SURFACE CHARGE OF GIANT AXONS OF SQUID AND LOBSTER

JOHN R. SEGAL

From the Neurophysiology-Biophysics Research Unit of the Veterans Administration Hospital, Boston, Massachusetts 02130

ABSTRACT A method is described for the determination of the electrophoretic mobility of single, isolated, intact, giant axons of squid and lobster. In normal physiological solutions, the surface of hydrodynamic shear of these axons is negatively charged. The *lower limit* of the estimated surface charge density is -1.9×10^{-8} coul cm^{-2} for squid axons, -4.2×10^{-8} coul cm^{-2} for lobster axons. The electrophoretic mobility of squid axons decreases greatly when the applied transaxial electric field is made sufficiently intense; action potential propagation is blocked irreversibly by transaxial electric fields of the same intensity. The squid axon recovers its mobility hours later and is then less affected by transaxial fields. Eventually, a state is reached in which the transaxial field irreversibly reverses the sign of the surface charge. In contrast, there is no obvious effect of electric field on the mobility of lobster axons. The mobility of lobster axons becomes undetectable in the presence of Th^{4+} at a concentration which blocks the action potential, and in the presence of La^{3+} at a concentration which does not affect propagation. Quinine does not alter lobster axon mobility at a concentration which blocks action potential conduction. Replacement of extracellular Na^+ by K^+ is without effect upon lobster axon mobility. The electrophysiological implications of the results are discussed.

INTRODUCTION

The hypothesis that fixed charges determine the electrical behavior of excitable membranes is attractive because a wide variety of important macroscopic membrane phenomena can arise and be explained as the simple and immediate consequence of the presence of a high density of immobile charged groups within the membrane (Teorell, 1953, 1959 *a*, 1959 *b*, 1961). The possibility that fixed (negative) charges are, in fact, electrophysiologically relevant is suggested by the axonal cation permselectivity inferred from radioisotopic flux measurements (Hodgkin and Huxley, 1953; Caldwell and Keynes, 1960; Tasaki, Teorell, and Spyropoulos, 1961; Tasaki, 1963; Brinley and Mullins, 1965) and the relationship between resting potential and electrolyte concentration (Teorell and Spyropoulos; Tasaki, Watanabe, and Lerman, cited in Tasaki, 1967). However, this evidence does not constitute sufficient proof

of the role of fixed charges. The absence of an unequivocal demonstration of the presence of fixed charges prompted the present attempt to determine, in a direct and unambiguous manner, whether or not the axonal surface is charged.

The method (e.g. Abramson, Moyer, and Gorin, 1964) employed consists of the measurement of the movement of viable, intact axons when placed in an electric field applied perpendicularly to their long axis. The direction and magnitude of such electrophoretic mobilities are related to the electrical potential at the plane of hydrodynamic shear, or slippage plane, between the axon and the surrounding solution. This potential—the zeta-potential—is the consequence and thus the measure of the sign and density of charges borne by the axon at the surface of shear.

The plane of hydrodynamic shear is not necessarily located at the interface between the most peripheral anatomical layer and the extracellular solution (Elul, 1967) because the surface of the axon may be highly permeable to water (Nevis, 1958; Tasaki, Teorell, and Spyropoulos, 1961). Thus, in the absence of an experimental determination of the actual plane of shear of the axon, it would be premature to identify it with a particular one of the many anatomical surface layers (axolemma, Schwann cell, basement membrane, etc.). Therefore, the most specific conclusion that can be drawn about the measurements described below is that they are of the properties of the surface of the axon at the plane of shear between it and the surrounding solution.

It shall be demonstrated that the shear surface of squid and lobster giant axons bears fixed electrical charges and that these can be related to the electrophysiological state of the axon.

METHODS

Biological Material

The experiments reported here were performed on single giant axons of the squid, *Loligo pealii*, or the lobster, *Homarus americanus*. Axons were isolated by conventional techniques from the hindmost stellar ganglion and the circumoesophageal connective, respectively. The axon diameters were: squid, 400–500 μ; lobster, 75–110 μ.

Electrophoresis Chamber

The chamber, illustrated in Fig. 1, was designed so that an axon could be suspended in physiological solutions, an electric field applied perpendicularly to its long axis, and the ensuing movement detected. A 1.5 cm length of axon was attached to a frame bent from 0.4 mm diameter glass rod with 10 μ diameter silk filaments tied to its ends. The tension of these filaments was adjusted (visually) so that the axon was free to move laterally, but yet was not so loosely suspended as to be unduly deflected by environmental mechanical disturbances. The glass frame with attached axon was transferred to the electrophoresis chamber within a fluid-filled carrier which eliminated the passage of the axon through an air-water interface. The glass frame was affixed eccentrically to a plexiglass plug which fitted a matching hole in the central portion of the chamber. By rotating the plug within this hole, it was possible to alter the position of the axon across the short dimension of the chamber.

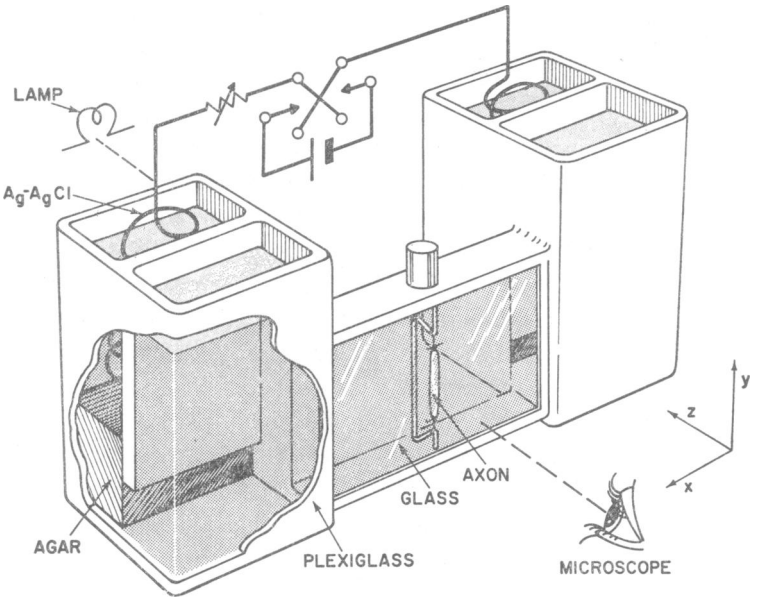

FIGURE 1 Schematic representation, not to scale, of the apparatus employed to measure the electrophoretic mobility of intact giant axons. The thermostatically controlled water bath in which the entire electrophoresis chamber was immersed is not illustrated. See text for full details.

The electrophoresis chamber was constructed of plexiglass and glass. The portion which contained the axon consisted of two plates of glass, 7.5 × 5.0 (high) cm, separated along the top and bottom by 0.48 cm thick plexiglass spacers. The ends of the rectangular parallelepiped, so defined, joined two plexiglass compartments open to the atmosphere, of dimensions 4.6 × 5.9 × 6.6 (high) cm. Each of these was connected to plexiglass electrode compartments (100 cm^3 volume) through 4% agar plugs 8 cm thick. The agar isolated the axon from the possible effects of electrode products and gassing. Otherwise, the latter, when present, can cause spurious movements. The electrodes were coils of silver wire 0.25 × 40 cm electrolytically coated with AgCl. The same solution was used to fill all the compartments and to prepare the agar. A constant electric field was produced and maintained in the central, glass-walled compartment by passing a constant current between the two electrodes. The major portion of the total electrical resistance of the chamber was that due to the fluid between the glass plates.

The whole chamber was suspended in a thermostatically controlled water bath at 4.0°C (the temperature at which the change of density of water with temperature is at a minimum). This was essential, as measurements carried out at room temperature were completely obscured by convection due to the Joules heating of the solution. The rise in temperature of a chamber initially at 4.0°C was about 1°C for the largest fields employed.

The axon was viewed through a microscope of 125 × magnification. The lateral position of the axon was determined with a filar micrometer eyepiece; its distance from the glass wall of the electrophoresis chamber by measuring the change in plane of focus of the microscope (corrected for refraction) with a dial indicator.

Sensitivity

The reproducibility of the measurements was limited by the backlash and resolution of the microscope-filar micrometer combination—about $\pm 0.3\ \mu$—and the lateral, random movements of the axon due to building vibrations, etc.—about 10 μ peak-to-peak. The effect of the latter was reduced by either waiting for the brief quiescent periods or by visually averaging the position of the axon. The final error in determining the position of the axon was not more than $\pm 0.75\ \mu$.

It should be possible to increase the sensitivity of these measurements at least 100-fold with effective isolation of the axon from vibration and a more sensitive detector of axonal movement (e.g. Hill, 1950). This is worth achieving as it would then be feasible to study the effect of a variety of agents on the surface charge of a single axon. It would also be possible to detect changes of electrophoretic mobility, should they occur, during the course of an action potential prolonged, for example, by tetraethylammonium chloride (Tasaki and Hagiwara, 1957).

Solutions

The squid axon physiological solution was artificial sea water which consisted of (mM): 460 NaCl, 55.0 $MgCl_2$, 11.0 $CaCl_2$, 10.0 KCl, and 0.6 $KHCO_3$. The lobster axon physiological solution was Coles solution (Cole, 1941) which had the following composition (mM): 445 NaCl, 24.9 $CaCl_2$, 15.1 KCl, 8.8 H_3BO_3, 8.0 $MgCl_2$, and 4.0 Na_2SO_4. The solutions were oxygenated and brought to pH 7.3 with NaOH.

Transmembrane Current

When an axon is placed in a transverse electric field, as in the present case, current will flow between the axoplasm and extracellular solution. This transmembrane current must be kept within the normal physiological range of the axon if the electrophoretic measurements are to be physiologically meaningful. That this condition was met is shown by the following calculation of transmembrane current due to the applied electric field.

For DC currents, the axon is equivalent (Cole and Curtis, 1944) to a homogeneous cylinder of resistivity $R = r + R_m/a$ where r is the resistivity of the axoplasm, R_m is the resistance of a unit area of the surface membrane, and a is the radius of the axon. When a cylinder of resistivity R, is suspended in a uniform electric field, E, applied perpendicularly to its long axis, the radial component of the field at a point within the cylinder at its outer edge is $2E\lambda/(\lambda + R^{-1}) \cdot \cos\theta$ where λ is the conductivity of the medium in which the cylinder is placed and θ is the angle between the field and the radius drawn to the point in question (e.g. Henry, 1931). The current which crosses the boundary betweeen cylinder and medium–the transmembrane current–is the field divided by the resistivity of the cylinder, i.e. $2E\lambda/(R\lambda + 1) \cdot \cos\theta$. R for a squid axon in artificial sea water is 4×10^4 ohm cm ($r = 50$ ohm cm (e.g. Schmitt, 1955), $R_m = 10^3$ ohm cm^2 (e.g. Hodgkin, 1964), $a = 0.025$ cm); λ was measured at 4°C and found to be (32 ohm cm)$^{-1}$. Thus, the maximum transmembrane current is $5 \times 10^{-5} \times E$ amp cm^{-2}. As E was no more than 1.56 v cm^{-1} (Fig. 2), the maximum membrane current of the squid axon under the conditions of these experiments was 8×10^{-5} amp cm^{-2}–less than one-tenth the peak current density during excitation (Hodgkin and Huxley, 1952). The significant differences between the parameters of lobster axon–Coles solution and squid axon–artificial sea water are that the lobster axon radius is one-fifth that of the squid axon and its membrane resistance is eight times that of the squid axon (Brinley, 1965). Therefore, the

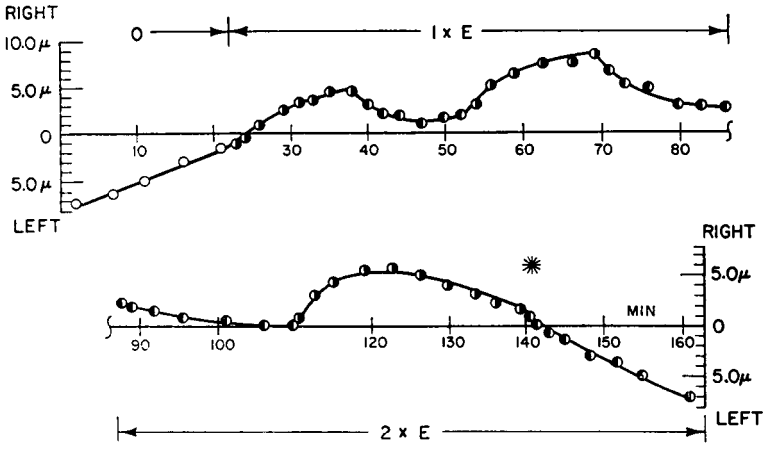

FIGURE 2 Displacement of a squid axon in the direction perpendicular to its long axis, as a function of time, due to an electric field applied perpendicularly to its long axis. The meaning of the symbols in this and all subsequent figs. is: ○, no field applied; ◑, the electric potential of the right compartment (Fig. 1) was positive with respect to that of the left; ◐, the electric potential of the left chamber (Fig. 1) was positive with respect to that of the right. The intensity of the applied field is denoted by the symbol between the horizontal arrows; the time of exposure of the axon to the field is indicated by the horizontal distance between the arrow heads. In this and all subsequent figs. pertaining to the squid axon, the field intensities were: $1 \times E$, 0.39 v cm^{-1}; $2 \times E$, 0.78 v cm^{-1}; $4 \times E$, 1.56 v cm^{-1}. The axon was immersed in artificial sea water at 4°C. It was located 1.25 mm from the glass wall nearest the microscope (Fig. 1). Note the marked decline in electrophoretic mobility evident at the reversal of the direction of the field labeled with the asterisk (a full description of this phenomenon is given within the text). Axon No. 1.

maximum transmembrane current density of the lobster axon under the conditions of these experiments is about one-fortieth that of the squid axon.

RESULTS

When a squid or lobster giant axon is placed in an electric field applied perpendicularly to its long axis, it moves in a direction opposite to that of the field—as would be the case if the shear surface were negatively charged—Figs. 2, 3, and 4. Before any biological significance can be attributed to these results it is necessary to demonstrate that these movements are due, in fact, to a surface charge. Potential causes of the movement, other than electrophoresis of the axon, which must be considered, are: convection, electroosmotic flow of the surrounding medium, and electrophoresis of the suspensory silk filaments. These three phenomena will now be shown to have had a negligible effect upon the axons.

VALIDATION OF THE METHOD

Convection

The inevitable Joules heating of the fluid within the chamber when an electric current flows leads to convective movement of the fluid. This and/or the decrease in

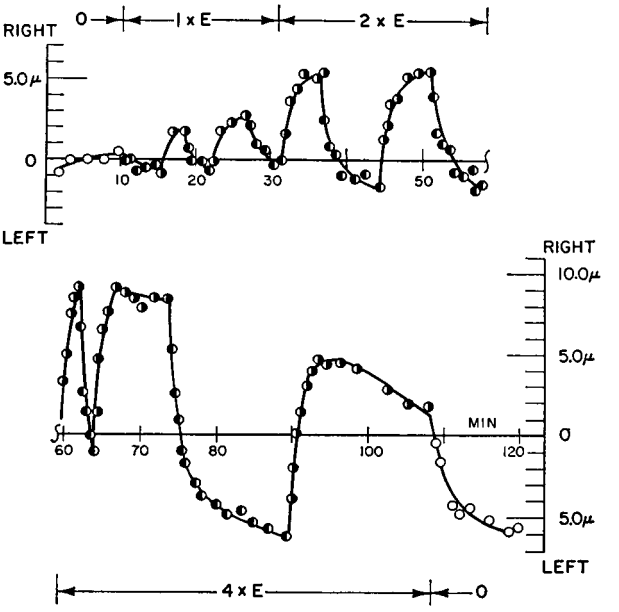

FIGURE 3 Displacement of a lobster giant axon in the direction perpendicular to its long axis, as a function of time, due to an electric field applied perpendicularly to its long axis. The graphical conventions and meaning of the symbols are the same as for Fig. 2, with the following exception. The intensity of the applied fields was: $1 \times E$, 0.43 v cm^{-1}; $2 \times E$, 0.87 v cm^{-1}; $4 \times E$, 1.73 v cm^{-1}. The axon was immersed in Coles solution at 4°C. It was located 1.12 mm from the glass wall nearest the microscope (Fig. 1).

density of the fluid, per se, could cause the axon to move laterally. However, Joules heating and hence convection are independent of the direction of current flow. The fact that the direction of the axon movement reverses when the polarity of the field is reversed is proof that the movement is not due to convection.

The effect of convection is evident in the figures as a slow drift of the zero-field position of the axon.

Convective movements did, on occasion, exceed and obscure the field-dependent movements. In these instances the axon moved in a single direction at a relatively rapid rate. The direction did not reverse when the field was reversed, nor did it cease immediately when the field was removed.

Movement of Fluid Medium and Suspensory Filaments

As the glass-water interface can be charged, there might be an electroosmotic flow of fluid (e.g. Davies and Rideal, 1961) and, hence, of the axon along the long axis of

the chamber when an electric field is applied in that direction. The direction of this flow cannot be predicted for the present cases: it is quite possible that the usually negative surface charge of the glass is reversed in solutions of such high ionic strength as artificial sea water and Coles solution. Thus, electroosmosis cannot be dismissed as the cause of the axonal movements simply on the basis of its being in the "wrong" direction. An indirect measurement of the fluid velocity was made which shows it to be too small to have been a significant factor.

Before proceeding it is necessary to establish, theoretically, the pattern of fluid flow within the chamber arising as the consequence of electroosmotic flow along the walls. This analysis will show that, although the side compartments are open to the atmosphere, the over-all geometry of the chamber is such that it behaves hydrodynamically as though it were closed to the atmosphere. Furthermore, the alteration in level of the fluid within the side compartments due to electroosmosis would be too small to be detected, necessitating the indirect evaluation of electroosmosis employed.

The velocity of a fluid, u, flowing in the x-direction between two parallel plates located in the xy-plane is (Lamb, 1932)

$$u = u_W + 1/2\eta \cdot (z^2 - h^2) \cdot \partial p/\partial x$$

where u_W is the velocity at the walls, η is the viscosity of the fluid, $\partial p/\partial x$ is the gradient of pressure along the x-axis, z is the distance from the central plane of the plates, $2h$ is the separation between the two plates, and x, y, z, are the rectilinear coordinate components (Fig. 1). u_W is, for the present situation, the electroosmotic velocity of the fluid at the plane of slip between fluid and glass wall. Initially, $\partial p/\partial x$ is zero, but following application of an electric field, there is a net flux of fluid into one of the side compartments, the level rises, and $\partial p/\partial x$ increases. This continues until an equilibrium state is attained in which $\partial p/\partial x$ is great enough to prevent further entry of fluid—the net flux becomes zero. The flux of fluid per unit cross-sectional area is $\int_{-h}^{+h} u\, dz$; this is zero when $\partial p/\partial x = 3u_W\eta/h^2$. In the present electrophoresis chamber $\partial p/\partial x$ is constant everywhere within the glass-walled region; it is equal to the pressure difference between the side compartments divided by the length of the glass plates.

If the zeta-potential of the fluid-glass interface is (generously) estimated as 100 mv then u_W is 8 μ sec^{-1} for the largest fields employed (1.7 v cm^{-1}) (Davies and Rideal, 1961). It follows from the above expression that the pressure gradient which reduces the net flux to zero is 6×10^{-4} dyne cm^{-3}. This corresponds to a difference in fluid level between the side compartments of only 450 A—too small to be detected without special techniques.

The time required to reduce the net flux to zero is calculated in the following manner. The net flux is zero when the pressure difference between the side compartments is $3u_W\eta l/h^2$ where l is the length of the glass plates (7.5 cm). The volume

of fluid which must enter one compartment and leave the other to produce this pressure difference is $3Au_w\eta l/2h^2$, where A is the area of the fluid-air interface of the side compartment (27 cm^2). The initial net flux of fluid, upon establishing the electric field, is, assuming plug flow, $2hu_wg$, where g is the height of the glass plates (5.0 cm). The time required to reduce the net flux to zero is of the order of $(3Au_w\eta l/2h^2)(2hu_wg)^{-1}$, i.e. 0.03 sec in the chamber employed. Thus, the net flux due to electroosmosis would be reduced to zero within a negligible period of time in terms of the time scale of the present measurements. Therefore, for all practical considerations, the electrophoresis chamber acts as though it were closed to the atmosphere.

Under that condition the net flux is zero and the velocity of fluid as a function of position between the glass plates is (e.g. Abramson, Moyer, and Gorin, 1964)

$$u = (3z^2/h^2 - 1)u_w/2. \tag{1}$$

Thus, the flow reverses direction at $z = \pm h/\sqrt{3}$, i.e. 1.01 mm from the glass walls in the present chamber.

We can now show that the electroosmotic movement of the fluid was too small to have significantly affected the axon. For this purpose the movement of a silk filament was determined as a function of its distance from the glass walls. The electrophoretic component of motion of the filament is independent of position—the electric field is independent of location—but that due to electroosmosis is not—it varies according to equation 1. Thus, if there is significant electroosmosis, the silk filament will move with a velocity which varies with the distance of the filament from the glass wall.

If the movement of the silk filament is to be employed as a measure of the possible effect of electroosmosis on the axon, then it is necessary that they both be similarly affected by the motion of the fluid. The extent to which axonal displacements are coupled to the motion of the fluid was first determined in the absence of an electric field. A known velocity was impressed upon the fluid (Coles solution) by adding fluid to one side compartment at a constant rate; the concomitant equilibrium displacement of a lobster axon, showing large field-dependent movements, was measured. A length of the silk filament, identical to that used to suspend the axons, was then attached to the glass frame in place of the axon. The tension of the filament was adjusted so that its equilibrium displacement for a given fluid (Coles solution) velocity was the same as for the axon. Thus, electroosmosis of the fluid would have the same effect on both axon and silk filament.

With the filament 1.1 mm from the wall a field of 1.7 v cm^{-1} was applied; there was no detectable movement of the filament. Therefore, either the electrophoretic movement of the filament was exactly opposite to that due to electroosmosis of the fluid or both components of motion were zero.

The latter is shown to be the case by the results obtained when the distance between silk filament and the wall was increased to 2.1 mm. At this position the electro-

osmotic component of motion should have increased by a factor of 8.0 with respect to the prior location, according to equation 1. The filament-fluid coupling was determined and found to be the same as previously. Again, the filament did not move when an electric field of 1.7 v cm^{-1} was applied. Thus, both the electrophoretic mobility of the filament and the effect of electroosmotic flow were negligible.

Since the coupling between silk filament movement and fluid flow had been made identical to that between the lobster axon and fluid, it follows that electroosmotic flow, even if present, had no significant effect upon the axon.

The same conclusion was reached when these measurements were made of a silk filament and squid axon in artificial sea water.

Convection, electroosmosis, and electrophoresis of the silk filaments are thus excluded as the cause of the field-dependent axonal movements. These movements are, therefore, necessarily the consequence of the axonal shear surface being charged.

Biological Results

Normal Physiological Conditions

The electrophoretic behavior illustrated in Figs. 2, 3, and 4 was typical of the five squid axons and five of the six lobster axons studied in artificial sea water and Coles solution, respectively. The single atypical lobster axon showed no measurable movement—the axon may have been too tautly suspended.

The patterns illustrated demonstrate that the surface at the plane of slip between axon and fluid is negatively charged. In support of this conclusion, observe (Fig. 3 in particular): (*a*) the axon moves in a direction opposite to that of the field, (*b*) upon reversing the direction of the field the initial rate of movement is approximately proportional to the field intensity, and (*c*) the equilibrium displacement of the axon is approximately proportional to field intensity.

The velocity of the axon and its equilibrium position are determined by the balance between the electrophoretic force acting on the axon and the opposing elastic forces of the silk filaments and axon. An equilibrium displacement is attained when the magnitudes of these two counteracting sets of forces becomes exactly equal. Since the axon is under some tension even in its initial, field-free position, it follows that the maximum observed mobilities were always less than the true electrophoretic mobility of the axon. How much less the observed mobility is than the actual one can only be decided after a detailed evaluation of all the mechanical forces acting on the axon.

The *maximum* observed mobilities of the individual axons were the following: $-(0.2\text{–}2.6)\ \mu\ min^{-1}\ v^{-1}\ cm$ (mean $= -1.9$) for five squid axons during the initial measurements in artificial sea water; and $-(0.8\text{–}6.8)\ \mu\ min^{-1}\ v^{-1}\ cm$ (mean $= -3.6$) for five lobster axons in Coles solution.

The most likely reason for the wide range of mobilities is a variation, from axon to axon, of the tension of the suspensory silk filaments. Because of this uncontrolled

factor, no significance can be attributed to the difference between the mean maximum mobilities of squid and lobster axons. The interpretation of these mobilities in terms of zeta-potential and surface charge density is deferred to the Discussion.

Thus, the shear surface of the giant axons of squid and lobster are negatively charged under normal physiological conditions—a property common to a wide variety of cell types (e.g. Brinton and Lauffer, 1959; Elul, 1967). Most pertinent is the latter author's demonstration that cells of cultures of neural cell bodies, glia, and muscle fibers are negatively charged.

Field-Induced Diminution and Reversal of Mobility

When the intensity of the field in which a squid axon was suspended was increased from 0.39 v cm^{-1} to 0.78 v cm^{-1}, the mobility decreased markedly, almost completely disappearing (Figs. 2 and 4 *a*). This occurred while the axon was exposed to the more intense field with a mean (five axons) delay of 30 min (range: 15–53 min) following the increase in field intensity.

It was not established if there is a true threshold field intensity for the loss of electrophoretic mobility or if it simply occurs as the cumulative effect of a maintained transaxial field. The axons had been exposed to the 0.39 v cm^{-1} field for 15–100 min (mean: 50 min) without an obvious change in their mobilities. There appeared to be no corrrelation between the time of exposure to the 0.39 v cm^{-1} field and the time required for mobility diminution during the application of the 0.78 v cm^{-1} field. However, the data is too limited to reach any definite conclusions about the field intensity-exposure time relationship for the decline of mobility.

The loss of electrophoretic mobility was reversible. Axons left for 9–26 hr, with no electric field applied, were found to have recovered their mobilities (Fig. 4 *b*). (This was true of all four axons examined for recovery.) However, at this stage the mobility was affected very much less by transaxial electric fields than initially (Fig. 4 *b*). There were signs of field-induced mobility diminution (not illustrated), but only after exposure to the most intense field (1.56 v cm^{-1}) and not to the degree of Figs. 2 and 4 *a*.

Three of these same axons were next examined 24–41 hr after the start of the experiments. At this stage the applied transaxial electric field caused a reversal of the sign of the charge of the shear surface—it became positive. This field-induced charge reversal is illustrated in Fig. 4 *c*; it was found in all three axons examined. As shown in Figs. 4 *c* and 4 *d*, when the shear surface has become positively charged, it remains so irreversibly. The surface charge of the axon of Fig. 4 was still positive 100 hr after the start of the experiment, but the magnitude of the mobility was considerably less than at the 75 hr measurements of Fig. 4 *d*.

The preceding description of the alteration with time of the relationship between electric field and axonal electrophoretic mobility must be regarded as tentative, in view of the limited number of measurements. However, the data *is* sufficient to con-

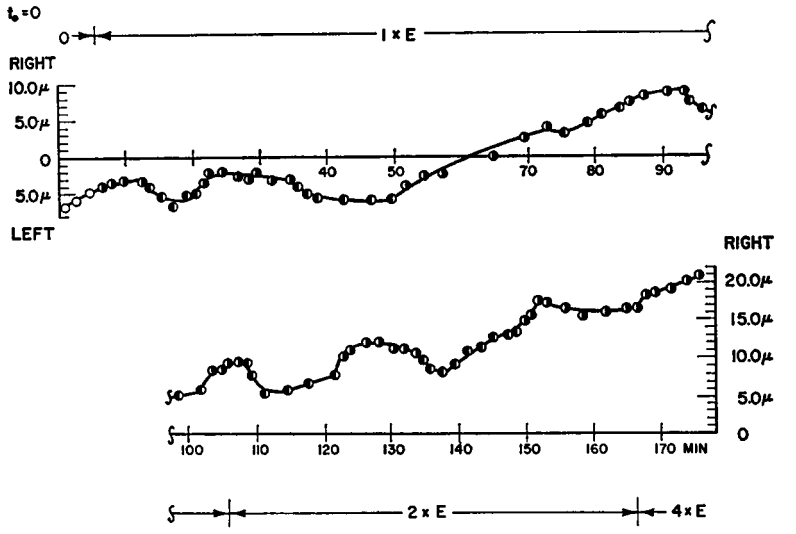

FIGURE 4 *a* Initial determination of the electrophoretic mobility. Observe the great decline of mobility evident after 130 min.

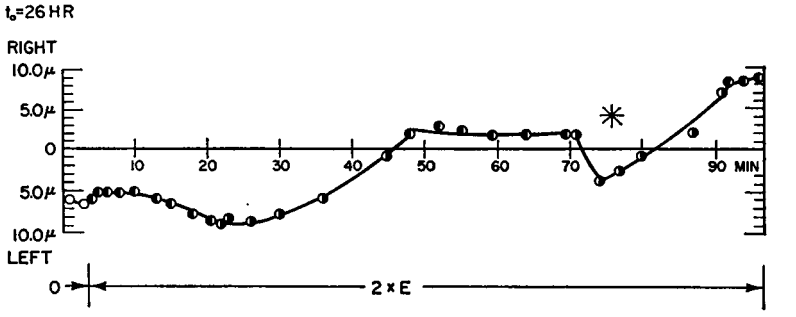

FIGURE 4 *b* Electrophoretic movements of the same axon 26 hr after the start of the record of Fig. 4 *a*. No field was applied during the period between the two records. Note that the magnitude of the mobility is relatively constant, in contrast to Fig. 4 *a*. The axon appears to have become temporarily positively charged at the time marked by the asterisk. When other axons at this stage were subjected to a field of $4 \times E$, there was some diminution of mobility, but less than that illustrated in Figs. 2 and 4 *a*.

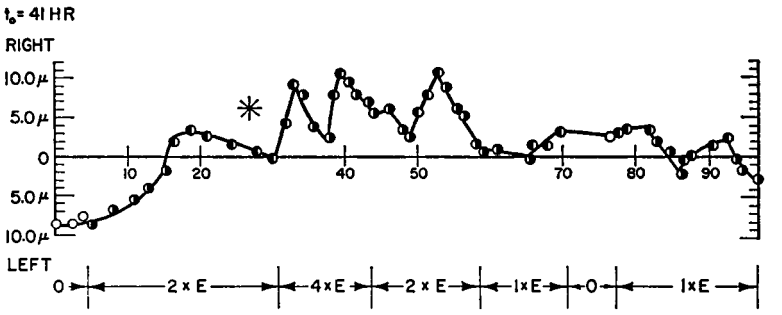

FIGURE 4 *c* Electrophoretic movements of the same axon 41 hr after the start of the record of Fig. 4 *a*. No field was applied during the period between this record and the one of Fig. 4 *b*. Note that the axon was initially negatively charged but when the intensity of the field was doubled (asterisk) it became positively charged.

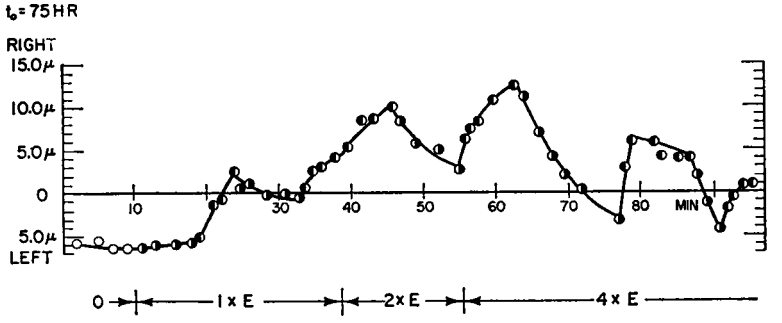

FIGURE 4 *d* Electrophoretic movements of the same axon 75 hr after the start of the record of Fig. 4 *a*. No field was applied during the period between this record and the one of Fig. 4 *c*.

FIGURE 4 Electrophoretic movements of a squid giant axon immersed in artificial sea water at 4°C. The axon was located 1.55 mm from the glass wall nearest the microscope (Fig. 1). The graphic conventions and meaning of the symbols are identical to those of Fig. 2. Axon No. 2.

clude that both the magnitude and sign of the mobility of a squid axon and, hence, its surface charge can be altered by an applied transaxial electric field.

The possible correlation between squid axon electrophoretic mobility and the ability of an axon to propagate an action potential was explored. In separate experiments, the effect of transaxial electric fields on action potential conduction, alone, was examined. These measurements were performed at room temperature with a different chamber from that employed for the electrophoresis studies. A field was applied perpendicularly to the axis of a cleaned axon along 6.4 mm of its length. The propagation of the action potential through this region was monitored by conventional techniques. The polarity of the transaxial field was reversed about every ten min, as in the electrophoresis measurements. A field of 0.32 v cm^{-1} was applied to each of three axons. Conduction in one of these was irreversibly blocked after 42 min. Propagation of the remaining two was unaffected by this field after 75 and 85 min, respectively. When the field was increased to 0.64 v cm^{-1}, irreversible conduction block occurred in the former after 2 min, and in the latter after 18 min.[1]

Thus, action potential propagation ceases and electrophoretic mobility decreases markedly upon exposure to transaxial electric fields. The intensities of the fields and the times required for the two effects to appear are about the same. The quantitative differences which do exist may be due to the fact that the action potential propagation block measurements were made at a higher temperature—the potency of agents which affect propagation increases with temperature (Spyropoulos, 1957; present author, unpublished). These results are therefore evidence that field-induced action potential block and mobility diminution occur concomitantly. The significance of this finding is discussed below.

Lobster giant axons did not display the squid axon pattern of field-induced mobility diminution and/or reversal even during prolonged exposure to the most intense fields. There were isolated instances when the surface charge reversed temporarily (for about 20 min), but no axon became permanently positively charged as did

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[1] There is no immediately obvious reason why action potential conduction block should occur when a transaxial electric field is applied and maintained for a prolonged period. The magnitude of the membrane currents due to the fields employed should not be very great in terms of those which flow during the course of an action potential. According to the computation detailed in Methods, the maximum membrane current associated with fields of 0.32 v cm^{-1} and 0.64 v cm^{-1} should be 1.6×10^{-5} amp cm^{-2} and 3.2×10^{-5} amp cm^{-2}, respectively–small fractions of the peak current density of about 4×10^{-3} amp cm^{-2} occurring during an action potential (Hodgkin and Huxley, 1952). However, the membrane is apparently rendered irreversibly inexcitable when such currents flow for tens of minutes rather than milliseconds.

The block of action potential propagation cannot be attributed to accumulation of either electrode products or some (unknown) substance leaving the axon: substitution of fresh artificial sea water around a blocked axon did not restore propagation, nor was the conduction velocity of a new axon affected when it was bathed with the fluid which had surrounded a blocked axon.

Propagation block cannot have been the result of destruction of the axon by the increase in solution temperature owing to the Joules heating of the solution. The temperature in the vicinity of a blocked axon had risen by no more than 1°C.

the squid axon. It was not determined if action potential conduction in the lobster axon is irreversibly blocked by a maintained transaxial field.

The electrophoretic mobility of both squid and lobster axons eventually disappears completely, taking as long as a week to do so. This may be due to the cumulative effect of the fields to which the axons had been subjected, or it might occur simply as a concomitant to the inevitable complete physiological deterioration of an isolated axon.

Lobster Axon Mobility and Action Potential Conduction

The possible relationship between the surface charge of the lobster axon and its ability to propagate an action potential was explored by comparing the electrophoretic and electrophysiological effects of a variety of cations when added to Coles solution.

The chemicals selected for study were: $Th(NO_3)_4$, $LaCl_3$, and quinine-HCl. The relative effects of these on the extracellularly recorded action potential of unisolated giant axons of desheathed circumoesophageal connectives was measured at 4°C. At a concentration of 25 mM, $Th(NO_3)_4$ blocked propagation in 25 min (50 mM $NaNO_3$ had had no detectable effect after 100 min); 25 mM $LaCl_3$ had had no detectable effect after 5 hr (cf. Takata, Pickard, Lettvin, and Moore, 1966). Quinine-HCl blocked propagation within 33 min at a concentration of 5 mM (pH adjusted to 7.3). Thus, there was the opportunity to study the electrophoretic effects of large concentrations of two heavy metal ions, one blocking the action potential and the other not. The fact that quinine was a more potent blocking agent than Th^{4+} meant that the electrophoretic behavior of an inexcitable axon could be studied while in the presence of a much lower concentration of foreign cation.

The effect of Th^{4+}, La^{3+}, and quinine on the electrophoretic mobility of lobster axons was determined. No Coles solution measurements were made: the isolated axon was placed directly into the chamber filled with the modified Coles solution. This was done to avoid the possibly deleterious effect of prolonged exposure to electric fields which otherwise would have been necessary.

The results of these experiments are:

(*a*) When Th^{4+} is added to Coles solution at a concentration which blocks action potential conduction, there is no detectable electrophoretic movement of the axon (three axons in Coles solution plus 35 mM $Th(NO_3)_4$).

(*b*) When quinine-HCl is added to Coles solution at a concentration which blocks the action potential, the electrophoretic mobility is not affected (three axons in Coles solution plus 5 mM quinine-HCl, pH adjusted to 7.3).

(*c*) Electrophoretic movement is greatly diminished by a concentration of La^{3+} which does not affect action potential conduction (four axons in Coles solution plus 25 mM $LaCl_3$: three of these showed no detectable electrophoretic movement; that of the fourth was the same as for axons in Coles solution).

When interpretating this data, it must be remembered that the present method is not quantitative. Here, the failure of an axon to move in an electric field is not evidence that its shear surface is uncharged. All that can be said of the loss of electrophoretic mobility is that it indicates a diminution to below the level detectable by this method. Whether the surface charge of an immobile axon is positive, negative, or zero is not known.

The effects of Th^{4+}, La^{3+}, and quinine are evidence of three qualitatively different relationships between electrophoretic mobility and action potential conduction: diminution of mobility with action potential block (Th^{4+}), diminution of mobility without action potential block (La^{3+}), and action potential block without loss of mobility (quinine). There is thus no evidence of a relationship between electrophoretic mobility and the ability of a lobster axon to propagate an action potential. The interpretation of this finding in terms of the charge density of the shear surface is discussed below.

Effect of Resting Potential

The possibility of a dependence of surface charge and electrophoretic mobility on transmembrane potential was examined by determining the mobility of lobster axons immersed in Coles solution in which all the NaCl has been replaced by KCl—such a substitution should eliminate the resting potential of the axon. Measurements of the mobilities of three axons in the concentrated potassium solution revealed no obvious differences from that obtained for axons in Coles solution. Thus, there is no evidence that the properties of the shear surface are altered by the loss of resting potential, per se.

DISCUSSION

Prior to discussing the electrophysiological significance of these results, it is necessary to first interpret the electrophoretic mobilities in terms of the zeta-potential of the shear surface. When that has been done an estimate can then be made of the surface charge density and its possible consequences.

The relationship between zeta-potential and mobility depends upon the conductivity of the electrophoresing particle relative to that of the fluid medium (Henry, 1931, 1948; Booth, 1948). For a given zeta-potential, the effect of a finite conductivity of the particle is to reduce its mobility with respect to that of a perfectly insulating particle. For example, the mobility of a cylinder of (homogeneous) specific conductivity equal to that of the fluid medium, moving in a field applied perpendicularly to its long axis, is one-half that obtained when the conductivity of the cylinder is zero. When the conductivity of the particle is very great compared to that of the solution, the mobility vanishes. When the conductivity of the particle is small compared to that of the solution, the relationship between mobility, U, and zeta-

potential, ζ, is

$$U = D\zeta/4\pi\eta, \tag{2}$$

where D is the dielectric constant of the solution. This limiting relationship for insulating particles has been found to be applicable, theoretically (Henry, 1931) and experimentally (e.g. Abramson, Moyer, and Gorin, 1964), to particles of widely diverse sizes and shapes—in particular, cylinders whose radius and length are as large as those of axons.

In the case of an axon, the conductivity term in the zeta-potential–mobility equation would be a function of the specific conductivities of axoplasm, Schwann cell layer, endoneurium, etc. There appears to have been no theoretical treatment of such a complex situation; in any case, the respective conductivities of the various axonal surface layers are unknown. For the present, all that can be done is to treat the axon as a perfectly insulating cylinder and apply equation 2 to the mobility measurements to calculate ζ. Clearly, the axonal surface layers must be significantly conductive—if they were not, the large transsurface ionic movements which occur physiologically would not be possible. Thus, equation 2 necessarily leads to an underestimate—the lower limit—of the zeta-potential of the axonal shear surface.

As already noted, the experimentally observed mobilities are less than the true electrophoretic mobilities which would be obtained if the motion of the axons was not constrained as it is by the suspensory silk filaments. We shall therefore consider only the largest single value of U found for axons under normal conditions to calculate ζ from equation 2. This yields the *lower limit* of the estimate of ζ. The greatest mobility of a squid axon during its initial measurement in artificial sea water was $-2.6\ \mu\ \text{min}^{-1}\ \text{v}^{-1}\ \text{cm}$; that of a lobster axon in Coles solution was $-6.8\ \mu\ \text{min}^{-1}\ \text{v}^{-1}\ \text{cm}$. When the bulk phase values of the quantities in equaton 2 are employed ($D = 80$, $\eta = 1.57 \times 10^{-2}$ poise), it follows that ζ is at least -1.0 mv for squid axons; the minimum value of ζ of the lobster axon is -2.5 mv.

The density of fixed surface charges responsible for these zeta-potentials can be obtained from the Gouy-Chapman equation—the theoretical relationship between surface charge density, the concentration and type of ions within the solution phase, and the potential at the surface (e.g. equation 1, Haydon, 1961). When ζ is as small as in the present cases, it is equal to the surface potential (Haydon, 1960). Then, according to the Gouy-Chapman equation, the minimum density of charges affixed to the shear surface of the squid axon is -1.9×10^{-8} coul cm^{-2}; that of the lobster axon is -4.2×10^{-8} coul cm^{-2}. This is equivalent to one electronic charge per 290 $(\text{A})^2$ of squid axon shear surface and 200 $(\text{A})^2$ of lobster axon shear surface, respectively.

What could be the electrophysiological consequences of such a surface charge? First, the sign—negative—is that which would account (Teorell, 1953) for the fact that the rate of movement of cations between axoplasm and extracellular solution

is considerably greater than that of anions. This is not to imply that the shear surface must be the major diffusion barrier of the total axonal surface. Even if the shear surface contributed nothing to the total electrical resistance of the axon surface membranes, the structure as a whole could still behave as an ion-exchanger membrane. Anions would be excluded from the layer(s) bearing the negative charges and would thereby be prevented from passing into and out of the axoplasm. The total membrane complex would thus be cation permselective.

The sign of the surface charge is consistent with axonal cation permselectivity, but to decide if it is actually the cause requires a physicochemical characterization of the shear surface and underlying structure which is not presently available. This becomes evident in the following attempt to calculate the effect on intra-extracellular ionic transport of the estimated density of surface charges. For this purpose it is necessary to specify the volume density of fixed charges of the structure whose surface gives rise to the zeta-potential. If the distribution of charges throughout this structure is such that the charge density within any plane perpendicular to the shear surface is identical to that of the shear surface, then 1 cm^3 contains 6.6×10^{-3} coul in the case of the squid axon and 21×10^{-3} coul in the case of the lobster axon. If the structure bearing the charges is assumed to be completely accessible to the solution which contacts it—equivalent to assuming the structure to be volumeless—then the concentration of fixed charges would be 6.8×10^{-5} M for the squid axon and 22×10^{-5} M for the lobster axon. Such concentrations of fixed charges are orders of magnitude too small to account for the cation permselectivity found experimentally.

However, the assumption that the volume of the structure bearing the fixed charges can be neglected in calculating the volume charge density is completely arbitrary. How far the axon deviates from this idealization is, of course, not known. Any such deviation will *increase* the calculated volume charge density and the inferred enhancement of cation transport with respect to that of anions. Thus, until there has been an experimental determination of the effective volume of the structure supporting the shear surface, the computation of the previous paragraph cannot serve as a basis for rejecting the possible role of the shear surface fixed charges in determining the electrical transport properties of the axon.

An opportunity does exist for a test of the role of the shear surface in conferring ion-exchanger properties on the axon which does not require quantitative knowledge of the actual volume charge density. This is provided by the reversal of charge phenomenon found for the squid axon. If the shear surface is the cause of axonal permselectivity, then when it is positively charged the transmembrane flux of anions should be greater than that of cations—the axon should be anion permselective. If the permselectivity of the axon is reversed by fields which reverse the sign of the charge of the shear surface, it would be evidence of a physiological function of the surface charge.

Are the fixed charges of the axonal extracellular surface directly involved in the generation of the action potential? The present evidence is not sufficient to provide a definite, unequivocal, answer to this question. On the one hand, the seemingly concomitant action potential block and loss of electrophoretic mobility of the squid axon is suggestive of a direct relationship between surface charge and the ability of the axon to propagate an action potential. But, on the other hand, the cationic effects on propagation and mobility of the lobster axon suggest that surface charge and ability to propagate are not directly related. However, neither of these apparently contradictory results is, in fact, necessary proof of the respective interpretations.

On account of the unassessed effect of surface conductance, the field-induced diminution of electrophoretic mobility of the squid axon cannot be interpreted unambiguously. The loss of mobility may be due to: an increase in surface conductance, a decrease in surface charge density, both, or the combination of an increase of one with a decrease of the other. The loss of mobility is consistent with an alteration of surface charge, but to prove it so and determine its nature requires a measure of axonal surface conductance. The exact significance of the mobility loss in terms of the properties of the shear surface is, thus, not known; its implications regarding the electrophysiological function of shear surface charges cannot be decided for the present.

When foreign cations are added to Coles solution every possible relationship is found between lobster axon mobility and its ability to propagate an action potential. This finding suggests the absence of a causal connection between surface charge density and action potential conduction. However, the validity of this interpretation rests on the prior demonstration that surface conductance is not altered by the added cations. Only then is the mobility of an axon a measure of the effect of the cations on the surface charge density. The present lack of information about the lobster axon surface conductance prevents drawing an unqualified inference of the effect of the cations on surface charge. As in the case of the squid axon data, no unambiguous conclusion can be reached from these experiments about the possible function of lobster axon surface charge in the generation of the action potential.

Thus, further experimental information is required for a full assessment of the electrophysiological role of the negative charges of the shear surface. The discovery of an agent which reverses the sign of the mobility, and hence the sign of the surface charge, without affecting the action potential would be unambiguous proof of the absence of a causal connection between the sign of the surface charge and excitation. Alternatively, the finding that all agents which reverse the surface charge also block conduction would be strong evidence of the necessity of negative surface charges for electrophysiological activity.

The most intriguing of the present experimental results is the finding that squid axons reach a stage at which the sign of the charge of the shear surface can be reversed by transaxial current flow. This occurs about 20 hr after the time when the evidence suggests the axons had been rendered irreversibly inexcitable by the applied field. Nevertheless, this is not proof that reversal of surface charge is therefore electrophysiologically irrelevant. The fact that an axon is inexcitable does not imply a total degradation of its electrophysiological properties. Nor is there reason to be-

lieve that the attributes of an inexcitable axon are unrelated to those of an excitable one. Thus, the reversal of charge phenomenon may be a clue to the mechanisms underlying the action potential.

This possibility is suggested by the fact that the calculated magnitude of the transmembrane current associated with charge reversal is within the range occurring during normal physiological activity of the axon. Thus, the density and/or sign of the surface fixed charges may alter during the course of an action potential and be a cause of it. It may be that the positively charged axon found under the present, possibly extreme, experimental conditions is in a permanent form of a state which exists only transiently during normal electrophysiological activity.

This suggestion is necessarily speculative as it is unknown if surface charge density alters during the course of an action potential; or how such a change might be responsible for the electrophysiological behavior of the axon. However, it is clear from these results that transmembrane currents of physiological intensity can drastically alter a microscopic membrane property. Thus, the axonal surface is not an inert, passive, structure, but one whose intrinsic physicochemical attributes can be affected by the operative external forces.

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