces seems to be of paramount importance as a gating mechanism. The ordered structure of the unmodified lipid bilayer, as evidenced by the high electrical resistance after stabilization, was drastically disturbed by the added protein. It is reasonable to suggest that either the antigen, or the parts of the antibody molecule which combine with the antigen, protrude through the membrane, so that an antigen-antibody-complement structure is formed across the membrane to give an increased ionic flux. It should be noted that in passive immune hemolysis antigen molecules that are fixed at the surface of erythrocytes combine with antibody and complement in solution to lyse the erythrocytes.

The circuit shown in Fig. 2 is a modified version of that used by del Castillo (*1*). It does not allow distinction between changes in resistance and potentials which are due to ionic gradients across the membrane (Nernst potentials). This is not a serious limitation in the experiments reported here. A more complete investigation should include the current-voltage relationship of the membrane (*4*).

Clinical use of the effect described in this report will require a reproducible method for the formation of bilayers. The average initial resistance for 24 membranes was 6.26×10^8 ohm, and the standard deviation (S.D.) was 3.96×10^8 ohm. After 30 minutes, the resistance had leveled off at a higher value, and the average for 20 remaining membranes was 9.83×10^9 ohm with S.D. = 9.40×10^9 ohm. The average ratio between the resistance 30 minutes after membrane formation and the initial resistance was 23.7 with S.D. = 33.74. The large uncertainties in these figures can indicate that not all of the available membrane area is occupied by a bilayer at any instant of time, so that "lenses" of lipid or hydrocarbon can float around in the orifice (*7*).

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References and Notes

1. J. del Castillo, A. Rodriguez, C. A. Romero, V. Sanchez, *Science* **153**, 185 (1966).
2. P. Mueller and D. O. Rudin *Nature* **213**, 603 (1967).
3. ———, *Biochem. Biophys. Res. Commun.* **26**, No. 4, 398 (1967).
4. ———, *Nature* **217**, 713 (1968).
5. We thank Dr. J. Mead and Mrs. D. Long for assistance with the thin-layer chromatography.
6. Sphingomyelin (lot 4619) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; DL-α-tocopherol (lot 72394) and free base histidine (lot 64418), from Calbiochem, Los Angeles, California; *n*-decane, from Matheson, Cole, and Bell, New York, N.Y.; egg-white lysozyme (three times crystallized, No. 4403, lot 107965), from California Corporation for Biochemical Research, Los Angeles, California; bovine insulin (ten times recrystallized, lot 16666), from Novo Therapeutisk Laboratorium, Copenhagen, Denmark; and ribonuclease-A from bovine pancreas (type XII-A, lot 27B-8550), from Sigma Chemical Corporation, St. Louis, Missouri.
7. F. A. Henn and T. E. Thompson, *J. Mol. Biol.* **31**, 227 (1968); J. Taylor and D. A. Haydon, *Disc. Faraday Soc.* **42**, 51 (1966).
8. C. Huang, L. Wheeldon, T. E. Thompson, *J. Mol. Biol.* **8**, 148 (1964); T. Hanai, D. A. Haydon, J. Taylor, *J. Theoret. Biol.* **9**, 433 (1965).
9. The time constant is the time it takes the voltage across a resistance (R) and a capacitance (C) in parallel to reach 63 percent of its final value after a current step has been applied (Fig. 2).
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11. E. R. Arquilla, J. Hamlin, S. Hamashige, A. Miller, *J. Exp. Med.* **120**, 841 (1964).
12. E. R. Arquilla and J. Finn, *ibid.* **122**, 771 (1965).
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