BIOELECTRIC EFFECTS OF IONS MICROINJECTED INTO THE GIANT AXON OF LOLIGO*

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INTRODUCTION

The bioelectric phenomena of excitable tissues involve a complex series of events associated with ionic movements across the labile relative barrier of the cell which is termed the excitable membrane. Present day theories postulate that the resting potential is essentially determined by asymmetry in the distribution of different ions between the interior of the cell and its exterior, that asymmetry being produced at least partly by Donnan effect (17), and partly by specific, metabolically energized secretory activity (34, 46). The magnitude of the resting potential is believed to be complexly determined by the ratios of the distribution of K^+, Na^+, and Cl^- and by their effective mobilities (25). The sign of the potential is in the direction that would be determined by the tendency of the ion of greatest effective mobility (K^+) to diffuse from the more to the less concentrated phase (17).

Activity superimposes upon this electrochemical system an alteration of electromotive force which is determined by a characteristic response of the membrane. According to the theory of Hodgkin and Huxley (22) this response

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§ Fellow of the Henry L. and Grace Doherty Charitable and the John Simon Guggenheim Memorial Foundations (1950–52). Now Research Associate on Grant B-400 to Professor H. Merritt, from the National Institute of Neurological Diseases and Blindness.

is initiated by a relatively small decrease of the resting potential and causes striking, specific, and sequential alteration in the permeance of the membrane first to Na^+ and then to K^+. The influx of Na^+ acting upon the capacity of the axonal membrane causes a new, oppositely directed electromotive force which in turn affects that influx (21). When reversal of the membrane electromotive force increases as a consequence of flux of sodium ions in the direction of their concentration gradient, Na^+ permeance is diminished and halted by an "inactivation" process. After the increase in flux of Na^+, K^+ begins to flow outward and this flow outlasts the earlier Na^+ influx, restoring the potential to the resting value. Hodgkin and Huxley (22) have incorporated, with conspicuous success, a large number of experimental results into a semiempirical analysis in terms of these processes.

The specificity of the sequence of ionic processes disclosed in the response implies specific molecular alterations in the excitable membrane. Hodgkin and Huxley (22) offer suggestions regarding the nature and kinetics of such alterations in discussing the physical significance of the mathematical equations of their theory. Estimates of the maximum thickness of the excitable membrane (about 100 A) scarcely permit a structure of more than a few molecular layers. Furthermore, probably only a small part of the surface of the membrane is involved in the reactions which result in response (45). Within these dimensional limitations, which imply involvement of relatively few molecules, the distinction formerly sought between chemical and physical components of bioelectric activity loses its meaningfulness.

Evidence as to the nature of the bioelectric generators has depended heavily on the changes in activity produced by altering the ionic composition of the fluid surrounding the tissue. Thus, Hodgkin and Katz (25) were able to account for the dependence of the resting potential in the squid giant axon on the external K^+ (5) by adapting the "constant field" equation of Goldman (9). Data on resting potentials of muscle fibers (30, 36, 39) and myelinated frog nerve fibers (29) are in agreement with this analysis (17).

Alteration of the external ionic environment was also a principal tool in the explanation provided by Hodgkin and Katz (25) for the overshoot of the axonal spike (5, 18), as well as in the development of the theory of Hodgkin and Huxley (19–24) mentioned earlier. Other data (15, 41) which describe the impedance changes of squid axons during activity under various conditions of external environment can also be explained by Hodgkin and Huxley's formulation. Indeed, a phenomenon observed for the first time in the course of these experiments, the temporary rise in membrane resistance during the terminal phase of the spike, named (41) the "initial after-impedance" also appears in the calculations of Hodgkin and Huxley (22), though smaller than the measured value.

The microinjection technique (14), on the other hand, permits alteration

of the internal ionic milieu of the axon. In combination with electrical recording, it can therefore be used to study the effects on the bioelectric generators, both of rest and of activity, caused by internal changes of various ion species. The present report details the results of two series of experiments on this subject carried out in 1952 and 1953 at the Marine Biological Laboratory, Woods Hole.[1]

The experiments involved measurements of the effects of microinjected ions on the resting and action potential as recorded with inserted microelectrodes.[2] In some of the experiments (included in Table III, and illustrated in Fig. 10) two or three microelectrodes were inserted into the axon in order to determine the bioelectric changes at different distances from the site of injection, as well as at the latter. The ions involved were: K^+, Rb^+, Na^+, Li^+, Ba^{++}, Ca^{++}, Mg^{++}, Sr^{++}, and Cl^-, NO_3^-, HCO_3^-, $SO_4^=$, aspartate, and glutamate.

Methods

1. Preparation.—It is not necessary to remove the small fibers of the stellar nerve from the squid giant axon in order to insert micropipettes (14) or microelectrodes (47) into the latter. The axon is then less liable to damage which may be caused by the lengthy cleaning process, and can also be studied soon after removal from the animal, before significant alterations may have occurred in its ionic constituents (33).

The hindmost stellar nerve of the Woods Hole squid, *Loligo pealii*, or sometimes the next nerve forward, was placed upon a plexiglass carrier mounted on a mechanical stage. An array of Ag-AgCl wire electrodes was fixed on this carrier for stimulating and recording from the nerve (Fig. 1 *A*). Drying of the nerve was prevented by covering it with a layer of sea water. The volume of the external fluid was large compared with that of the axon and the fluid was frequently renewed. Thus, alterations in its composition, due to exchange with the axonal interior or due to evaporation were small. Pipettes and microelectrodes, maneuvered into position with micromanipulators, were then inserted into the giant axon under observation through a high power dissecting microscope. The experiments were carried out at room temperatures which ranged from 21–28°C.

2. Micropipettes and Microelectrodes.—The injection arrangement was of the Chambers type employing a non-expansible system (4). The injection micropipette also served as a microelectrode, the dual function being accomplished by using a plexiglass pipette holder with a side arm (Fig. 1 *B*). A 125 μ chloride-coated silver wire extended from the side arm through the front end into the lumen of the micro-

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[1] We wish to thank the Director and staff of the Marine Biological Laboratory for their cooperation and assistance in making this work possible.

[2] In agreement with general usage in electrophysiology, the microcapillary salt bridge between the interior of the axon and the external metallic electrode (in this case Ag-AgCl) is here called a "microelectrode." It may be pointed out that this usage, though not encouraged by electrochemists, is no more inconsistent than is that of the term "glass electrode."

pipette. The entire system was filled with air-free mineral oil up to the tip of the micropipette which was then placed in a drop of the solution to be injected. The solution, which had been previously colored by chlorophenol red (0.05 to 0.1 per cent final concentration) and filtered, was slowly taken up into the pipette by pulling on the syringe plunger. Provided the solution was relatively clean, no great difficulty was encountered in this process. The solution extended well into the shaft of the micropipette where it bathed and thus made electrical contact with the Ag-AgCl wire. The use of the indicator had a twofold purpose. The colorant permitted ob-

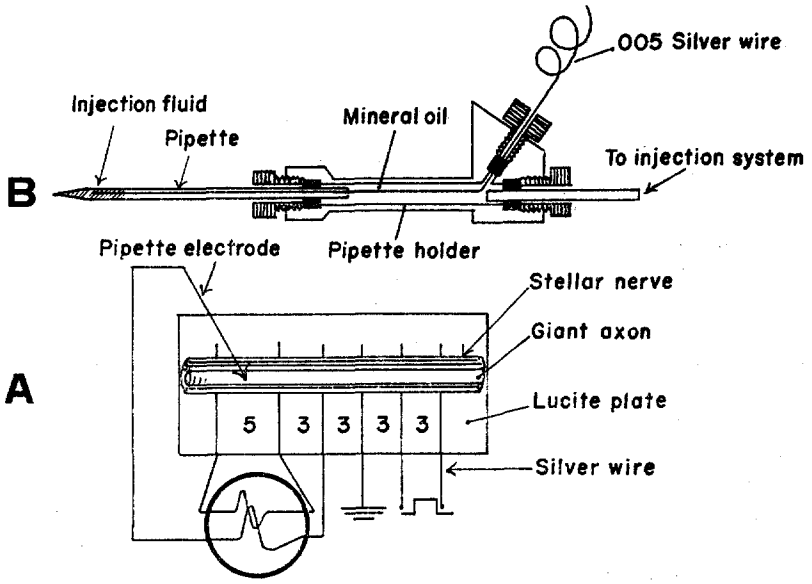

FIG. 1. Diagrams—*A*, of the stimulating and recording arrangement. Distance between wires indicated in millimeters, and *B*, of the combined micropipette-electrode. Not drawn to scale.

servation of the instant of fluid ejection from the pipette tip, its rate of flow, and the approximate length and region of the injection into the axon. In addition, the meniscus at the oil-electrolyte interface in the shaft of the micropipette was more easily visualized thereby so that its excursion could be measured with a calibrated ocular micrometer to indicate the amount of electrolyte injected into the axon. Additional microelectrodes for simultaneous multiple recording were drawn from the same type of capillary tubing as that used for micropipettes[3]. The capillary was first filled (as a closed system) with 3 M KCl (39) and the tip then formed on a pipette puller. This method of drawing "prefilled" electrodes (31) is more convenient than is that of

[3] The capillary tubing used had remarkably uniform dimension, an outside diameter of 0.8 mm. and a thin wall. It was furnished by Mr. James D. Graham, Haddonfield, New Jersey.

later filling by boiling the empty microcapillary in the electrolyte solution (36). Each microelectrode was mounted in a holder similar to that used for the pipette (Fig. 1 *B*), but without a connection to the syringe system. These holders were filled with 3 KCl and electrical connection with the amplifier input was made by a Ag-AgCl wire.

Insertion of the micropipette-electrode was either axially (5, 18) when a rather large shaftlet and tip opening could be used, or radially through the axonal membrane (47) when the pipette tip was of the order of 2 μ. The former method was used in an early series of these experiments. While the results were entirely similar, the radial impalement method proved more convenient and was used in all later experiments. The additional microelectrodes for multiple recording usually had tips of the order of 0.5 μ. However, a 2 μ tip diameter can be safely used as judged by the fact that resting potentials and spikes of high amplitudes were obtained with pipette-electrodes having tips up to 8 μ diameter and that the potentials remained steady up to 20 minutes after impalement (Fig. 3). The electrodes used in the present work had low resistance compared with the values given by Nastuk and Hodgkin (39) or other values in the literature, their resistance being only of the order of several megohms.

3. Amplifying, Recording, and Stimulating Systems.—In one series of experiments the amplifying and recording system was a dual channel oscillographic unit. The preamplifiers were highly differential, directly coupled, with symmetrical cathode follower inputs[4]. The input tubes were used as probes mounted close to the preparation, but because of the low resistance it was not necessary to minimize the electrode capacity as was done by Nastuk and Hodgkin (39). The frequency response of the system was measured in each experiment essentially as described by the aforementioned workers. Usually the electrodes attenuated a 0.1 msec. square pulse 5 per cent or less. The grid current of the input tubes was of the order of 10^{-11} amperes.

For the experiments using multiple microelectrodes each beam of the two-channel oscillograph was split into 2 by an electronic switch operating at 100 kc. This permitted simultaneous registration of as many as four independent traces. Each microelectrode fed into a new type of negative capacity amplifier[5]. The rise time of the recording system was 20 to 80 μsec.

The pipette-electrode was inserted into the axon in the region between a pair of external recording electrodes (Fig. 1 *A*). It was connected into the directly coupled recording system operating at a sensitivity of 2 mv./mm. at the tube face. The same recording sensitivity also applied to the additional microelectrodes when these were used. The external recording electrodes were led to a capacitatively coupled amplifier at higher gain (up to 40 μv./mm. at the tube face).

Initially the external and microelectrode recording systems were adjusted so that the oscillograph traces coincided when the pipette-electrode was in the sea water surrounding the axon. The beam coupled to the external electrodes thereafter served as the base line for the resting and action potentials recorded after insertion of the pipette-electrode into the axon (Figs. 1 *A*, 3, 4, 5, 10). The same procedure was also

[4] Designed in this laboratory by Mr. Ernest Amatniek.

[5] Designed by Mr. Amatniek, and to be described elsewhere.

used in the experiments with multiple microelectrodes. Checks were made at the end of each experiment for the drift of the directly coupled recording systems.

Stimuli were delivered to the axon through a low capacity circuit (40) from a sweep synchronized square pulse generator. Pulse durations were usually 0.1 msec., and the stimulus strength only sufficiently above threshold to give minimal latency. In addition to visual monitoring, continuous recording of the oscillograph traces was made with a Grass camera and a "slave" oscillograph, at a repetition rate of 1, 2, or 5 per sec., starting just before insertion of the pipette-electrode and continuing during and after injection of the axon. The beginning of injection and its end were reported by one of us who performed the injection, while another monitored the presentation of the electrical activity. A signal of the injection was also incorporated into the photographic records by a marking light.

4. Injection Technique.—An important factor for successful microinjection into the giant axon is the injection rate. A rapid gush of fluid from the micropipette may cause instantaneous and irreversible damage to the nerve fiber as evidenced by immediate block of propagation and decrease in resting potential. Slow injection, however, is well tolerated (Fig. 3). The rate of injection was measured in a number of experiments and averaged 0.01 to 0.03 $mm.^3$ of fluid per sec.

To make the data for effects of different ion species comparable we attempted in the first series of experiments (1952) to inject approximately comparable volumes of fluid (of the order of 0.1 to 0.5 $mm.^3$), and to observe the bioelectric effects for several minutes after the injection had ended. Therefore, the concentrations of the different injected electrolytes were adjusted in the course of the series of experiments, to produce their observed effects within this range of volumes. For example, K^+ was injected in solutions ranging from 0.2 to 2 M, but because, as will be shown later, increase of internal K^+ is relatively ineffective in altering the bioelectric properties, it usually exerted an action only in concentrations of 0.5 M and higher. For the purpose of the present experiments, therefore, injections without effect of large volumes of 0.2 to 0.4 M KCl served as control experiments in which as much as 10 $mm.^3$ of such solutions were sometimes introduced (Fig. 3, Table II).

In the series of experiments carried out in 1953 the aim was to study immediate effects of the ions. Therefore, the injection was usually continued until the spike recorded at the pipette-electrode had decreased below the level of the resting potential, an indication of incipient block (see Figs. 4 and 5). Although the techniques differed as described, the results of the two series were essentially similar (Table III).

To study the action of K^+ without the possible complication introduced by Cl^-, potassium aspartate and glutamate were used at pH 7.8 with, or without additional Cl^- (Table III). Na^+ was injected in various concentrations, also with Cl^-, glutamate, or aspartate; Rb^+ and Li^+ were injected as chlorides. Since the divalent cations exert their effects when only small quantitites are injected into the axon, these ions were used in low concentration (Table III) as the chlorides. Osmotic effects were then minimized in some of these experiments by appropriate concentrations of glucose, with or without additional 0.3 M KCl. The effects of other anions were studied by using KNO_3, K_2SO_4, and $KHCO_3$. The detailed compositions of the solutions are given in Table III.

It is useful to grasp the volume and concentration dimensions involved in the in-

jection of about 0.1 mm.^{3} of fluid into an axon. Most of the axons had diameters in the range of 400 μ. One millimeter length of such an axon has a volume of 0.125 mm^3. Injection of 0.1 mm.^{3} of electrolyte would therefore have filled completely a volume approximately corresponding to 1 mm. length of nerve which contains the following amounts of certain ions (calculated from reference 17): K^+ 2.1 μg., Na^+ 0.1 μg., Cl^- 0.17 μg., Ca^+ 2.5×10^{-3} μg.[6]

With injections of 0.1 to 0.5 mm.^{3} the colored electrolyte was usually seen to spread over at least 3 to 5 mm. of the axon, more or less symmetrically in front and behind the pipette tip. The initial variation in length and form of the column appears largely determined by the fibrillar structure of the axon as described by Chambers and Kao (3). The injection fluid first runs in elongated channels, and diffuses within a few seconds (unlike injected oil) through the cross-section of the region probably because it is freely miscible in the aqueous portion of the axon, which constitutes about 95 per cent of the interior (1). The change in electrolyte concentration produced in the axon by the microinjection may be calculated approximately. It will be shown later (Figs. 4 and 8) that 29.2 μg. of K^+ ions as 0.38 mm.^{3} of 2 M KCl acting over 2 min. caused propagation block. If, during this time the ion had been uniformly distributed through the volume enclosed by 5 mm. of the axon, the microinjection would have caused approximately a threefold increase in the internal K^+. Actually, in the region of the pipette tip, the concentration must have been higher. The boundary of the injected region also was probably more indefinite than has been assumed above.

RESULTS

1. Resting and Action Potentials before Injection

The experiments on injection of various ions were designed to study the effects of these upon the resting and action potentials. It is germane, therefore, to summarize the measurements of the potentials before these had been affected by microinjection. Altogether 542 measurements were made on 411 nerves.

(*a*) *The Resting Potential.*—The resting potential ranged from 27.5 to 68 mv. (Fig. 2 *A*, *C*). These values are uncorrected for theoretical "junctional" potentials. The mean value was 48.57 mv. and the mode 48.67 mv. (Fig. 2). At comparable temperatures Hodgkin and Katz (26) obtained an average of 45.5 mv. and Curtis and Cole (5) 51 mv. In our experiments, microelectrodes filled with electrolytes of very different concentrations all recorded resting potentials of about the same range of variation (Table III) contrary to expectations derived from theoretical considerations regarding effects of junctional variations (5, 25).

There seemed to be no direct correlation between the initial resting poten-

[6] The value for Ca^{++} is calculated on the basis of Hodgkin and Katz' (27) conclusion that the squid axoplasm probably contains 3×10^{-4} M Ca^{++}. The axonal concentration of Mg^{++} is unknown.

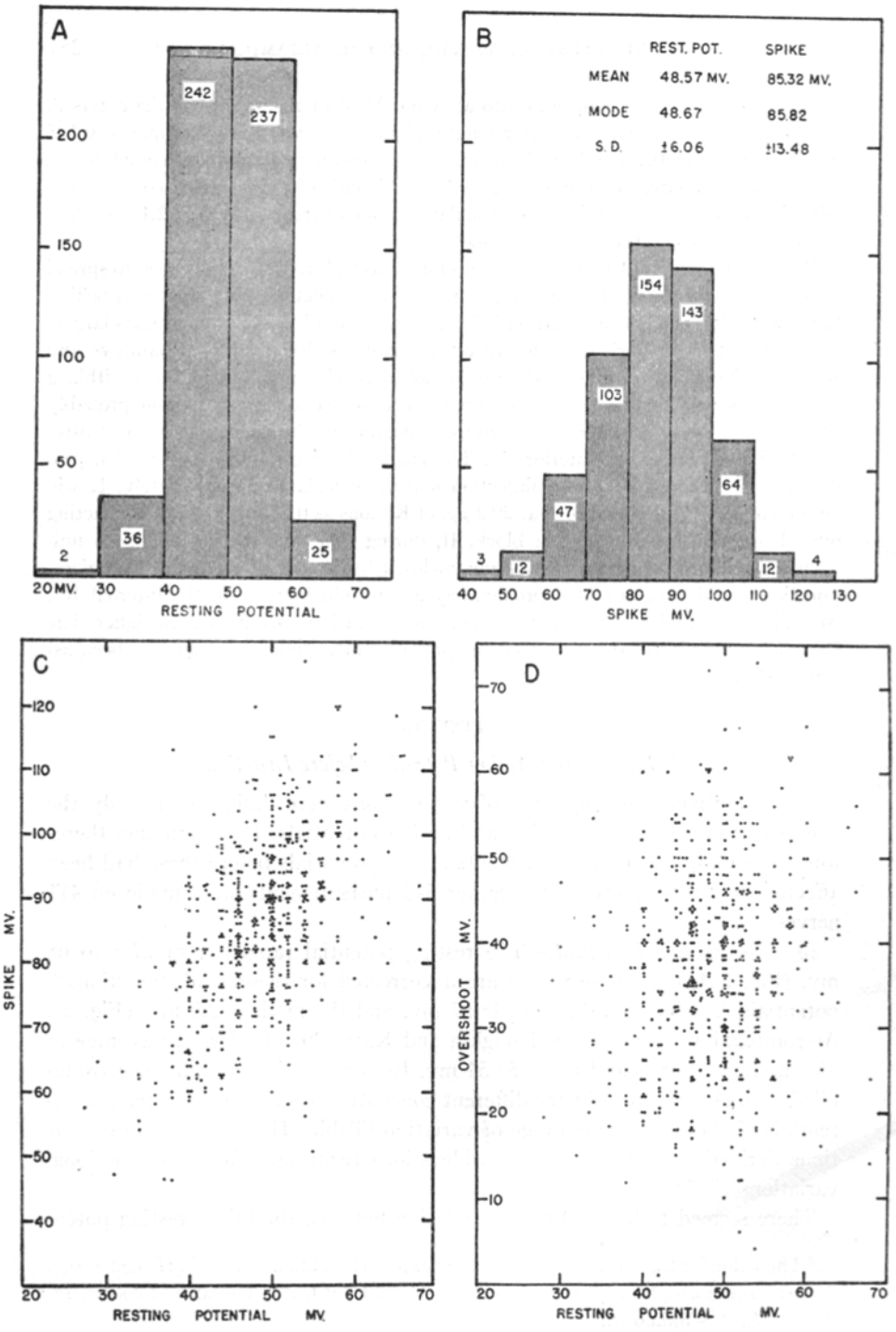

FIG. 2. *A* and *B:* Distribution of the measured values of resting potentials and spikes in 411 squid giant axons. *C* and *D:* Relation between spike amplitude and resting potential, and between overshoot and resting potential in 411 axons.

tial and the time elapsed after isolation. Most of the nerves in the present experiments were studied within 1½ hours after removal from the animal. Table I, however, shows the values of resting potential and spike height obtained from nerves which had been kept in sea water for many hours after dissection. The potentials of the paired nerve, examined immediately after removal from the animal, are also shown for two experiments. In a nerve surviving as long as 18 hours, the resting potential and spike were about as high as in the paired nerve studied when first excised.

(*b*) *The Spike Amplitude.*—This corrected when necessary—as noted earlier—for amplifier response, showed high variability (Fig. 2 *B*) and little cor-

TABLE I

Resting Potential and Spike Height in Squid Axons Kept in Sea Water for Various Times after Removal from Animal

Experiment No. (Series D 53)	Temperature	Axon diameter	Time	Resting potential	Spike amplitude
	°C.	μ	*hrs.*	*mv.*	*mv.*
176 paired	24	432	1	48	95.5
177 paired	23.8	456	18	46	87.6
198 paired	25	456	0:30	49	97.0
199 paired	21	444	12	54	97.0
202	22.5	408	5:10	47	108.0
165	26	408	5:10	45.5	98.5
166	26	456	5:40	53	99.0
150	25	480	12	44	94.0
195	25	576	12	52.5	103.5
151	25	430	13:25	54	98.0
152	26	430	13:40	61	104.0
187	24	480	14	46	88.6

relation with the resting potential (Fig. 2 *C*, *D*). Hodgkin, Huxley, and Katz (24, p. 432) discarded axons which had spikes smaller than 85 mv. Since our preparations were not subjected to the cleaning procedure required for the experiments of Hodgkin and his colleagues, and its possible source of damage, we have not felt justified in discarding low values of either resting or spike potentials. The ability of nerves with resting potentials below 30 mv. to produce propagated spikes should be noted.

The absolute values for the spike amplitude in our experiments ranged from 46 to 127 mv. (mean 85.32 and mode 85.82 mv., Fig. 2 *B*, *C*). At comparable temperatures Hodgkin and Katz (26) obtained a mean value of 74.5 mv. and Curtis and Cole (5) 104 mv. The overshoot varied in a range of nearly 70 mv. (Fig. 2 *D*). Hodgkin and Huxley (19, p. 455) report a variation of V_{Na} from 95 to 119 mv., or a range of 24 mv., despite the selection which had been exercised with reference to the potentials.

(*c*) *Spike Duration and Conduction Velocity.*—The extensive series of measurements also provides data on the duration of the internally recorded propagated spike. At the temperatures of the experiments, the duration from the onset of the spike to the time the falling phase crosses the base line varied from 0.35 to 0.5 msec. The positive potential lasted 4 to 5 msec. These values are in agreement with those of Hodgkin and Katz (25). The mean conduction velocity measured for a smaller series was 19.2 M. P. S. in satisfactory agreement with the more accurate measurements of Hodes (16).

2. Effect of Injecting an Inert Substance

A number of controls were carried out in order to ascertain that microinjection by itself does not significantly affect the resting potential or the spike. One particularly striking type will be illustrated here. The pipette-electrode was filled with 0.4 M glucose containing 0.3 M KCl as the electrolyte.[7] Fig. 3 shows the time course of the resting and action potentials, as recorded every half-second before, during, and after injection of a total of 7.3 mm.^{3} of the fluid. The entire axon was colored by the dye since the amount of injected fluid would have filled completely 5.6 cm. of a cylinder of axonal diameter (406 μ). The nerve was distended and turgid at the end of the experiment, yet the amplitudes of both the resting and action potentials remained unaffected during the 20 min. of the experiment.

Of specific interest for the present report is the fact that all the ions in the axoplasm except Cl^- were probably diluted by the injection. The Cl^- was increased approximately tenfold. In fifteen of twenty-three experiments, in which 2 to 10 mm.^{3} of 0.3 M KCl were injected, changes in the resting potential were absent or small (Table II). On the basis of the Donnan theory (reference 25, and Fig. 6) the resting potential in these cases would be expected to have decreased by at least 30 mv. The massive injections decreased spike amplitudes to various extents but did not block propagation. These control experiments involved injections 4 to 100 times greater in volume than the experiments testing the effects of ions. They also extended over periods 2 to 10 times longer than the test series. The small changes in the resting potential, when present, may therefore be ascribed to the severity of the control conditions. In the remaining eight control experiments involving injections of 2.6 to 7.9 mm.^{3} of fluid, resting potentials decreased within the 1st minute. These changes are ascribable to low tolerance to the nerves for the massive volumes of fluid. The control experiments therefore establish the validity of the microinjection technique as used in the test experiments.

[7] In this experiment the injection fluid also contained 0.05 μg./mm.^{3} of butyryl choline. In this concentration the drug had no effect on the axon.

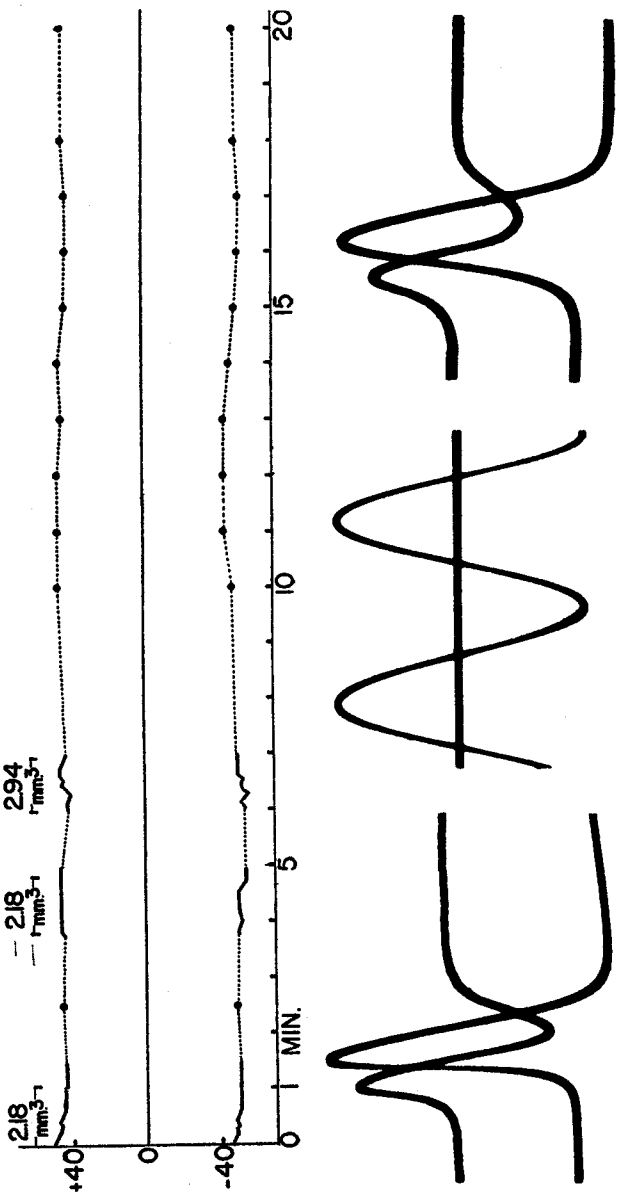

FIG. 3. Absence of effect on resting potential and spike amplitude after injection of 0.3 M KCl in 0.4 M glucose (Experiment D-53-206, temperature = 24°C., 406 μ fiber). *Above:* The upper line of the graph shows the spike amplitude (about 40 mv. overshoot) during 20 minutes of observation. Times and volumes of the three injections are indicated above this. The lowest line shows the resting potentials during the course of the experiment. *Below:* The spike recorded by the microelectrode is the large deflection, that by the external electrode is the smaller, diphasic response recorded on the zero potential base line of the internal electrode. The responses at the beginning of the experiment are shown on the left and at the end, on the right. Changes in duration of the spike such as those seen here were produced by a variety of experimental conditions. Calibration 100 mv. and 1000 c.p.s. Pipette tip 5 μ diameter. Injection rate averaged 0.03 mm.^{3}/sec. Spike amplitude not corrected for amplifier response.

3. Changes in the Spike during and after Injection

Samples of the bioelectric changes occurring during the course of an experiment with injection of 2.0 M KCl are shown in Fig. 4. The same experiment is detailed in the curves of Fig. 8. Records *A* to *C* of Fig. 4 show the resting and action potentials before, at the beginning, and end of an injection which introduced into the axon 28 μg. of K^+ as 0.36 mm^3 of 2.0 M KCl. As the internally recorded spike decreased in height the second phase of the externally recorded response was slightly delayed. The delay was reflected in a broadening of the

TABLE II

*Effects of Massive Injection of 0.3 M KCl with 0.4 M Glucose**

Experiment No. Series D 53)	Axon diameter	Injection volume	Duration of experiment	Resting potential		Spike amplitude	
				Initial	Final	Initial	Final
	μ	$mm.^3$	*min.*	*mv.*	*mv.*	*mv.*	*mv.*
66	432	2.9	20	47	40	97	90.5
80	384	4.14	9	44	40	98	78.5
121	480	4.30	7	46	44	92.5	92.0
159	384	3.75	11:45	53	46	99	80.5
164	504	2.20	8	59	50	96	83.0
176	432	2.18	8:45	48	43.5	95.5	83.5
189	408	3.76	4:30	46	40	91	78.0
199	444	10.1	20	54	48	97	80.0
200	648	7.53	17:15	53	44	100	76.0
201	432	2.18	24	52.5	48	108.5	103.0
203	456	2.18	18	50	50	110.5	88.0
204	528	2.18	20	50	50	104.2	106.0
206	406	7.30	20	46	49	103.2	101.2
207	384	8.55	14	46	41	93	81.0
209	480	6.79	12	50	48	98	94.0

* The solutions used in these experiments also contained ineffective concentrations of prostigmine, eserine, butyryl choline, or acetylcholine. Spike amplitudes corrected for amplifier response when necessary.

peak of the internal spike (*D* and *E*) and was manifested more clearly still by formation of a notch (*F* and *G*) designated as the delayed spike (d.s.) in the curves of later figures. The notched responses are similar in appearance to the records obtained in incipiently blocking medullated nerve fibers (*e.g.* reference 43). As the delayed spike moved later (*H* to *J*) it also appeared, but somewhat earlier, on the second phase of the diphasic external response. The time difference may be due to the delay line of electrotonic propagation from the distal spike to the site of the microelectrode. It is, however, also possible that decrementing activity propagated through the site of injection was unable to excite part of that altered axonal region while its local circuit was still able to

cause full response in a more distal, unaffected site. The more powerful local circuit of the latter might then have initiated activity retrogradely in the previously unexcited zone of the injection site, and this would appear as a delayed response recorded by the internal electrode.

When the experimental conditions permit slow onset of block, as in Fig. 4, it is seen that the latter occurs only when the amplitude of the transmembrane spike has fallen approximately to the magnitude of the resting potential. Block of propagation is signalized by the disappearance of the delayed spikes

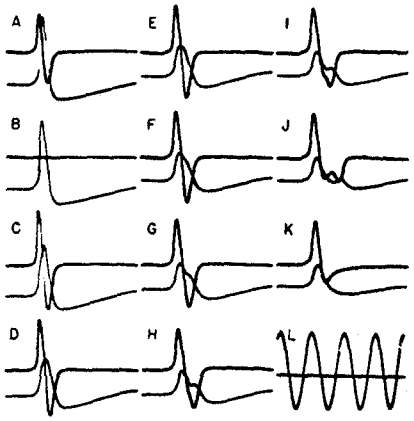

FIG. 4. Effects of injecting 2 M KCl (Experiment 52-163, also illustrated in Fig. 8). The first injection (1.2 μg./K^+) had practically no effect as seen in Fig. 8. The responses before, at the beginning, and at the end of the second injection (0.36 mm.^{3}) are shown in *A* to *C*. In *D* (7 sec. after *C*) the internally recorded spike has a double peak, which is more pronounced in *E* (15 sec. after *C*). Records *F* to *K*, made 30 sec., to 45 sec., 1 min., 1 min. 30 sec., 1 min. 45 sec., and 1 min. 47 sec. after *C*, are illustrative samples from the sequence at 2/sec., lasting more than 2 min., on which Fig. 8 is based. Calibration 100 mv. and 1000 cycles.

(*K*), but frequently, as in this record, the externally recorded spike exhibits, and for a long time, a diphasic artifact which indicates that local activity still persists in the interelectrode stretch of the axon. This is also demonstrated by the persistence at the microelectrode of a potential of the order of 30 mv. When recovery from propagation block occurs, the delayed spike reappears, at first late in the response, then moves earlier, and eventually may merge with the initial response which has increased in amplitude. These changes are at the same time reflected in the second phase of the external spike.

4. Accuracy of Detection of Local Bioelectric Changes with the Microelectrode

The axon both at rest and during activity represents continuously distributed parallel generators. It is therefore necessary to examine whether the

potential recorded by the microelectrode is falsified because the electromotive forces of the axonal generators in the region affected by the microinjection are swamped out by the parallel electromotive forces of the adjacent unaffected generators. This is probably not the case for the experimental conditions of the present work.

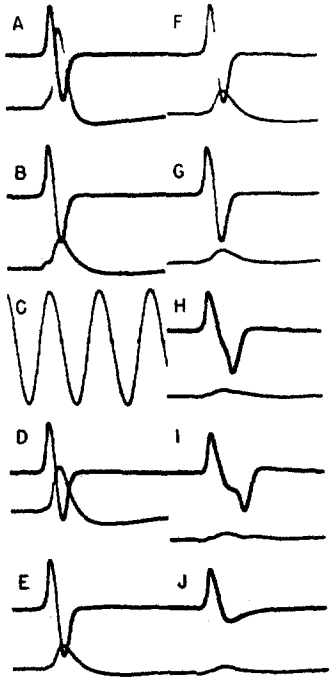

FIG. 5. Local changes in potential recorded by the internal microelectrode (Experiment 52-166, temperature 25.5°C., 460 μ fiber). Injection fluid 30 μg./ml. mytilon. The internal spike (*A*) was sharply decreased in 3 sec. by injection of 0.05 mm.^{3} of fluid (*B*). *D*, 4 min. 45 sec. later when a second injection introduced 0.008 mm.^{3} *E*, 5 sec. after the end of this injection. *F*, 1 min. 30 sec. later, when a third injection began and introduced 0.58 mm.^{3} in 1 min. *G*, at 30 sec. and *H* at end of the injection. *I* and *J*, 2 and 3 sec. later. Calibration in *C* 100 mv., 1000 cycles. The small initial deflection of the internally recorded spike is due to the use of the proximal external recording electrode as the reference for the internal electrode.

(*a*) In nearly all the experiments reported here, the injections were made in volumes not smaller than 0.1 mm.^{3} and up to 10 mm^3. Thus the length of nerve affected was at least 1 mm. and usually much more.

(*b*) The injections of the experiment illustrated in Fig. 3 (7.3 mm.^{3}) would have filled completely a 5.6 cm. cylinder of axonal diameter. Thus the axonal ionic composition had been radically altered in this experiment in the entire

nerve approximately symmetrically about the tip of the microelectrode. Since the length constant of the squid axon in sea water is about 5 mm., the maximum contributions of some "unaffected" parallel generators several centimeters away from the recording site would therefore have been very small indeed. It will be shown (Fig. 6) that on the basis of present theory the resting potential in the experiment of Fig. 3 should have decreased by about 30 mv. However, as seen from the figure there was no change in the resting potential.

(*c*) Many experiments with injection of various drugs produced results illustrated in Fig. 5. An initial injection of 0.05 mm.[3] of mytolon chloride[8] (30 μg./ml.) probably involved no more than 0.5 to 1 mm. of the 460 μ axon. Within 3 sec., when diffusion could not have been appreciable, the spike had decreased by 65 per cent (*B*). Nearly 5 min. later, after some recovery of spike amplitude (*D*), a second injection added only 0.008 mm.[3] of fluid. Within 5 sec. (*E*) the internally recorded spike had decreased further, though the length of nerve affected by the added substance must have been of the order of 0.1 mm.

As seen from the upper traces of the records in Fig. 5, the velocity of propagation through the injected region was unchanged (records *A* to *F*). This result is to be expected if the effects of the injection were localized to a region approximately 1 mm. long. When propagation block was developing as a result of a larger injection the response originally observed by the internal electrode (*H*) was less than 10 per cent of the initial spike height. At the same time, the late electrotonically recorded reflection of the delayed propagated response corresponded to less than 5 per cent of the initial internal activity (*H* and *I*). When propagation was blocked (*J*) the internal electrode still recorded a small response in the tempo of the initial spike. This remnant activity must therefore be interpreted as the diminished local response of the blocked nerve in the region of the microelectrode.

(*d*) The experiments with impalement of the axon by two or three microelectrodes furnish convincing proof of the ability of the electrodes to record different amplitudes of resting potentials and spikes in nearby regions of the axon (Fig. 10 and Table III).

5. Effects of Microinjection on the Potentials

(*a*) *Theory.*—

The basis of the resting potential in terms of the asymmetric distribution of ions between the axon and its external fluid has been described above. The equation used by Hodgkin and Katz (25) permits an approximate calculation of the changes in the resting potential which might be expected to occur if the internal ionic composition were altered with respect to various ions. Such

[8] Kindly supplied by Winthrop-Stearns, Inc. Mytolon is 2,5-bis-(3-diethylaminopropylamino)-benzoquinone-bis-(benzyl chloride).

calculations with reference to the internal concentration of K^+ are shown in Fig. 6.

The curves were obtained on the basis of the initial ionic concentrations and the coefficients used by Hodgkin and Katz but for a temperature of 25.5°C. The figure expresses the theoretical changes of the resting potential in terms of the relative increase or decrease of internal K^+ produced in several ways. Injection of 2 M potassium

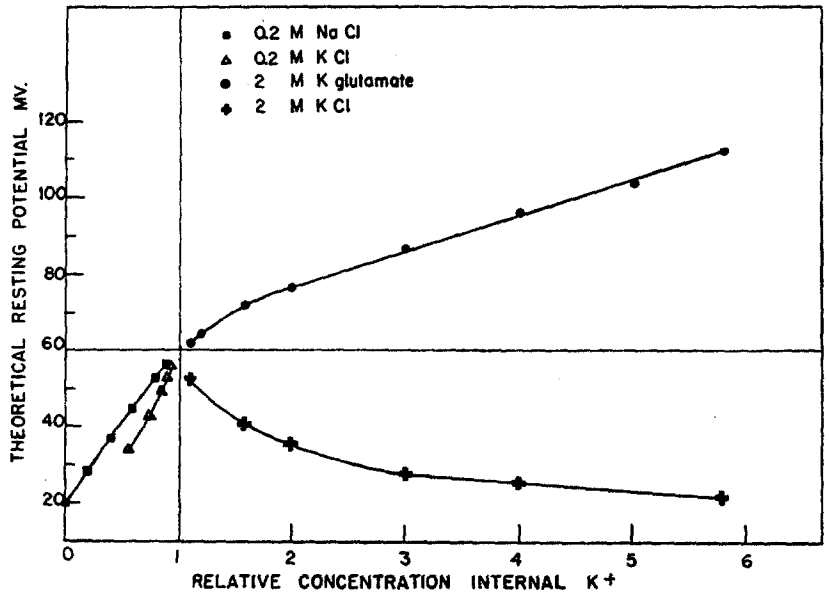

FIG. 6. Changes expected in the resting potential on changing the internal concentration of K^+ by injection of different solutions, as described in the text. The calculations are based on the equation (21)

$$\text{Resting potential} = 59 \text{ mv} \cdot \log_{10} \frac{(K_i^+) + 0.04(Na_i^+) + 0.45 \cdot 540}{10 + 0.04 \times 455 + 0.45(Cl_i)}$$

glutamate or aspartate would increase K^+, but dilute Na^+ and Cl^-. Injection of 2 M KCl, on the other hand, would simultaneously increase Cl^-. Injection of 0.2 M KCl would dilute K^+ while increasing Cl^-, but injection of 0.2 M NaCl would dilute K^+ even more, at the same time increasing Na^+ and Cl^-. The terminal points of the four curves of Fig. 6 represent the resting potentials that should be attained when the axonal ionic composition becomes that of the respective injection fluids.

It is seen from Fig. 6 that doubling K^+ by injecting 2 M potassium glutamate should, on the basis of Donnan theory, increase the resting potential by 17 mv., while a similar injection of 2 M KCl should decrease the potential by 24 mv. Massive injection of these solutions should cause nearly a fivefold increase

in internal K^+ with the resting potential increased by 53 mv. in the first case and decreased by 38 mv. in the second.

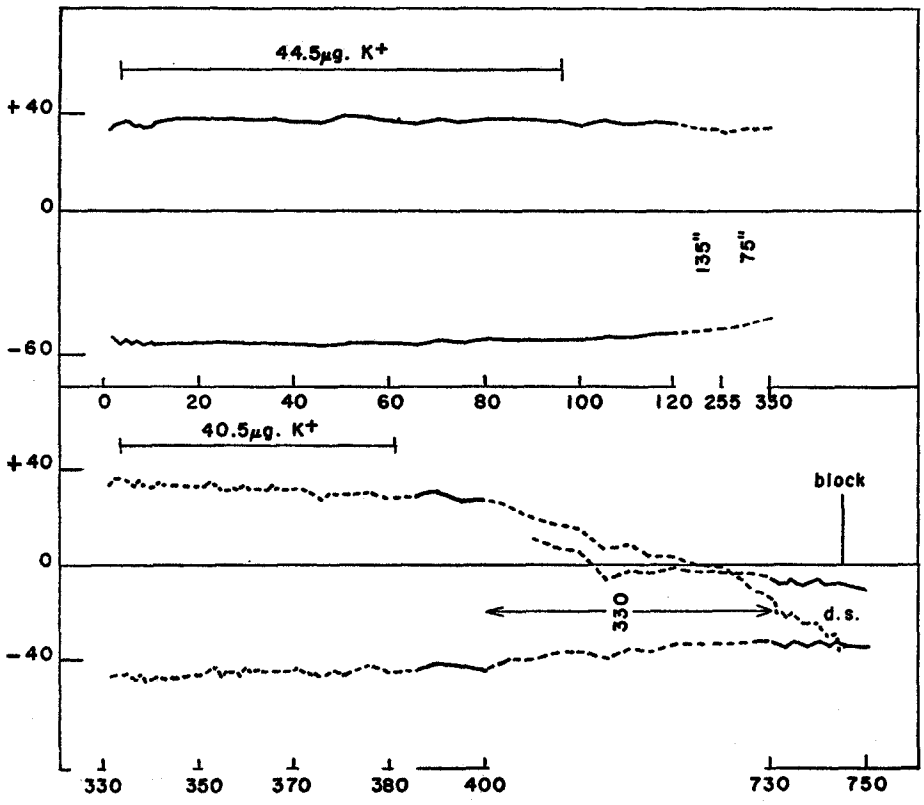

FIG. 7. Effect of injecting 1.3 M potassium aspartate in 0.2 M KCl (Experiment 52-189, temperature 27.5°C., D = 460 μ). During the first injection of 0.75 mm.^{3}, containing 44.5 μg. K^+, the spike increased by 4 mv. The resting potential remained unchanged, but after 3 min. had elapsed, diminished by 10 mv. Small progressive decrease in both spike and resting potential occurred during the second injection of 0.5 mm.^{3} or 40.5 μg. K^+ (lower part of the figure). Block occurred 6 min. after the end of the second injection. The line marked d.s. represents the appearance and amplitude of the small delayed spike illustrated in Fig. 4 and described in text. The time scale is drawn only in the early portion of the figure, interruption in the time base indicates changes in scale.

(*b*) *Observed Effects.*—

A summary statement of the results will simplify the presentation of the rather large number and variety of experiments.

Effects on Resting Potential.—(*i*) The resting potential was found to be in-

sensitive to ionic changes which might be expected either to raise (*e.g.*, as by injection of potassium aspartate) or to lower (*e. g.*, as with KCl or NaCl) this potential (Figs. 3, 7 to 9, and Table III).

(*ii*) The sequence of bioelectric changes which did occur was essentially similar no matter what the ionic alterations of the interior were. These changes first involved a decrease in spike amplitude. When the latter had fallen nearly

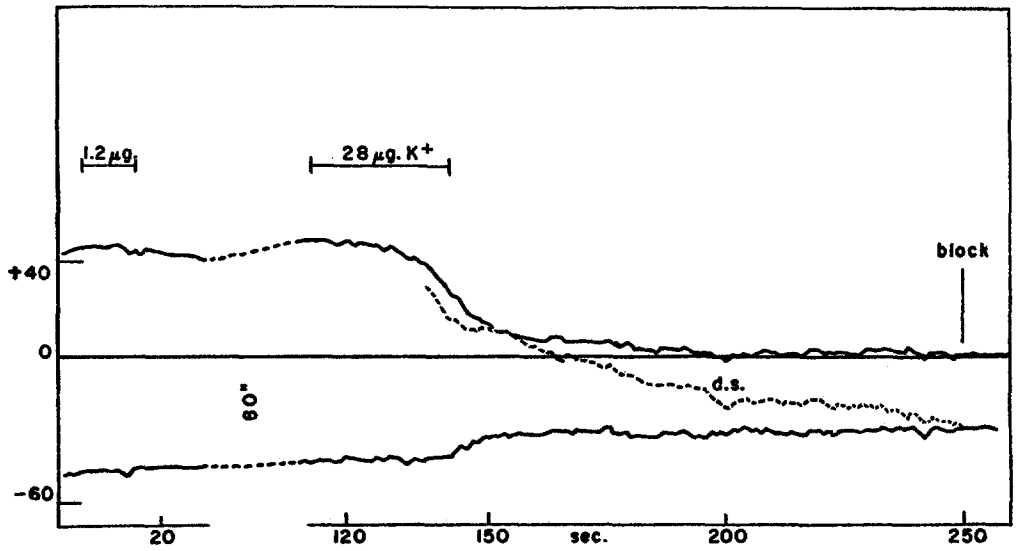

FIG. 8. Time course of changes in resting and action potential of the giant axon on injecting 2 M KCl (Experiment 52–163, temperature 25.5°C., 340 μ fiber). The first injection was 0.016 mm.^{3}, containing 2.3 μg. KCl or 1.2 μg. K^+. This had no effect. The volume of the second injection delivered in 30 sec. was 0.36 mm^3 containing 53 μg. KCl or 28 μg. K^+. The spike began to decline at the 18th sec. during the injection, but the resting potential remained unchanged for another 13 sec. Block as recorded at the distal external electrode occurred about 110 sec. after the end of the injection. Other details as in Fig. 7.

to the level of zero overshoot, and propagation block was incipient, the resting potential decreased (Figs. 7 to 9 and Table III).

(*iii*) At block the resting potential usually diminished by 30 to 50 per cent of the initial value, although more profound depolarization occurred with microinjections of high concentrations of some ions (Table III).

(*iv*) The decrease in resting potential was usually largest at the site of injection (Table III and Fig. 10).

Comparisons of the Action of Different Ions.—(*i*) K^+, injected with a variety of inorganic and organic anions, was relatively ineffective in producing the

bioelectric changes described above. This is seen in the detailed data of Table III and in summary form in Table IV.

(*ii*) A few experiments indicated that Rb^+, injected as the chloride, had similar ineffectiveness (Tables III and IV).

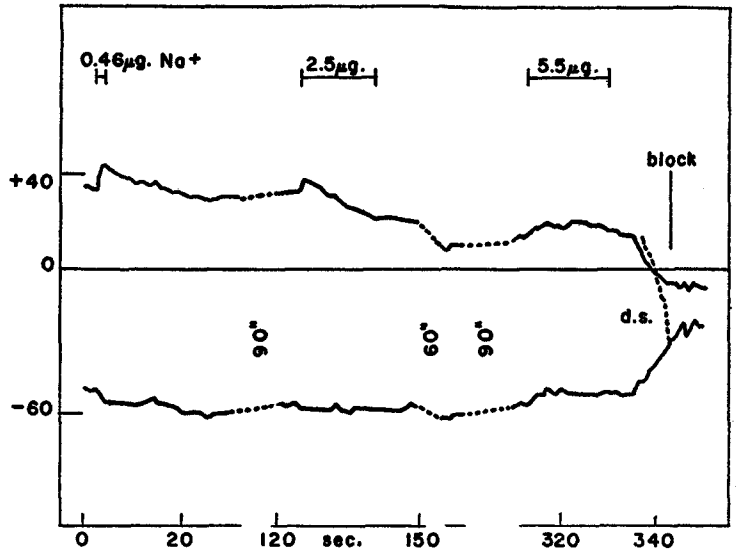

FIG. 9. Effects of injecting 2 M NaCl (Experiment 52-170, temperature 25.5°C., D = 410 μ). At the beginning of the first two injections spike height increased somewhat (10 mv. and 6 mv. respectively). The first, brief injection (0.01 mm.^{3}, containing 0.46 μg. Na^+) was accompanied by a small increase in the resting potential. The larger second injection (0.06 mm.^{3} containing 2.5 μg. Na^+) caused a decline of the spike, after the initial increase, but no change in the resting potential. A third injection of 0.12 mm.^{3} (5.5 μg. Na^+) eventually caused propagation block. The resting potential which had returned to the original value rapidly decreased to a very low level as block developed.

(*iii*) Na^+, injected with chloride, glutamate, or aspartate, was 5 to 10 times more effective in decreasing spike height and resting potential than were K^+ or Rb^+ (Fig. 9, Tables III and IV).

(*iv*) Li^+, injected as the chloride, appears to be as effective as Na^+ (Tables III and IV).

(*v*) The divalent cations Ba^{++}, Ca^{++}, and Mg^{++} are much more potent (Tables III and IV). Sr^{++} appears to be less effective, but this matter has not been further examined.

(*vi*) The anions used (Cl^-, HCO_3^-, NO_3^- SO_4^-, glutamate, and aspartate) do not seem to contribute any marked specific effects on the bioelectric potentials (Tables III and IV).

TABLE III

Bioelectric Effects of Various Ions Microinjected into Giant Axons

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		mm.³	$\gamma m. \times 10^{-6}$		mv.	mv.	mv.
KCl (0.5 M K^+)									
52-138	26	374	H_2O	0.54	10.5		80	50	30
2.0 M									
52-162	25.5	340	H_2O	0.1	7.8		88	54	26
-163	25.5	340	"	0.38	29.2		94	52	30
-165	25.5	442	"	0.6	46.6		87	48	27
2.56 M									
53-31	24.2	417	H_2O	0.62	61.7	II (1.4)	102	54	44
						III	92	60	60
-32	25.0	430	"	0.52	52.4	II (1.55)	106	64	42
						III	90	56	44
-33	24.2	442	"	0.41	41.0	II (3.84)	102	56	28
						III	90	50	42
K H CO_3 (2.1 M K^+)									
53-93	27.0	540	0.1 M KCl	0.44	36.	I (2.7)	70	48	32
						II (3.6)	74	54	34
						III	80	50	32
-94	27.0	447	"	0.26	21.6	I (2.3)	74	54	34
						II (3.7)	72	47	30
						III	82	44	36
-95	27.5	442	"	0.36	29.6	I (2.3)	80	50	32
						II (3.0)	76	50	26
						III	76	44	26

* When the experiment involved insertion of two or three electrodes, II indicates the pipette, I, the electrode proximal, and III, distal to the stimulating electrodes. Distances between the electrodes are given (in millimeters) in parentheses.

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		$mm.^3$	$gm. \times 10^{-6}$		mv.	mv.	mv.
			KNO_3 (2.1 M K^+)						
53-107	26	540	0.1 M KCl	0.73	56.7	I	90	54	28
						(3.7)			
						II	90	54	16
						(3.2)			
						III	86	48	26
-108	26	491	"	0.37	29.0	I	72	48	26
						(1.8)			
						II	82	48	*ca.* 6
						(2.8)			
						III	82	46	20
			K_2SO_4 (0.7 M K^+)						
53-103	25	491	0.1 M KCl	0.80	22.6	I	92	44	22
						(1.5)			
						II	92	50	26
						(2.5)			
						III	84	50	34
-104	25	344	"	0.97	27.3	I	74	50	30
						(1.9)			
						II	90	50	30
						(3.2)			
						III	74	50	30
			(1.54 M K^+)						
53-105	25	518	0.1 M KCl	1.96	118	I	90	50	34
						(2.3)			
						II	92	48	30
						(3.2)			
						III	82	50	30
-106	25	442	"	1.96	118	I	86	50	40
						(1.3)			
						II	90	50	30
						(3.1)			
						III	82	48	34

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		$mm.^3$	$gm. \times 10^{-6}$		mv.	mv.	mv.
Potassium glutamate (0.3 M K^+)									
53-85	27	490	H_2O	1.36	16	I	72	41	34
						(2.24)			
						II	76	34	30
						(4.05)			
						III	70	42	34
53-86	27	492	H_2O	1.4	16.5	I	58	54	38
						(2.8)			
						II	84	50	34
						(3.5)			
						III	70	46	34
-87	26	470	"	1.47	17.3	I	92	46	40
						(2.7)			
						II	90	46	20
						(2.0)			
						III	88	50	40
(0.53 M K^+)									
53-88	26.5	418	0.1 M KCl	1.14	19.1	I	86	46	36
						(3.7)			
						II	88	46	36
						(2.3)			
						III	60	50	42
-89	27.0	442	"	3.38	55.8	I	88	46	38
						(3.4)			
						II	92	50	40
						(5.4)			
						III	90	58	34
(2 M K^+)									
53-152	26	354	H_2O	0.1	8.0		62	42	42
-153	26	425	"	0.18	13.6		56	54	52
-158	27	510	0.02 M NaCl	0.29	22.6		82	48	30
-159	27	425	"	0.29	22.6		82	53	36
-160	27	370	"	0.27	20.6		83	53	34
-161	27	—	"	0.58	45.6		80	54	30

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		mm.³	gm. × 10^{-6}		mv.	mv.	mv.
Potassium aspartate (0.23 M K^+)									
53-77	26.5	418	H_2O	1.9	17.1	I	70	52	36
						II	74	46	16
						III	66	40	38
-79	26.5	590	"	0.4	3.6	I (2.76)	80	56	42
						II (3.4)	72	54	26
						III	82	56	46
-80	28.0	492	"	0.4	3.6	—	—	—	—
-83	26.5	370	"	0.53	4.8	I (2.89)	76	42	30
						II (3.53)	64	46	24
						III	78	46	32
-84	26.5	565	"	1.54	13.8	I (3.6)	86	48	34
						II (2.1)	64	52	28
						III	80	50	32
(1.5 M K^+)									
52-187	27.5	400	0.2 M KCl	0.4	23.9		80	50	39
-188	27.5	510	"	0.8	48.0		86	54	39
-189	27.5	460	"	1.3	75.8		90	52	36
Rb Cl (1.17 M Rb^+)									
53-58	26	442	H_2O	0.67	67.1	I (2.2)	83	46	30
						II (4.4)	77	40	30
						III	65	46	37
-59	26.2	418	"	0.72	72	I (2.5)	85	45	33
						II (3.7)	85	50	28
						III	85	51	47
-60	26.2	442	"	0.60	60.5	I (2.9)	75	43	36
						II (4.3)	80	45	30
						III	86	52	52

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		$mm.^3$	$gm. \times 10^{-6}$		mv.	mv.	mv.
Na Cl (0.43 M Na^+)									
53-37	24.5	344	H_2O	0.67	6.9	II (2.6)	90	54	—
						III	92	52	—
-38	25.0	344	"	0.13	1.3	II (1.0)	102	60	48
						III	91	54	46
-39	25.0	246	"	0.9	9.0	II (1.6)	80	62	53
						III	80	52	40
(0.5 M Na^+)									
52-155	26	357	H_2O	0.19	2.2		71	50	38
-156	26	391	"	0.48	5.6		112	66	54
-157	26	460	"	0.28	3.2		92	68	—
(2.0 M Na^+)									
52-168	25.5	458	H_2O	0.1	4.6		78	52	40
-169	25.5	510	"	0.13	6.0		84	52	39
-170	25.5	408	"	0.19	8.8		84	52	30
(4.34 M Na^+)									
53-34	25	467	H_2O	0.16	16.7	II (2.1)	100	56	44
						III	100	50	38
-35	25	295	"	0.11	11.0	II (1.3)	91	46	36
						III	92	49	36
-36	25	270	"	0.03	3.2	II (1.0)	58	52	37
						III	68	52	36

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		mm.³	gm. × 10^{-6}		mv.	mv.	mv.
Sodium glutamate (0.51 M Na^+)									
53-90	24.5	419	0.1 M NaCl	0.7	8.3	I	82	42	38
						(3.1)			
						II	84	44	40
						(2.7)			
						III	84	44	40
-91	24.5	541	"	1.3	15.0	I	86	48	32
						(2.9)			
						II	82	48	24
						(4.9)			
						III	82	50	30
-92	24.5	540	"	2.1	24.0	I	92	52	36
						(2.9)			
						II	90	50	30
						(3.3)			
						III	91	48	38
(1.0 M Na^+)									
53-68	26.5	442	0.1 M NaCl	0.15	3.5	I	66	40	40
						(1.0)			
						II	80	44	34
						(2.2)			
						III	84	46	46
-69	26.5	440	"	0.77	17.6	I	72	44	34
						(2.1)			
						II	70	50	24
						(2.9)			
						III	76	40	34
-70	25	490	"	0.7	16.0	I	80	46	38
						(2.6)			
						II	70	44	28
						(1.3)			
						III	70	40	32
-71	26	308	"	0.17	4.0	I	78	42	38
						(2.4)			
						II	66	40	22
						(2.0)			
						III	64	40	34
-72	26	368	"	0.12	2.7	I	76	52	44
						(3.5)			
						II	82	48	—
						(3.2)			
						III	80	40	40

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		$mm.^3$	$gm. \times 10^{-8}$		mv.	mv.	mv.
(2.2 M Na^+)									
52-186	26.5	442	0.1 M NaCl	0.12	5.6		80	56	26
Sodium aspartate (0.11 M Na^+)									
53-73	27	540	0.1 M NaCl	1.04	2.7	I (1.8)	82	40	38
						II (1.8)	80	38	26
						III	64	50	50
-74	27	565	"	0.97	2.5	I (2.5)	74	40	38
						II (1.7)	72	42	24
						III	64	46	40
-75	27.5	470	"	1.05	2.7	I (2.3)	76	44	38
						II (3.0)	80	—	30
						III	84	48	48
-76	27.5	394	"	0.92	2.4	I (2.2)	82	42	40
						II (3.0)	76	40	30
						III	86	52	44
(2.31 M Na^+)									
52-190	27.5	475	0.1 M KCl	0.38	20.1		87	50	42
-191	27.5	425	"	0.41	22.1		86	54	46
Li Cl (1.44 M Li^+)									
53-54	25	470	H_2O	0.4	4	I (1.7)	92	52	42
						II (—)	86	64	30
						III	82	—	—

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		*mm.*³	*gm.* × 10^{-6}		*mv.*	*mv.*	*mv.*
Li Cl (1.44 M Li^+)—*Continued*									
-55	25	392	H_2O	0.27	2.7	I	84	54	38
						(2.1)			
						II	90	44	16
						(1.5)			
						III	92	56	34
-56	26	418	"	0.63	6.3	I	92	56	38
						(1.9)			
						II	90	50	26
						(1.3)			
						III	90	50	32
-57	26.5	491	"	0.29	2.9	I	96	50	42
						(3.1)			
						II	92	52	36
						(2.8)			
						III	90	50	50
Ba Cl_2 (7.3×10^{-3} M Ba^{++})									
53-47	25	442	0.5 M glucose 0.3 M KCl	0.65	0.65	II	100	52	38
						(2.3)			
						III	102	54	52
-48	26.5	492	"	0.69	0.69	II	96	50	32
						(2.4)			
						III	90	46	42
-49	26.5	443	"	1.02	1.02	II	90	50	40
						(3.2)			
						III	96	50	46
-50	27	470	H_2O	0.08	0.08	II	94	52	20
						(1.6)			
						III	100	58	40
-51	26.5	442	"	0.31	0.31	II	96	52	18
						(2.4)			
						III	90	56	48
-52	26.5	442	"	0.2	0.2	II	102	50	26
						(2.6)			
						III	96	50	50

TABLE III—*Continued*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		mm.³	gm. × 10^{-6}		mv.	mv.	mv.
				(7.3 × 10^{-2} M Ba^{++})					
53-44	26	443	H_2O	0.1	1.0	II (1.8)	84	58	28
						III	84	54	42
-45	26	467	"	0.03	0.3	II (1.5)	62	44	30
						III	70	46	46
-46*a*	26	417	"	0.04	0.4	II (2.1)	94	54	32
						III	90	54	54
-46*b*	26	470	"	0.06	0.6	II (1.0)	82	42	38
						III	62	44	40
				Ca Cl_2 (2.5 × 10^{-3} M Ca^{++})					
52-231	26	390	0.2 M KCl	0.17	0.017		76	46	27
-232	26	340	"	0.18	0.018		59	38	36
-233	26	458	"	0.27	0.027		62	40	25
-234	26	475	"	0.45	0.045		61	36	27
				(2.5 × 10^{-2} M Ca^{++})					
53-24	26	368	0.4 M glucose 0.3 M KCl	0.17	0.17	II (1.7)	92	50	30
						III	95	56	36
-25	24	295	"	0.17	0.17	II (1.5)	96	53	30
						III	80	58	50
-26	24	443	"	0.23	0.23	II (2.6)	112	56	22
						III	112	56	50
-27	24.5	394	0.3 M KCl	0.19	0.19	II (1.6)	102	58	36
						III	106	64	48

TABLE III—*Continued*

Experimental No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		mm.³	$\frac{gm.}{10^{-6}} \times$		mv.	mv.	mv.
(2.5 × 10^{-2} M Ca^{++})—*Continued*									
-28	25.0	443	0.3 M KCl	0.51	0.51	II (1.3)	104	60	36
						III	98	46	48
-29	25.0	394	"	0.45	0.45	II (3.8)	110	58	30
						III	114	60	50
-30	24.5	394	"	0.15	0.15	II (4.9)	96	56	22
						III	100	60	50
(2.5 × 10^{-1} M Ca^{++})									
53-22	26	344	0.4 M glucose 0.3 M KCl	0.06	0.6		—	—	—
-23	26.2	344	"	0.06	0.7		—	—	—
$MgCl_2$ (4.1 × 10^{-1} M Mg^{++})									
53-4	26.5	420	H_2O	0.15	1.5	II (6)	80	50	32
						III	40	46	42
-5	26.5	394	"	0.06	0.6	II (2.1)	64	—	12
						III	84	—	36
-7	26	418	0.4 M glucose 0.3 M KCl	0.16	1.6	II (1.6)	78	56	20
						III	90	56	38
-8	26	418	"	0.08	0.8	II (1.5)	80	54	32
						III	100	58	46
-9	26	394	"	0.10	1.0	II (0.9)	76	50	26
						III	98	52	36
-10	26	325	"	0.08	0.8	II (1.4)	84	52	54
						III	80	64	50

TABLE III—*Concluded*

Experiment No.	Temperature	Axon diameter	Solvent	Volume	Amount of ions	Multiple electrodes*	Potential		
							Initial		Final RP
							SH	RP	
	°C.	μ		$mm.^3$	$\frac{gm.}{10^{-6}} \times$		*mv.*	*mv.*	*mv.*
			$SrCl_2$ (0.114 M)						
53-64	27.5	417	H_2O	0.4	4	I	88	46	28
						(2.0)			
						II	88	46	21
						(2.0)			
						III	90	54	32
-65	27.5	590	"	0.725	7.3	I	96	48	38
						(2.4)			
						II	100	—	14
						(2.9)			
						III	96	51	38
-66	27.5	394	"	0.35	3.5	I	60	40	40
						(2.4)			
						II	84	50	14
						(2.9)			
						III	80	54	38
			(1.14 M)						
53-61	25.8	394	H_2O	0.02	2	II	58	36	26
						(1.6)			
						III	70	35	16
-62	25.8	440	"	0.12	12	I	92	52	21
						(1.0)			
						II	98	43	11
						(3.6)			
						III	101	58	25
-63	25.8	418	"	0.014	1.4	I	88	43	23
						(2.4)			
						II	82	45	11
						(2.6)			
						III	90	51	33

The blocking effectiveness of a given ion species and concentration shows considerable variation (Table III). The reasons for the variation are not clear. There is evidence from experiments with drugs that injection into the core of the axon is less effective than injection into the peripheral region. This may be due to differences in diffusion rate or to different degrees of disturbance of the axoplasm by the injections. Specific differences in different nerves may also play a role, although if present these were not correlated with the length of survival of the axon. In early experiments (12) injections of very small amounts of Mg^{++} caused block. This finding was not confirmed in the later series, and the early experiments have therefore not been included.

TABLE IV

Effectiveness of Different Cations in Causing Propagation Block in Squid Giant Axons

Cation	Substance	No. of experiments	Average blocking quantity of cation	
			μg.	
K^+	K Cl	7	35.6	
	K HCO_3	3	29	
	K NO_3	2	42.6	33.0
	K_2SO_4	4	71.5	
	K glutamate	11	23.4	
	K aspartate	8	23.8	
Rb^+	Rb Cl	3	66	
Na^+	Na Cl	12	5.9	
	Na glutamate	10	9.7	7.8
	Na aspartate	6	8.6	
Li^+	Li Cl	4	4.0	
Ba^{++}	Ba Cl_2	10	0.5	
Ca^{++}	Ca Cl_2	13	0.3	
Mg^{++}	Mg Cl_2	6	1.0	
Sr^{++}	Sr Cl_2	6	5.0	

Differences in Effects of Different Cations.—Aside from the differences reflected in the amounts of the various cations needed to depress the spike and the resting potential, only two types of effect were observed which may be considered as specific to ion species.

(*i*) Injection of 0.5 M Na^+ in addition to depressing spike amplitude, also causes marked prolongation of the duration, as seen in Fig. 10. This prolongation occurs first and most markedly at the site of injection and often to such an extent that the spike in this region outlasts the later response in a nearby region more distal to the stimulating electrodes. This change occurs prior to decrease of resting potential. Li^+, though approximately as effective as Na^+ in blocking activity, does not cause as marked a prolongation of the spike. The other cations exert even less action of this type. On the other hand, some drugs

when injected in high concentration also prolong the spike, but usually this action takes place before there is marked alteration in spike height (reference 10, and unpublished data).

(*ii*) The divalent cations, which are particularly potent in depressing the spike and the resting potential, usually decrease the latter much more than do the monovalent ions (Tables III and IV).

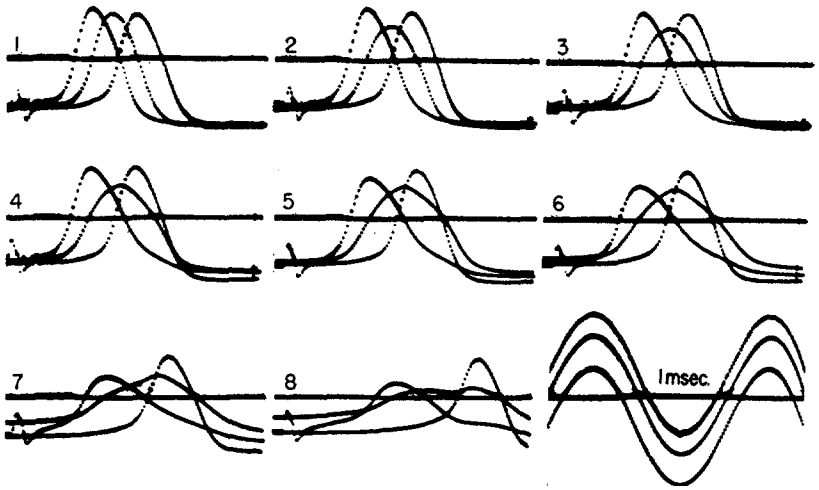

FIG. 10. Local differences in effects of injecting 0.5 M Na^+ (Experiment 53-91, temperature 24.5°C., 541 μ fiber). The three microelectrodes were 4.9 and 2.9 mm. apart. The spike at electrode I is first in the record, II is the pipette. *1*, beginning of experiment. *2*, 17th sec. of an injection lasting 45 sec. *3*, 30th sec. *4*, 42nd sec. A pause of 1 min. then ensued, there being relatively little change in the responses. *5*, beginning of a second injection lasting 1 min. *6*, 23rd sec.; *7*, end of the injection; *8*, 30 sec. later. Elapsed time of the records is about 3 min. 30 sec. Calibrations 100 mv. and 1000 C.P.S.

DISCUSSION

(*1*). *Resting Potential.*—Although the 411 axons studied in these and related experiments were all capable of impulse propagation, considerable variation was observed in their resting potential (Fig. 2, Table III). Wide scatter of resting potential values is also found in cardiac muscle fibers. Thus, in the dog heart the range is 71 to 102 mv. (reference 6, Fig. 7), 66 to 103 mv. (reference 28, personal communication), 36 to 91 mv. in cat auricular fibers (2), and 40 to 112 mv. in the frog ventricle (48). The potentials recorded were essentially independent of the type of junction used in the measurement (Table III), and of the period elapsed after isolation of the axon (Table I). Keynes (32) and Keynes and Lewis (33) reported that giant axons of *Sepia* tend to lose K^+ and

gain Na^+ at a rapid rate.[9] That nerves kept in sea water for many hours have resting potentials and spikes of the same magnitudes as nerves studied freshly after removal would indicate that the resting potential is not principally determined by the K^+i/K^+o ratio. This appears to be the case also from the microinjection experiments described in detail in section 5 *b* of Results. Large alterations in K^+i and Na^+i occur in excised muscle fibers of the spider crab (42). These, however, are not reflected in parallel changes of the resting potential which remains nearly constant. The conclusion indicated by these various findings is that the resting potential is not determined by a Donnan equilibrium condition in which K^+ and Cl^- play the dominant roles as freely diffusible ions. Additional evidence for this conclusion is summarized elsewhere (11).[10]

The microinjection experiments reported here, present a number of other obstacles to the Donnan theory of the resting potential. Not only in this potential insensitive to internal alteration of K^+ or Cl^-, but in addition all changes in internal ionic composition lead to the same result, namely, a decrease of the resting potential following a decrease of the spike amplitude to the range of zero overshoot. Therefore the resting potential appears to be linked in some way unknown at the present time with the ability of the cell to respond as an action generator. Furthermore, the decrease in resting potential when it occurs may be confined to a relatively small region of the axonal site of the injection. Finally, these local changes can be produced by injections of very small amounts of divalent cations, when the existing levels of concentrations of K^+, Cl^-, or Na^+ are little affected.

In the light of this accumulated evidence that the resting potential is not the result of a Donnan equilibrium system an alternative view as to the origin of the potential has been proposed (11). This view is not at present framed in quantitative and analytical terms, but may serve as a working hypothesis for future experiments. The explanation is based on the generally accepted existence of a specific pump extruding Na^+ which tends to enter the resting cell under the drive of the normal gradient, both chemical and electrical. It assumes that the "intrinsic" driving force of the sodium pump (*i.e.*, the intrinsic ability

[9] Keynes and Lewis (33) state that these changes begin immediately after death of the animal. However, examination of their Figs. 7 and 8 reveals (11) that this tendency is not marked during the first 4 hours except for nerves 2 cm. or less in length, in which end-effects may have predominated.

[10] For example, Mauro (38) finds that a resting potential of a magnitude nearly that recorded with a saline-filled microelectrode is obtained also with a reversible Ag-AgCl internal electrode. On the basis of the Donnan theory of the resting potential, the latter electrode should record no potential differences across the resting membrane. Lorente de Nó (37), Ling (35), and Teorell (44) discuss alternatives to the Donnan theory of the resting potential.

of the pump mechanism to eject Na^+, unmodified by the steady state conditions of the cell) is large in comparison with the driving force in the opposite direction of the chemical concentration gradient alone. Under this condition of "intrinsic" asymmetry in ionic fluxes, a potential must develop which would operate to balance the inward and outward fluxes of Na^+ in the steady state. This potential would tend to decrease the outward flux and increase the inward, and would produce internal negativity as in the resting potential. It would have the properties of a diffusion potential, but its primary characteristics would be: (*i*) that it is dependent, not on ratios of any one ion species, but on the mode of operation of the sodium pump, and (*ii*) that the origin of the potential would not lie in the differences in concentrations across a barrier membrane, but in the membrane structure itself.

A number of findings discrepant with the concept of a Donnan origin for the resting potential are easily explained on the basis of the alternative view:

(*a*) Independence of the resting potential from the type of recording electrodes (*i.e.* different concentration junctions, reversible electrodes) follows from the assumption that the diffusion potential is caused by a specific configuration of the membrane which is implied in the term "sodium pump."

(*b*) As long as this configuration is not altered drastically, ionic changes inside or outside the axon would not greatly affect the resting potential. This is the case clearly obtained in the results with microinjection. The apparent dependence of the resting potential on alterations of external K^+ may also be explained in the same manner. Decrease of external K^+ or its increase up to 2 or 3 times the normal value, has little effect on the resting potential. Hodgkin and Katz (25) have explained this result on the basis of the mathematical formulation of Goldman (9) and have invoked three constants (P_K,P_{Na},P_{Cl}). Only after the spike height is considerably decreased, as takes place when the external K^+ is 3 or more times higher than normal, does the cell begin to act as a Donnan system, in which the rate of change of the resting potential has a slope of 58 mv. per tenfold change of K^+. In the microinjection experiments reported here the results are similar. Only when the spike amplitude is decreased to the level of zero overshoot does the resting potential decrease significantly. The effects on the resting potential produced by external changes in K^+ can therefore be assimilated with those of internal changes of various ions. The cell and its membrane begin to respond as a Donnan system only after depression of the spike, when the sodium pump mechanism is probably affected.

(*c*) The alternative view explains the marked effectiveness of divalent cations in depressing the resting potential as a consequence of specific interference with the membrane configuration.

(*d*) Since the seat of the resting potential is viewed as lying in specific structures of the membrane, local alterations of this would lead to localized differences in resting potentials, as found in the present experiments (Table III, Fig. 10).

(*2*) *The Spike.*—The range of overshoot of the spike observed in the present series of measurements is far broader than has been reported (17). It is likely that this unselected series includes some nerves damaged during dissection or insertion of electrodes. Thus the few axons with resting potentials below 40 mv. and overshoots below 30 mv. might easily fall into this category. The constellation of resting potentials above 50 mv. with overshoots below 30 mv. would imply that damage to the action generator had occurred without affecting that of the resting potential. The reverse would have to be suggested for axons with low resting potentials and large overshoots, although this combination might be ascribed to leakage about the site of insertion of the electrode (7). It is difficult to ascertain whether or not electrical leakage may have occurred in any given case. However, the seal around the micropipette in all cases appeared to be complete with respect to the injection fluid. Thus, it was occasionally observed that, in the course of the injection, fluid escaped from cut branches of the axon, but such leakage was not observed at the site of impalement, although the concentration of fluid and its color intensity were highest at the site of injection. Furthermore, the range of the overshoot is about equally large at all observed values of the resting potential (Fig. 2 *D*), a fact which indicates that the variation in amplitude of the overshoot is inherent in the differences of the axons. Hodgkin and Huxley (18) reported in a series of ten axons a range of 24 mv. of variation in V_{Na}, which corresponds to the overshoot. However, the nerves used in these experiments had been selected for spike amplitude of 85 mv. or more.

Axons with resting potentials of about 30 mv. were able to respond with propagated activity. These resting potentials correspond to a steady depolarization of about 20 mv. and depolarizations of this magnitude increase inactivation enough to eliminate sodium influx (21). However, the relative independence of overshoots and resting potentials found in the present experiments is contrary to the relation between membrane potential and inactivation or indicates that the relation between spike amplitude and electrochemical potentials may be a rather loose one.

The general effect of the alteration of the internal ionic composition of the axon with respect to any ion is to decrease the spike amplitude. This result is not surprising, since it must be assumed that the complex functional organization of the bioelectric action generator is probably delicately poised with respect to the ionic environment.

This poising at first glance would seem to be rather insensitive to internal K^+, as judged by the finding that large amounts of this ion must be introduced into the axon before the spike is affected. However, in terms of the relative change of the normal state with respect to K^+, the excess amount of this ion needed to cause block is probably not greater than is the relative excess of Na^+ or of the divalent cations which cause block. The internal concentration of K^+ is about 10 times that of Na^+ and at least 1000 times that of Ca^{++}. The ratios of

the blocking amounts of these ions are approximately in the proportion (Table IV), 100:20:1, or expressed in terms of blocking effectiveness 1:5:100. However, a decrease in spike amplitude is effected rapidly by injection of Na^+ or the divalent cations, whereas the effect of excess K^+ is more gradual (Figs. 7 to 10).

The similarity of the blocking effectiveness of Rb^+ to that of K^+, and of Li^+ to that of Na^+ is in agreement with results obtained in other types of experiments. Thus, Feng and Liu (8) observed that externally applied Rb^+ causes the same type of change in the resting potential as does increase of K^+. Hodgkin and Katz found that Li^+ may be substituted for Na^+ in the external medium (25).

Prolongation of the spike, such as occurs with injection of Na^+, has also been observed in other types of microinjection experiments (10, and unpublished data), but not the decrease of the spike amplitude. Closer experimental analysis of the underlying phenomena may therefore provide further information on the membrane events associated with the response.

Differences in form and amplitude of the spike can occur at loci in the axon a few millimeters apart (Fig. 10). These differences can be produced by localized microinjection, and the response in one region can outlast activity downstream. These findings have led to the suggestion (13) that the process of activity, once initiated in a small region proceeds along a time course and to an extent which is relatively independent of activity in immediately neighboring loci.

The changes in resting potential which are produced by microinjection of various cations in most cases appear when the overshoot of the spike becomes small and block is incipient (Figs. 7 to 10). Similar results have been obtained in the action of chemical blocking agents (unpublished data). It has been suggested above (p. 278 *ff.*) that the resting potential has its origin in the sodium pump system, which is presumably located in the membrane. Since the mechanism causing the spike is most probably also located in the excitable membrane, it would not be unlikely that the two generators were interlocked in some manner. Under such circumstances, alterations in the spike-producing mechanism, caused by injection of cations and reflected in decrease of the spike amplitude, might also affect the system causing the resting potential, but secondarily as a consequence of their action on the spike generator.

SUMMARY

1. A technique is described for recording the bioelectric activity of the squid giant axon during and following alteration of the internal axonal composition with respect to ions or other substances.

2. Experimental evidence indicates that the technique as described is capable of measuring changes in local bioelectric activity with an accuracy of 10 to 15 per cent or higher.

3. Alterations of the internal K^+ or Cl^- concentrations do not cause the change in resting potential expected on the basis of a Donnan mechanism.

4. The general effect of microinjection of K^+, Rb^+, Na^+, Li^+, Ba^{++}, Ca^{++}, Mg^{++}, or Sr^{++} is to cause decrease in spike amplitude, followed by propagation block.

5. The resting potential decreases when the amplitude of the spike becomes low and block is incipient.

6. The decrease in resting potential and spike amplitude may be confined to the immediate vicinity of the injection.

7. At block, the resting potential decreases up to 50 per cent, but injection of small quantities of divalent cations may cause much larger localized depolarization.

8. The blocking effectiveness of K^+, Na^+, and Ca^{++} expressed as reciprocals of the relative amounts needed to cause block is approximately 1:5:100. Rb^+ has the same low effectiveness as does K^+. Li^+ resembles Na^+. Ba^{++} and Mg^{++} are approximately as effective as Ca^{++}.

9. Microinjection of Na^+ may cause marked prolongation of the spike at the injection site as well as decrease in its amplitude.

10. The anions used (Cl^-, HCO_3^-, NO_3^-, SO_4^-, aspartate, and glutamate) do not seem to exert specific effects.

11. A tentative explanation is offered for the insensitivity of the resting potential to changes in the axonal ionic composition.

12. New data are presented on the range of variation, in a large sample, of the magnitude of the resting potential and spike amplitude.

BIBLIOGRAPHY

1. Bear, R. S., Schmitt, F. O., and Young, J. S., *Proc. Roy. Soc. London, Series B*, 1937, **123**, 520.
2. Burgen, A. S. V., and Terroux, K. G., *J. Physiol.*, 1953, **119**, 139.
3. Chambers, R., and Kao, C. Y., *Exp. Cell Research*, 1952, **3**, 564.
4. Chambers, R., and Kopac, M. J., *in* Handbook of Microscopical Technique, (C. E. McClung, editor), New York, Paul B. Hoeber, Inc., 3rd edition, 1950, 524.
5. Curtis, H. J., and Cole, K. S., *J. Cell. and Comp. Physiol.*, 1942, **19**, 135.
6. Draper, M. H., and Weidmann, S., *J. Physiol.*, 1951, **115**, 74.
7. Fatt, P., and Katz, B., *J. Physiol.*, 1951, **115**, 320.
8. Feng, T. P., and Liu, Y. M., *J. Cell. and Comp. Physiol.*, 1949, **34**, 33.
9. Goldman, D. E., *J. Gen. Physiol.*, 1943, **27**, 37.
10. Grundfest, H., *Arch. exp. Path. u. Pharmakol.*, 1953, **220**, 136.
11. Grundfest, H., *in* Electrochemistry in Biology and Medicine, (T. Shedlovsky, editor), New York, John Wiley and Sons, Inc., in press.
12. Grundfest, H., Altamirano, M., and Kao, C. Y., *Fed. Proc.*, 1953, **12**, 58.
13. Grundfest, H., Altamirano, M., and Kao, C. Y., *Fed. Proc.*, 1954, **13**, 63.
14. Grundfest, H., Kao, C. Y., Nachmansohn, D., and Chambers, R., *Nature*, 1952, **169**, 190.

15. Grundfest, H., Shanes, A. M., and Freygang, W., *J. Gen. Physiol.*, 1953, **37**, 25.
16. Hodes, R., *J. Neurophysiol.*, 1953, **16**, 145.
17. Hodgkin, A. L., *Biol. Rev.*, 1951, **26**, 929.
18. Hodgkin, A. L., and Huxley, A. F., *J. Physiol.*, 1945, **104**, 176.
19. Hodgkin, A. L., and Huxley, A. F., *J. Physiol.*, 1952, **116**, 449.
20. Hodgkin, A. L., and Huxley, A. F., *J. Physiol.*, 1952, **116**, 473.
21. Hodgkin, A. L., and Huxley, A. F., *J. Physiol.*, 1952, **116**, 497.
22. Hodgkin, A. L., and Huxley, A. F., *J. Physiol.*, 1952, **117**, 500.
23. Hodgkin, A. L., and Huxley, A. F., *Proc. Roy. Soc. London, Series B*, 1952, **140**, 177.
24. Hodgkin, A. L., Huxley, A. F., and Katz, B., *J. Physiol.*, 1952, **116**, 424.
25. Hodgkin, A. L., and Katz, B., *J. Physiol.*, 1949, **108**, 37.
26. Hodgkin, A. L., and Katz, B., *J. Physiol.*, 1949, **109**, 240.
27. Hodgkin, A. L., and Katz, B., *J. Exp. Biol.*, 1949, **26**, 292.
28. Hoffman, B. F., and Suckling, E. E., *Am. J. Physiol.*, 1952, **170**, 357.
29. Huxley, A. F., and Stämpfli, R., *J. Physiol.*, 1951, **112**, 496.
30. Jenerick, H. P., *J. Cell. and Comp. Physiol.*, 1953, **42**, 427.
31. Kao, C. Y., *Science*, 1954, **119**, 846.
32. Keynes, R. D., *J. Physiol.*, 1951, **114**, 119.
33. Keynes, R. D., and Lewis, P. R., *J. Physiol.*, 1951, **114**, 151.
34. Krogh, A., *Proc. Roy. Soc. London, Series B*, 1946, **133**, 140.
35. Ling, G. W., *in* Phosphorus Metabolism, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1952, **2**, 748.
36. Ling, G. W., and Gerard, R. W., *J. Cell. and Comp. Physiol.*, 1949, **34**, 383.
37. Lorente de Nó, R., A Study of Nerve Physiology, *Studies from the Rockefeller Institute for Medical Research*, 1947, **131** and **132**.
38. Mauro, A., *Fed. Proc.*, 1954, **13**, 96.
39. Nastuk, W. L., and Hodgkin, A. L., *J. Cell. and Comp. Physiol.*, 1950, **35**, 39.
40. Schmitt, O. H., *Science*, 1948, **107**, 432.
41. Shanes, A. M., Grundfest, H., and Freygang, W., *J. Gen. Physiol.*, 1953, **37**, 39.
42. Stephenson, W. K., *Biol. Bull.*, 1953, **105**, 368.
43. Tasaki, I., *Jap. J. Physiol.*, 1952, **3**, 73.
44. Teorell, T., *Progr. Biophysics*, 1953, **3**, 305.
45. Tobias, J. M., *in* Modern Trends in Physiology and Biochemistry, (E. S. G. Barron, editor), New York, Academic Press, 1952, 291.
46. Ussing, H. H., *Adv. Enzymol.*, 1952, **13**, 21.
47. Weidmann, S., *J. Physiol.*, 1951, **114**, 372.
48. Woodbury, L. A., Hecht, H. H., and Christopherson, A. R., *Am. J. Physiol.*, 1951, **164**, 307.