Proc. Nat. Acad. Sci. USA
Vol. 69, No. 1, pp. 93–97, January 1972

Genetic Modification of Electric Properties in an Excitable Membrane

(paramecium/calcium conductance/electrophysiological measurements/membrane mutant)

CHING KUNG* AND ROGER ECKERT

Department of Zoology and Brain Research Institute, University of California, Los Angeles, Calif. 90024

Communicated by T. M. Sonneborn, November 1, 1971

ABSTRACT **A behavioral deficiency produced by a single gene mutation in *Paramecium aurelia* was traced to impaired electric excitability of the cell membrane. Evidence is presented that the mutant membrane does not exhibit the normal depolarization-activated increase in calcium conductance responsible for regenerative depolarization in the wild type. Other electric properties characteristic of the wild-type membrane remain normal in the mutant.**

A major unsolved problem in neurobiology is the mechanism of negative resistance in electrically excitable membranes. After a sudden depolarization, the membrane exhibits a transient increased conductance to an ion, usually Na^+ or Ca^{++}, which then carries depolarizing charge into the cell. The additional depolarization results in a further increase in conductance, and thus the process becomes regenerative, driving the membrane voltage toward the equilibrium potential of the ion (1, 2). This regenerative behavior, which will be termed electric excitability, is responsible for the upstroke of an action potential. Although the electrical properties of excitable membranes have been studied intensively and have been described elegantly by the ionic hypothesis, virtually nothing is known about mechanisms of membrane excitation at the molecular levels of membrane structure and chemistry. One successful approach to the mechanism of biological functions has been to modify or delete a molecule responsible for one part of a complex function, and then to observe the effect on the function. Manipulations by gene mutation have been applied to studies of the nervous system by Benzer (3) in *Drosophila melanogaster*. This rationale is also behind the work of Kung (4, 5), which is directed at the genetic dissection of the excitable membrane of a ciliate, *Paramecium aurelia*.

Paramecium is admirably suited for a genetic approach to the study of membrane excitation. This cell is electrically excitable (6–8) and lends itself to intracellular recording and stimulating techniques (9, 10). The locomotor behavior of this ciliate has been shown to correlate with the electrical activity of the membrane (7, 11, 12). Behavioral correlates of membrane activity greatly simplify the problem of recognizing altered membrane function in mutants (4, 5). Since these unicells have no synapses or neural organization other than the cell membrane, analysis of altered membrane function is further simplified. Pure clones of various genotypes can be grown axenically (13) for biochemical analysis and comparisons and for immunological studies. The development of behavioral genetics in this organism rests on an extensive foundation of *Paramecium* genetics (15), and exploits the advantage of autogamy, a process of nuclear reorganization in *P. aurelia* that makes detection of mutants nearly as easy as in haploid organisms. Finally, a large fraction of the surface membrane of ciliates covers the cilia, and thus can be routinely harvested for fractionation (14). This paper reports electrophysiological studies on a mutant of *P. aurelia*, first selected for, and recognized through, its altered locomotor behavior (4, 5), in which the surface membrane has lost a single function essential for electric excitation.

The electric properties of the wild-type membrane of *Paramecium caudatum* are similar in basic principles of the ionic hypotheses (1) to those of other excitable membranes found in nerve and muscle. The electric excitability of *Paramecium* most closely resembles that of crustacean muscle membrane (8, 16–19). When it is depolarized by injected current, *Paramecium* produces a graded regenerative response in which the maximum amplitude varies with the extracellular calcium concentration by nearly the amount predicted by the Nernst relation for a pure calcium electrode (6). This, together with other evidence (19), indicates that the response in *Paramecium* results from a transient increase in calcium conductance evoked by depolarization. The increase in Ca^{++} conductance in response to depolarization will be termed "calcium activation" after the analogous "Na activation" exhibited by nerve membrane (1).

Depolarization of the membrane in the wild-type organism is followed by a shift in the direction of the ciliary power stroke (7, 11). This response, like excitation, depends on the presence of extracellular calcium ions (11, 20), and is graded with depolarization so that a strong depolarization produces a complete "reversal" of ciliary beating, causing the organism to swim in reverse. Recent evidence has implicated intracellular Ca^{++} in the activation of the ATPase enzyme that is active in producing ciliary reversal (21)†. Ca^{++} entering the cell through the surface membrane during excitation activates the reversal of ciliary beating (21a).

Behavioral patterns caused by ciliary responses to membrane activity were used in screening for membrane mutants (4, 5). Variants insensitive to cations that depolarize the wild type (9, 22) were isolated by the principle of chemotactic interference with geotaxis. Many such variants were found and their defects were shown to be genetic (4, 5). One group of mutants is of special interest, since they fail to swim backward in response to depolarizing stimuli. They are called

* Present address: Department of Biological Sciences, University of California, Santa Barbara, Calif. 93017.

† Naitoh, Y. (University of Tokyo), personal communication.

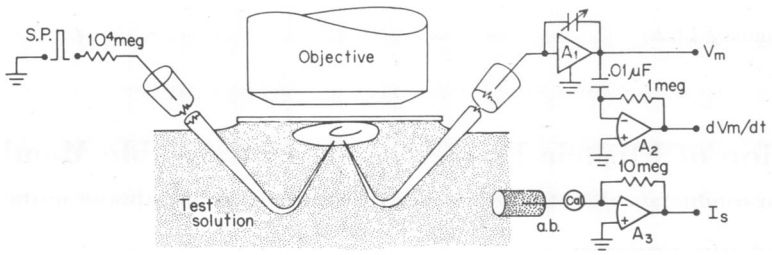

FIG. 1. Recording arrangement and instrumentation. Specimen impaled from below under visual control through coverglass. Stimulus pulse (*S.P.*) delivered through current-limiting resistor. Membrane potential, *Vm*, recorded with Amplifier 1 (Bioelectric Instruments NF1) with respect to virtual ground at summing junction of Amplifier 3 (Philbrick P25A), which also monitored the stimulating current, I_s. Output of *A1* was differentiated by Amplifier 2 (Philbrick P25A). All electrode wires Ag–$AgCl_2$. Bath connected to summing junction of A_3 through KCl–agar bridge (*a.b.*) and triggered calibrator (Bioelectric Instruments CA2).

"Pawns" because they swim only forward. Suprathreshold stimulation with potassium and other cations (20), as well as mechanical stimulation at the anterior end of the cell (which produces depolarization in the wild type, ref. 12), fail to evoke ciliary reversal. This is not due to a complete lack of responsiveness of the locomotor system itself, since mutants can orient toward the cathode of an applied electric field (4); this orientation depends on either increased frequency on the anodal side or ciliary reversal on the cathodal side of the cell (23). It was, therefore, reasonable to suppose that the control mechanism (presumably associated with the membrane), rather than the locomotor apparatus itself, was altered by mutation.

MATERIAL AND METHODS

All cells used in this work belong to syngen 4 of *P. aurelia*. From the different Pawn mutants, strain d4-95, carrying a pair of recessive alleles pw^B pw^B, was chosen for electrical studies. This mutant line (originally designated as pw 1-2-34) was derived from strain d4-85, which is behaviorally and physiologically normal in comparison to the wild-type stock 51, but which carries the marker gene *ts 111* in the original mutagenesis experiment. In the following description "Pawn" stands for d4-95 and "wild type" stands for the behavioral wild-type strain d4-85. It was necessary to use strain d4-85 as a control since all previous electrophysiological studies were made on the larger species *P. caudatum*. All cells were grown at room temperature in a modified Pringsheim solution (24) enriched with boiled egg yolk and bacterized. Specimens were adapted to 1 mM $CaCl_2$–4 mM KCl–1 mM Tris·HCl (pH 7.2) for 15 min or longer before experiments.

Care was taken to keep all conditions constant in electrical comparisons of Pawn and wild type. The electrical stimulating and recording techniques closely resembled those already described (7, 10). Input capacitance to the recording head stage was neutralized with positive feedback by "squaring up" a calibration pulse inserted between the bath and virtual ground of the operational amplifier used to monitor stimulating current. This was of special importance because of the high resistance (200–500 megohms) of the extrafine capillary electrodes in the test media of low ionic strength. Input leakage current was 0.2 pA. Test solutions in the bath were made with double glass-distilled water and contained 1 mM $CaCl_2$–4 mM KCl–1 mM Tris·HCl (pH 7.2). In some experiments, 4 mM $BaCl_2$ was substituted for the KCl in the bath. The essential features of electrical instrumentation are shown in Fig. 1. Electrical stimulation was limited to the physiological range. Thus, electrotonic depolarizations in excess of 25 mV were seldom applied. Cells requiring larger than normal currents (i.e. with low input resistances) were discarded as presumably damaged by insertion of the electrodes.

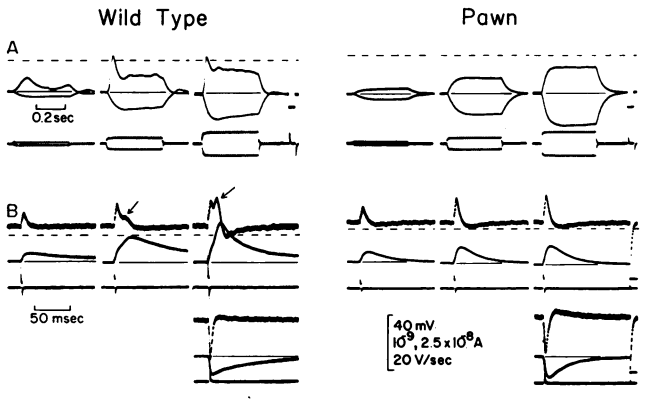

FIG. 2. Electric excitability of wild-type (d4-85) *P. aurelia* and mutant (d4-95) tested in 1 mM $CaCl_2$–4 mM KCl–1 mM Tris·HCl (pH 7.2). (*A*) Responses of wild type (*left*) and Pawn (*right*) to long depolarizing and hyperpolarizing currents. *Dashed lines* indicate the reference potential (V_m = 0). *Upper traces* give *Vm*, lower traces, I_s. Calibration pulse is seen at far right of *Vm* series in wild type and Pawn. (*B*) Responses of wild type and Pawn to brief current pulses. *Upper traces* give first time derivative of *Vm*, and *arrows* indicate derivative component associated with regenerative response. Single panel at *lower right* of each series shows response to hyperpolarizing pulse. Pawn shows none of the regenerative responses produced by the wild type.

RESULTS

The resting potentials of wild-type cells were −23.6 ± 1.1 mV, whereas that of Pawn was −24.9 ± 1.7 mV (±SE of measurements on eight specimens each). A *t*-test showed no significant difference.

Pulses of long duration (400 msec) were passed in both outward (depolarizing) and inward (hyperpolarizing) directions in wild type and Pawn (Fig. 2). Wild-type *P. aurelia* behave electrically very much like *P. caudatum* (9), showing graded regenerative depolarizing transients in response to depolarization, and only electrotonic potentials (with some delayed anomalous rectification) in response to hyperpolarizing current (Fig. 2*A*). In contrast, Pawn (in over 20 specimens tested)

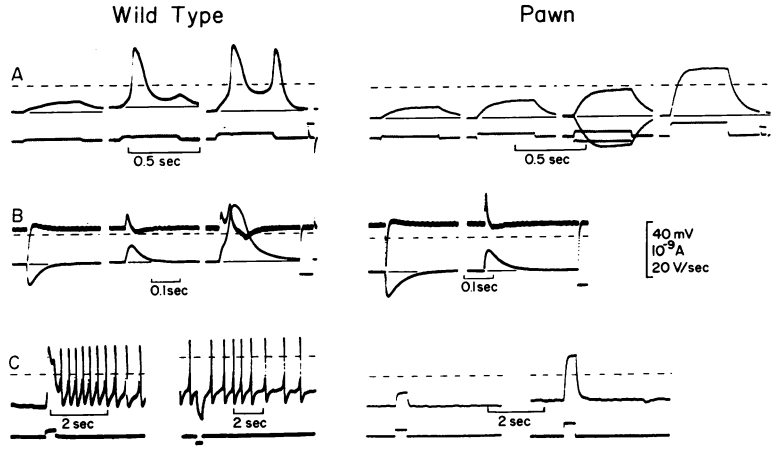

FIG. 3. Electric excitability of wild-type (d4-85). *P. aurelia* and mutant (d4-95) tested in 1 mM $CaCl_2$–4 mM $BaCl_2$–1 mM Tris·HCl. Wild type (*left*) shows characteristic all-or-none barium action potentials in response to long- (*A*) and short- (*B*) stimulus pulses, and as afterdischarges and spontaneously (*C*). The critical threshold for the barium spike produced by wild type is evident in series *B*, in which the same amount of current (4nA, 2 msec) produced no active response in the second frame and a full action potential in the 3rd frame. Pawn (*right*) shows no signs of local active responses or action potentials. Dashed lines indicate the reference ($Vm = 0$) potential. *Upper trace* is *Vm* and *lower trace* I_s in *A* and *C; upper trace* is dVm/dt and *lower trace Vm* in *B*.

had no clear regenerative response, showing little or no inflection on the upstroke of the RC component and no oscillation on the plateau.

Current pulses of short duration (2 msec) and higher intensity were passed and the potential changes produced were electronically differentiated (Fig. 2*B*). Pawn produced similar wave shapes in response to depolarizing and hyperpolarizing current pulses, (Fig. 2*B*, *right*). These electrotonic potentials, too, lack any sign of a regenerative component. Wild-type *P. aurelia*, like wild-type *P. caudatum*, responded with an inflection on the upstroke of the depolarization and a graded active component, which reaches an amplitude about twice the amplitude of the electrotonic hyperpolarization (Fig. 2*B*, *left*).

The graded electric responses of *P. caudatum* can be converted to all-or-none action potentials by the addition of Ba^{++} or Sr^{++} (8), similar to the effect of Ba^{++} and Sr^{++} on electric excitability in crustacean muscle (18). Ba^{++} increases membrane resistance, and also porduces a stronger regenerative current than does Ca^{++} (8). Barium renders the wild-type membrane of *P. aurelia* highly sensitive to depolarization, so that a depolarizing current as low as 10 pA can produce all-or-none action potentials in many cases (Fig. 3*A*, *left*). The action potential has a distinct threshold (Fig. 3*B*, *left*). In contrast, Pawn remains electrically inexcitable in Ba^{++} (Fig. 3*A* and *B*, *right*). Currents up to 20 times as high as the threshold current for wild-type specimens produced no response other than the passive electrotonic potential shift across the mutant membrane. In the presence of Ba^{++}, the wild-type membrane can produce an after discharge to strong depolarizing stimuli, and often shows spontaneous firing (Fig. 3*C*), as does the membrane of *P. caudatum* (25). Spontaneous firing in the wild type is correlated with spontaneous ciliary reversal (26). Pawn shows no spontaneous activity either electrically (Fig. 3*C*) or behaviorally (4).

One factor that can affect membrane excitability indirectly is the leakage conductance of the membrane. A low resistance to potassium can permit positive charge (i.e., K^+) to leak from inside the cell as regenerative current brings charge (Ca^{++} in *Paramecium*) into the cell, short-circuiting the current produced by Ca^{++} influx. For this reason, the input resistances of Pawn and wild type were compared. Potential displacements from 0–25 mV were produced by 400-msec current pulses of up to 300 pA delivered with an intracellular electrode. The steady-state potential displacements from the resting level were measured when the potential was steady toward the end of these pulses, and these were plotted against the intensities of the corresponding stimulating currents (Fig. 4). The steady-

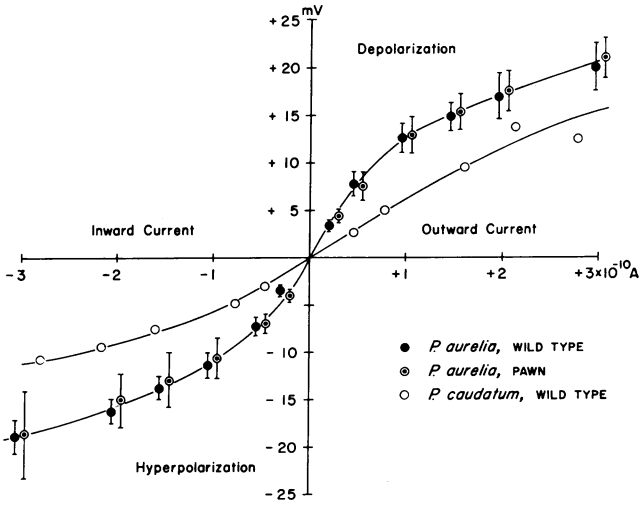

FIG. 4. Steady-state galvanostatic voltage–current relations of wild-type (d4-85) and mutant (d4-95) *P. aurelia* compared with those of the larger species *P. caudatum* (replotted from ref. 9). Test solution in all three cases was 1 mM $CaCl_2$–4 mM KCl–1 mM Tris·HCl. Wild-type and mutant membranes of *P. aurelia* show identical chord and slope resistances over ±20 mV displacements. Each *point* is the mean of measurements from eight specimens. *Vertical bars* indicate 95% confidence interval of the mean. Points representing wild type and Pawn are displaced slightly left and right, respectively, from current values to avoid overlap.

state current–voltage curves are virtually identical for wild-type *P. aurelia* and Pawn.

Membrane resistances at potentials close to rest were calculated from potential displacements of a few millivolts by weak currents. The input resistance of the wild type (143 ± 5 megohms) is not significantly different from that of Pawn (156 ± 7 megohms) (±SE of 32 measurements each). The input resistance of wild-type *P. caudatum*, replotted from Naitoh and Eckert (9), was lower, due at least in part to the larger surface area of *P. caudatum*.

Another factor that can reduce electric excitability is inactivation of the calcium (or Na^+) system by steady depolarization. To test the possibility that the Pawn membrane has a negative resistance like wild type, but that it is shifted substantially in the hyperpolarized direction (increased negativity), we hyperpolarized the membrane of Pawn up to 25 mV for 1 sec before depolarizing the membrane with stimuli ranging up to intensities sufficient to drive the electrotonic potential beyond zero. Hyperpolarization did not render the membrane electrically excitable, and only electrotonic potentials were evoked.

In summary, the membrane of Pawn gave no sign of electric excitation in the stimulus range used, whereas regenerative responses were routinely recorded from wild-type specimens of *P. aurelia* and *P. caudatum*.

DISCUSSION

We propose that the inexcitability of Pawn is due to an impairment of the mechanism for Ca^{++} activation (i.e., increased Ca^{++} conductance when the membrane is depolarized). Two major alternative possibilities are: (*a*) Pawn might have a reduced input resistance, which short circuits the inward calcium current, masking an unimpaired mechanism for calcium activation; and (*b*) the negative resistance region of the current–voltage relation might be shifted in Pawn to more negative membrane potentials so that the calcium mechanism, although present, would be nonfunctional unless the membrane was first hyperpolarized. Both of these alternative hypotheses were tested and ruled out, the first by the current–voltage measurements (Fig. 4), and the second by hyperpolarizing the membrane before stimulation.

The mutant membrane of Pawn behaves like that of the wild type in all measured electrical characteristics except one—it undergoes little or no increase in calcium conductance in response to depolarization. Impairment of the mechanism for Ca^{++} activation is further indicated by the failure of Pawn to undergo ciliary reversal when stimulated by KCl and other cations (4, 5). This conclusion follows because reversal of the ciliary power stroke results in the wild type from an influx of Ca^{++} in response to depolarization (21a), or in detergent-extracted models of *Paramecium* lacking a cell membrane in response to Ca^{++} concentrations above 0.1 μM†. The concomitant loss of both ciliary reversal (controlled by Ca^{++} influx) and regenerative depolarization (produced by Ca^{++} current) supports the conclusion that Pawn is defective in the mechanism that allows Ca^{++} to enter the cell through the membrane in response to a depolarization.

A role of nuclear genes in the development of electrical properties in excitable cell membranes has been indicated by the work of Minna *et al.* (27), in which hybrid cells with different degrees of electric excitability resulted from the fusion of excitable and inexcitable cell lines. The modification of membrane function (i.e., loss of Ca^{++} activation) in our study gains special significance from the evidence that Pawn differs from wild type by a single gene (5). Thus, it appears that the mechanism for calcium activation in *Paramecium* was impaired due to deletion or modification of a single (or small group of) gene product(s).

We gratefully acknowledge suggestions and advice from Drs. Akira Murakami and Richard Orkand, and most especially from Dr. Kenneth Friedman. This work was supported by NSF Grants GB 7999 and GB 20916.

1. Hodgkin, A. L. (1957) "Ionic Movements and Electrical Activity in Giant Nerve Fibers," in *Proc. Roy. Soc. Ser. B* **148**, 1–37.
2. Huxley, A. F. (1964) "Excitation and Conduction in Nerve: Quantitative Analysis," in *Science* **145**, 1154–1159.
3. Benzer, S. (1967) "Behavioral Mutants of Drosophila Isolated by Countercurrent Distribution," in *Proc. Nat. Acad. Sci. USA* **58**, 1112–1119.
4. Kung, C. (1971) "Genic Mutants with Altered System of Excitation in *Paramecium aurelia*, I. Phenotypes of the Behavioral Mutants," in *Z. vergl. Physiol.* **71**, 142–162.
5. Kung, C. (1971) "Genic Mutants with Altered System of Excitation in *Paramecium aurelia*, II. Mutagenesis, Screening, and Genetic Analysis of the Mutants," *Genetics* **69**, 29–45.
6. Eckert, R. & Naitoh, Y. (1969) Graded Calcium Spikes in *Paramecium*," in *Third Intern. Biophys. Congr. Intern. Union Pure and Applied Biophys.* Abstr. (Cambridge, Mass.), p. 257.
7. Eckert, R. & Naitoh, Y. (1970) "Passive Electrical Properties of *Paramecium* and Problems of Ciliary Coordination," in *J. Gen. Physiol.* **55**, 467–483.
8. Naitoh, Y. & Eckert, R. (1968) "Electrical Properties of *Paramecium caudatum:* All-Or-None Electrogenesis," in *Z. vergl. Physiol.* **61**, 453–472.
9. Naitoh, Y. & Eckert, R. (1968) "Electrical Properties of *Paramecium caudatum:* Modification by Bound and Free Cations," *Z. vergl. Physiol.* **61**, 427–462.
10. Naitoh, Y. & Eckert, R. (1971) "Electrophysiology of the Ciliate Protozoa," in *Experiments and Physiology and Biochemistry*, ed. Kerkut, G. A. (Academic Press, London), Vol. V.
11. Kinosita, H. (1954) "Electric Potentials and Ciliary Response in Opalina," in *J. Fac. Sci. Imp. Univ. Tokyo, Sec. 4*, **7**, 1–14.
12. Naitoh, Y. & Eckert, R. (1969) "Ionic Mechanisms Controlling Behavioral Responses of *Paramecium* to Mechanical Stimulation," in *Science* **164**, 963–965.
13. van Wagtendonk, W. J., Coldman, P. H. & Smith, W. L. (1970) "The Axenic Culture of Strains of Various Syngens of *Paramecium aurelia*," in *J. Protozool.* **17**, 389–391.
14. Gibbons, I. R. (1965) "Chemical Dissection of Cilia," in *Arch. Biol.* **76**, 317–352.
15. Sonneborn, T. M. (1970) "Methods in *Paramecium* Research," in *Methods in Cell Physiology*, ed. Prescott, D. (Academic Press Inc., New York), Vol. 14, pp. 241–339.
16. Fatt, P. & Katz, B. (1953) "The Electrical Properties of Crustacean Muscle Fibres," in *J. Physiol.* **120**, 171–204.
17. Fatt, P. & Ginsborg, B. L. (1958) "The Ionic Requirements for the Production of Action Potentials in Crustacean Muscle Fibres," in *J. Physiol.* **142**, 516–543.
18. Werman, R. & Grundfest, H. (1961) "Graded and All-Or-None Electrogenesis in Arthropod Muscle, II. The Effects of Alkali-Earth and Onium Ions on Lobster Muscle Fibers," in *J. Gen. Physiol.* **44**, 997–1027.
19. Naitoh, Y., Eckert, R. & Friedman, K. (1972) "Regenerative Calcium Responses in *Paramecium*," in *J. Exp. Biol.*, in press.
20. Naitoh, Y. (1968) "Ionic Control of the Reversal Response of Cilia in *Paramecium caudatum:* A Calcium Hypothesis," in *J. Gen. Physiol.* **51**, 85–103.

21. Naitoh, Y. (1969) "Control of the Orientation of Cilia by Adenosinetriphosphate, Calcium, and Zinc in Glycerol-Extracted *Paramecium caudatum*," in *J. Gen. Physiol.* **53,** 517–529.
21a. Eckert, R. (1972) "Bioelectric Control of Ciliary Activity," in *Science*, in press.
22. Kamada, T. (1934) "Some Observations on Potential Differences across the Ectoplasm Membrane of *Paramecium*," in *J. Exp. Biol.* **11,** 94–102.
23. Jahn, T. L. (1961) "The Mechanism of Ciliary Movement, I. Ciliary Reversal and Activation by Electric Current; the Ludloff Phenomenon in Terms of Core and Volume Conductors," in *J. Protozool.* **8,** 369–380.
24. Chapman-Andresen, C. (1958) "Pinocytosis of Inorganic Salts by *Amoeba proteus*," in *C. R. Trav. Lab. Carlsberg, Ser. Chim.* **31,** 77–92.
25. Kinosita, H., Dryl, S. & Naitoh, Y. (1964) "Spontaneous Change in Membrane Potential of *Paramecium caudatum* Induced by Barium and Calcium Ions," in *Bull. Acad. Pol. Sci.* **12,** 459–461.
26. Kinosita, H., Murakami, A. & Yasuda, M. (1965) "Interval between Membrane Potential Change and Ciliary Reversal in *Paramecium* Immersed in Ba-Ca Mixture," in *J. Fac. Sci. Imp. Univ. Tokyo, Sec. 4,* **10,** 421–425.
27. Minna, J., Nelson, P., Peacock, J., Glazer, D. & Nirenberg, M. (1971) "Genes for Neuronal Properties Expressed in Neuroblastoma x L Cell Hybrids," in *Proc. Nat. Acad. Sci. USA* **68,** 234–239.