Effects of Internal and External Ionic Environment on Excitability of Squid Giant Axon

A macromolecular approach

ICHIJI TASAKI, IRWIN SINGER, and TOSHIFUMI TAKENAKA

From the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Laboratory of Neurobiology, National Institutes of Health, Bethesda, Maryland. Dr. Takenaka's permanent address is the Tokyo Medical and Dental University, Department of Physiology, Tokyo, Japan

ABSTRACT The effects of ten cations and fifteen anions on the excitability of the squid giant axon were studied. The method of intracellular perfusion used in these investigations is described in detail. Empirical criteria were established for evaluating the relative favorability of any salt solution for maintaining the normal excitability of the membrane of the axon. It was found that both cations and anions could be ordered in sequences of relative favorability, which are directly related to the classic lyotropic sequences found for protein macromolecules and in colloid chemistry in general. The effects of concentration, salt mixtures, non-electrolyte carriers, enzymes, metabolic inhibitors, pH, and external media were also studied. The results are interpreted in terms of current concepts of the interactions between water structure, charged macromolecules, and their ionic environments. A macromolecular approach is given to the physicochemical nature of the "two stable states" of the excitable membrane, to describe the time-dependent potential changes observed.

INTRODUCTION

The surface layer of the squid giant axon is composed of proteins and phospholipids. Alteration of these macromolecular constituents of the membrane of the axon by various biochemical and physical means would be expected to profoundly affect its excitability. Recently it has been shown that various proteases and lipases, administered intracellularly either by injection (Rojas and Luxoro, 1963) or perfusion (Tasaki and Takenaka, 1964 *a*), suppressed excitability irreversibly. It is also possible that alteration of the state of the membrane macromolecules may be produced by the various salts used for intracellular perfusion, and this may be an essential factor in the maintenance of axon excitability.

These membrane macromolecules are ampholytes; *i.e.*, they carry both positive and negative charges. Phospholipids contain amino-, guanidino-, and imidazo- groups as possible sources of positive charge, and side-chain carboxyl groups as possible sources of negative charge. Strong electrochemical interaction may be assumed between adjacent charged groups in the membrane. Furthermore, such interaction should be strongly affected by various neutral salts introduced into the axon interior.

The physicochemical nature of the interaction between inorganic ions and macromolecules (*e.g.*, "complex formation" between mixtures of proteins, phospholipids, and inorganic salts) has been investigated by a number of colloid and polymer chemists (*cf.* Kruyt, 1949; Bungenberg de Jong, 1949). More recently, the nature of non-covalent bonds in proteins has been discussed extensively by Klotz (1958), Kauzmann (1959), Scheraga (1963), and others; Eisenman (1961) has investigated the factors determining the differences in affinity of various cations to fixed anionic sites in ion exchangers. With these recent findings it appears possible to propose a macromolecular interpretation of the influence of various intracellular salts on the physiological function of the axon.

This article describes the results of systematic investigation of the effects of various intracellular anions and cations on the excitability of the perfused squid giant axon. The results obtained clearly indicate that the effects of neutral salts are very different on the inner and outer surfaces of the axon membrane. The physicochemical basis of the lyotropic series and its importance in excitability phenomena are discussed.

METHODS

1. *Material* North Atlantic squid, *Loligo pealii*, were obtained at the Marine Biological Laboratory in Woods Hole. Giant axons were dissected out in natural sea water under darkfield illumination, and were then mounted in a lucite chamber containing natural sea water. The axons used were 35 to 50 mm in length, 350 to 650 μ in diameter (average, 458 μ for 122 axons), and were free from local injuries and cut branches. In most experiments axons were used without extensive cleaning.

2. *Perfusion System* The technique of intracellular perfusion used in this laboratory has been described previously (Oikawa, Spyropoulos, Tasaki, and Teorell, 1961); the details of the double cannulation procedure may be found in a previous report (Tasaki and Takenaka, 1963). These experimental techniques, as modified for the present study, are described below.

A cannula of about 180 μ in diameter was inserted into one end of the axon to serve as the inlet for the perfusion solution; a second cannula, of about 350 μ in diameter, was inserted into the other end as the outlet. The inlet cannula was connected through a glass valve and polystyrene tubing to reservoirs for the perfusion fluid. The glass valve permitted rapid alternation between two reservoirs with different

perfusion solutions. The dead space was less than 0.5 mm^3 in the inlet pipette when alternating perfusion solutions within the same axon. To prevent blockage of the outlet cannula, the major portion of the axoplasm was gently sucked out through the larger cannula prior to perfusion. The entire perfusion system from reservoirs to outlet was flushed with 12 per cent glycerol solution before any new perfusion solution was introduced into the reservoirs. The entire changeover from one perfusion solution to another was effected within 1 minute. Flow rates of 10 to 30 mm^3/min. were maintained by elevation of the reservoirs to a height of about 40 cm. The entire perfusion system was kept at room temperature, 21 to 23°C.

Variation in the height of the reservoir from 60 cm above the sea water level in the chamber to about 10 cm below the sea water level, was without effect on either the resting or action potential. However, under direct observation through a dissecting microscope, the axon was observed to increase by about 25 μ in diameter under high pressure, and to return to normal when the pressure was lowered.

3. *Stimulating Currents* Propagated nerve impulses were induced by electric shocks applied near the proximal end of the perfused zone of the axon by means of a pair of platinum electrodes and a Grass stimulator, in conjunction with an isolation unit (type SIU-4B). Generally, stimuli were of 0.1 millisecond duration, twice the threshold level of intensity, and 0.5 to 1 shock/sec. frequency. The "critical frequency," *i.e.* the frequency at which intermittent conduction block is produced, was determined with the intensity and duration above. When "high frequency" stimulation was employed, the frequency was varied continuously to maintain stimulation at a rate just less than the critical frequency; *i.e.*, at the maximum rate which would not impair conduction through the perfused zone.

4. *Recording System* Propagated action potentials were monitored with a second pair of platinum extracellular electrodes near the distal end of the axon, and were recorded on one beam of a dual-beam Tektronix oscilloscope (type 502). The intracellularly recorded action potentials were obtained with a uniform diameter (about 80 μ) glass micropipette electrode, placed concentrically within the drainage cannula so that the recording tip could be moved within the perfused zone. Isotonic KCl solution was used to fill the electrode. No significant differences were observed in records obtained with saturated KCl as the conducting medium within the electrode. The intracellular potentials were monitored through a Bak unity-gain cathode follower (Electronics for Life Sciences, Rockville, Md.) on the other beam of the oscilloscope.

This method of recording gives rise to a small artifact, which appears in the falling phase of the action potential. This artifact has been noted by others (Baker, Hodgkin, and Meves, 1964) and is produced by capacitative coupling between the electrolytic conductors inside and outside the recording electrode. There are two methods of eliminating this artifact. One method is to apply isotonic $MgSO_4$ solution to the distal (unperfused) portion of the axon (Tasaki and Luxoro, 1964); elimination of the action potential arising from the unperfused portion reduces the potential variation in the outflowing fluid in the cannula and decreases the artifact. An alternative method would be to use an electrode with a driven shield; in this case, care must be

taken to electrically isolate the shield from the surrounding conducting medium. In these studies no attempt was made to eliminate this artifact.

When resting potentials were measured, one oscilloscope beam was brought to represent the potential level of the glass micropipette electrode placed in the surrounding medium. Then the electrode was removed from the external medium and inserted into the axon. This internal potential level was recorded by the other oscilloscope beam. Resting potentials were then determined from the difference between the two potential levels. In almost all cases a large silver-silver chloride-agar electrode was placed in the external medium to serve as a ground. In all cases a Grass kymograph camera was used.

5. *Measurement of Membrane Resistance* For measurement of membrane resistance rectangular current pulses were obtained from Tektronix pulse generators (type 161). These currents were passed through an enamelled silver wire electrode of about 50 μ diameter, which had a bare area of 12 mm near its end. Another enamelled silver wire was twisted about the first wire, so that a bare region of 1 mm was located at the center of the bare area of the first wire. These two electrodes were inserted through the outlet cannula into the perfusion zone in place of the glass micropipette electrode. Current pulses delivered by the first wire were monitored by one oscilloscope beam, and were maintained at 1 μa in intensity and 10 milliseconds in duration. The membrane resistance was then determined from the potential variations produced by the inward current, simultaneously monitored by the other beam.

6. *Perfusion Solutions* Perfusion solutions consisted of three components: salt, buffer, and carrier. The uni-univalent salts (KF, K glutamate) generally were used in concentrations of 400 mM; the uni-divalent salts (K_2SO_4, K tartrate) were used in concentrations of 200 mM. Thus, the cation concentration was maintained at 400 mequivalents/liter for most studies. In studies of anion effects, the common cation was usually K, although the same results were obtained with Rb and other cations. In studies of cation effects, F, Cl, NO_3, and SO_4 were the most frequently used common anions. $RbNO_3$ and $CsNO_3$ were prepared from their hydroxides and HNO_3; propionates and butyrates of K were prepared from their acids and KOH. K isethionate was prepared by treating the Na salt with a cation exchange resin in hydrogen form (Dowex 50), and neutralizing the isethionic acid with KOH. All other salts were used in their commercially available forms, analytical or reagent grade.

In general, the carrier used was 12 volumes per cent glycerol solution. Variation of the glycerol concentration from 5 to 30 volumes per cent slightly altered the action potential shape; action potentials obtained with the more dilute glycerol carriers were of somewhat shorter duration and of slightly greater amplitude. Substitution of isotonic sucrose for the glycerol solution had no noticeable effects. The addition of sucrose to a salt-glycerol perfusion solution, to make that solution 1.0 M in sucrose as well, did not affect the results significantly.

The buffer used was generally a mixture of KH_2PO_4 and K_2HPO_4, in concentrations of less than 5 mM PO_4. In higher concentrations the buffer obscured the results by acting as a bulk anion. No differences were observed when a tris-HCl buffer was

substituted. Action potentials of normal amplitude were obtained with perfusion solutions consisting solely of buffer and carrier, with no additional salt; presumably the buffer itself acts as the bulk salt in this case. The pH of the perfusion solution was carefully maintained between 7.2 and 7.4 in all cases. A range of 6.0 to 8.2 was investigated for the effects of altering pH. Conduction was blocked quite rapidly when the pH was less than 6.4, regardless of which bulk salt was used; conduction was restored when the perfusion solution was rapidly changed to a solution of the same salt with more alkaline pH. Perfusion solutions of pH greater than 7.5 had a strong tendency to elicit spontaneous repetitive discharges from the axon. In general, repetitive firing could be suppressed by raising the divalent cation concentration in the external medium (see below).

In the enzyme studies either KF or K aspartate was used as the bulk salt; phosphate buffer and glycerol carrier were used in all cases. Several proteolytic and lipolytic enzymes, including trypsin (Worthington, twice crystallized), papain (Mann), ficin (Sigma, twice crystallized), phospholipases C and D (Calbiochem), lecithinase (Sigma, twice crystallized), and desoxyribonuclease (Mann) were used in concentrations of 0.02 to 1.0 mg/ml perfusion solution.

The effects of the proteolytic enzymes were observed directly by adding kaolin or talc particles, and dye molecules to the perfusion solution. In concentrations of 1 mg/ml, neither kaolin nor talc affected axon excitability or survival. Neither chlorphenol red nor methylene blue, in concentrations of less than 1 mg/ml perfusion fluid, affected either axon excitability or survival.

7. *External Media* The external medium employed generally was natural sea water as available in Woods Hole. In some cases a high Mg artificial salt mixture replaced the natural sea water; the most commonly used such mixture was 400 mM NaCl, 100 mM $MgCl_2$, and 33 mM $CaCl_2$. The pH was maintained at 8.0 with tris-HCl buffer. This salt mixture was very effective in preventing or suppressing spontaneous repetitive firing of nerve impulses. No significant differences were observed when $SrCl_2$ was substituted for either or both of the usual divalent cations. However, substitution of $BaCl_2$ for the usual divalent cations rapidly blocked conduction; this block could be reversed readily by replacing the external medium with natural sea water or the artificial salt mixture described above. Substitution of SO_4 or Br for the external Cl was without noticeable effect. The external NaCl was totally replaced in one series of experiments by tetraethylammonium (TEA) chloride. Complete elimination of Na ion in the external medium was insured by using a Na-free agar gel ground electrode. The perfusion solution was composed of K-free components: CsF (50 mM) as the bulk salt and 12 volumes per cent glycerol as carrier. Neural conduction could be maintained for more than 10 minutes in some axons under these conditions.

RESULTS AND INTERPRETATION

1. *Effect of Dilution of Electrolyte in Perfusion Fluid with Non-Electrolyte Solution.* Previous reports have demonstrated that excitability of the squid giant axon can be maintained during intracellular perfusion with solutions of low ionic strength (Tasaki, Watanabe, and Takenaka, 1962; Baker,

Hodgkin, and Shaw, 1962 *b*; Baker, Hodgkin, and Meves, 1964; Narahashi, 1963; Moore, Narahashi, and Ulbricht, 1964). A variety of unfavorable perfusion media, which produce conduction block rapidly in high concentrations, have little or no harmful effect at low concentrations (Tasaki and Takenaka, 1963). We wish to show that neural conduction suspended by perfusion with an unfavorable medium can be restored merely by diluting the perfusion fluid with isotonic non-electrolyte solution.

An example is presented in Fig. 1. A partially cleaned giant axon, immersed in natural sea water, was intracellularly perfused with 500 mM KCl solution (see Methods). Under continuous perfusion with this weakly buffered solution, both resting and action potentials remained practically constant for 10 to 20 minutes after the onset of perfusion. Following this period of apparently stationary conditions, there was a gradual decline in the action potential amplitude, followed by a rapid decrease of excitability. Conduction across the perfused zone was blocked in 30 to 40 minutes. The magnitudes of both resting potential and membrane resistance gradually declined with the decrease in excitability. When the 500 mM KCl perfusion fluid was quickly replaced with a 200 mM KCl solution at the moment of conduction block, conduction was immediately restored and the action potential recovered.

Recovery was more marked with greater dilution. When a period of 1 to 2 minutes elapsed after the onset of conduction block, recovery by perfusion with dilute solutions was either poor or absent. Dilution experiments of this type were carried out on several different axons, starting with 500 to 600 mM solutions of KSCN, KI (freshly prepared), KBr, and KCl. When these perfusion solutions were strongly buffered with isotonic phosphate solutions, conduction block was delayed markedly. Dilution studies were not carried out with KF, K aspartate, or K glutamate because conduction was not blocked for 1 to 2 hours.

Note that dilution restored the action potential without significant change in the resting potential when solutions were changed promptly (Fig. 2). This finding cannot be explained by the original formulation of the equivalent circuit theory, which would require a change in resting potential prior to a change in action potential (Hodgkin and Huxley, 1952). However, based on a consideration of the macromolecular state of the axon membrane, a qualitative interpretation may be given to the findings.

In the normal state, the membrane macromolecules may be assumed to form a close-knit complex system because of electrostatic and other molecular interactions. A high transmembrane resistance in the normal, resting state is ascribed to tight packing of the macromolecules, and to the unavailability of a large proportion of charged groups for transmembrane ion transport. Disruption of junctions between or within macromolecules, produced by the

added ions, could lower membrane resistance and eventually cause loss of excitability.

In systems of charged macromolecules and neutral salts, ions deriving from added salts tend to "screen" the electric charges of the macromolecules and

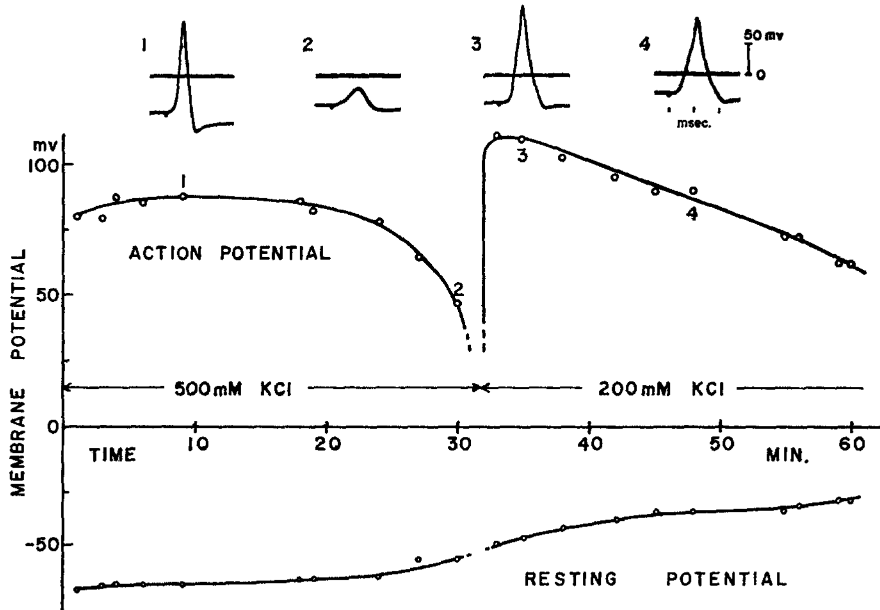

FIGURE 1. Effect of dilution of intracellular electrolyte. The time courses of the resting potential and action potential overshoot (obtained with 1 shock/sec. stimuli) are indicated by the lower and upper curves, respectively. The break in each curve represents the time during which conduction was blocked, and during which the perfusion solution was switched from 500 mM KCl to 200 mM KCl. Four representative oscilloscope records are shown at the top. The numbers refer to their respective positions in time along the action potential curve. Note that restoration of the action potential occurred within 1 minute without a simultaneous change in the resting potential, and that the large responses observed following the restoration were not maintained.

to reduce electrostatic interaction between charged groups (*cf.* Overbeek and Bungenberg de Jong, 1949, p. 226; Kauzmann, 1959, pp. 46 and 49). Since the magnitude of this effect is determined primarily by the concentration and valence of the ions concerned, dilution of the intracellular salt would tend to increase electrostatic attraction between oppositely charged groups in the membrane, to restore high membrane resistance, and to increase excitability (see Discussion).

The observed differences in physiological effects among different neutral salts of the same concentration and valence derive primarily from differences

in affinity among various inorganic ions to charged membrane groups. For a given common perfused anion, the cation with the stronger affinity to the negatively charged group should produce the most rapid conduction block. The order of cation affinity to carboxyl, sulfate, and phosphate groups follows classical lyotropic sequences (see Overbeek and Bungenberg de Jong, 1949, pp. 203 and 299). Since a similar series was expected to exist for anion affinity to positively charged groups (see Discussion), the effects of twenty different anions were compared in the following study.

2. *Survival Time and Membrane Conductance during Perfusion with Various K Salts.* Comparison of the effects of various perfusion fluid anions on axon excitability was made by two different methods. The first consisted of determining the time required to block conduction during continuous perfusion with solutions of various K salts. The second was based on comparing the "restoring ability" of anions; *i.e.*, the ability of one anion to restore excitability which has been depressed by another.

Results obtained by the first method are summarized in Fig. 2 for the halides. In the diagram at the left, the abscissa represents the time required to block conduction across the perfusion zone; this time is designated as the apparent "survival time," because conduction block is followed by irreversible loss of excitability (unless the intracellular perfusion fluid is replaced with a more favorable solution immediately). The ordinate represents the number of axons studied for each halide. Among the eighteen different axons perfused with 400 mM KCl solution, there were a few axons in which conduction block occurred within 20 minutes; the diameters of these axons were exceptionally small (less than 400 μ). The survival time increases in the following order: I $<$ Br $<$ Cl $\ll$ F. The survival time with KF was greater than 5 hours and could not be determined directly.

In the graph shown at the right of Fig. 2, average survival time was plotted against the classic lyotropic numbers of halide ions. These numbers were determined according to the tendency of anions to enhance or suppress precipitation of various sols (see Appendix). A linear relationship is found between survival time and lyotropic number. Many physicochemical properties of macromolecules are affected by added salts in the order represented by the lyotropic numbers (*e.g.*, swelling of gels, viscosity of sols, zeta potentials of colloids). The inset of Fig. 2 shows the relationship between the heats of hydration of halides and the lyotropic numbers (Voët, 1937). The exceptionally long survival time found for KF perfusion solutions might have been predicted from these relative anion positions.

The study of anion effects on excitability by the "restoration" technique yielded results consistent with those obtained by the survival time method. Typical results are shown in Fig. 3. In the example at the left, the axon was

perfused with a 400 mM KCl solution initially. The action potential developed in the perfusion zone remained unaltered for approximately 15 minutes during continuous intracellular perfusion. Following this apparently stationary stage, there was a gradual decline in the action potential. About 38 minutes after the onset of perfusion with KCl, conduction across the perfusion zone was suspended. When the perfusion fluid was promptly switched to a 400 mM

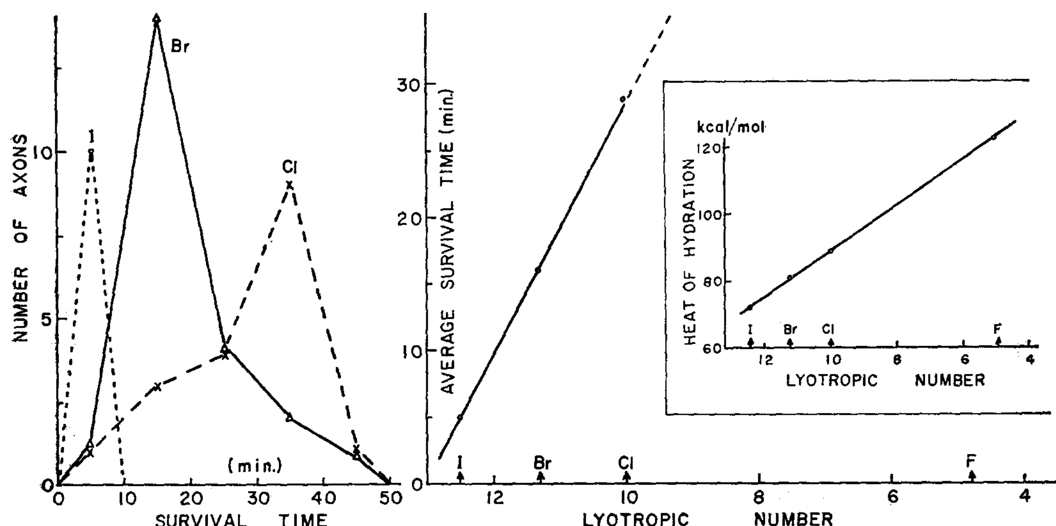

FIGURE 2. *Left.* Survival times for potassium halide perfusion solutions. The number of axons studied which fell within a given 10 minute interval are plotted at the midpoint of the interval. The average survival times were 4.9 minutes for iodide (ten axons), 16.9 minutes for bromide (twenty-six axons), and 27.4 minutes for chloride (eighteen axons). The distribution for fluoride is not included because the average survival time is greater than 2 hours. *Right.* Relationship between survival time and lyotropic number. The lyotropic numbers for the halide positions are indicated above the abscissa. The average survival time for each halide is plotted as the ordinate. Fluoride is not included for reasons already stated. *Inset.* Relationship between heat of hydration (ordinate) and lyotropic number (abscissa). The exceptionally long survival time found for fluoride might be predicted from this diagram (Voët, 1937).

KF solution, the action potential immediately recovered, and the resting potential increased in magnitude. In other cases of anion restoration, with no change in ionic strength, no change in resting potential was observed when the action potential recovered (Tasaki and Takenaka, 1963; Tasaki and Luxoro, 1964).

Following restoration of excitability with KF, the action potential amplitude remained at a constant high level. However, when the original 400 mM KCl solution was reintroduced into the axon, the action potential was immediately reduced. This finding can be understood if we assume that physico-

chemical changes in the membrane caused by KCl persist while the axon is perfused with KF. Apparently, this change in the membrane was not severe enough to discernibly reduce the action potential with a more favorable per-

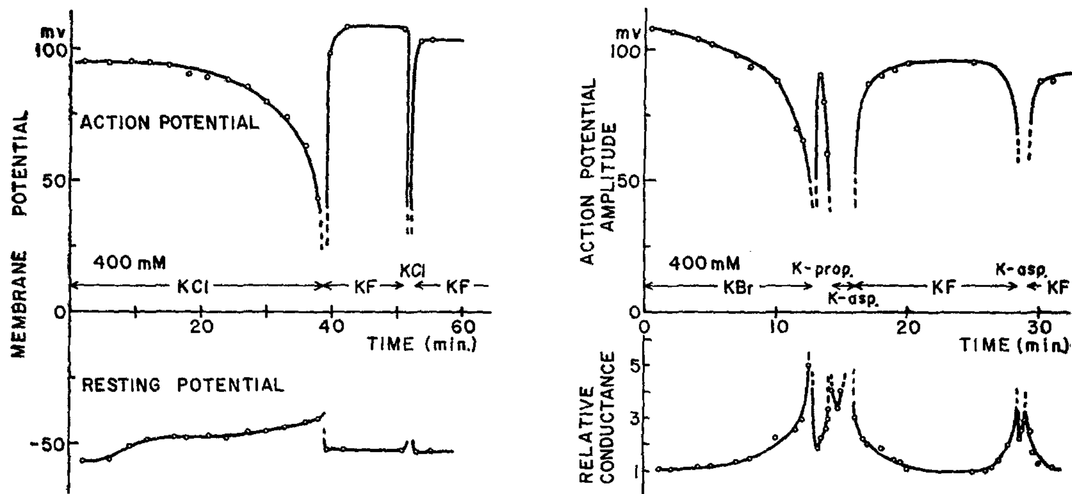

FIGURE 3. *Left.* Anion effect demonstrated by the restoration technique. The resting and action potentials (obtained with 1 shock/sec. stimuli) are indicated by the lower and upper curves, respectively. The breaks in each curve represent the times during which perfusion solutions (indicated just above the time scale) were changed. Within these intervals, perfusion solutions were not changed until the moment conduction was blocked. In each case the restoration of both resting and action potentials produced by changing to KF (from KCl, at 39 and 52 minutes, respectively) occurred within 1 minute. Note the rapid decline in both potentials produced by changing from KF to KCl (at 51 minutes, before the break in the curves). *Right.* Effect of anions on action potential (top) and membrane conductance (bottom). The action potential amplitude represents the peak of the action potential measured from the level of the resting potential. As in previous figures, the breaks in each curve represent the times during which perfusion solutions were changed. All values were obtained at a stimulus frequency of 1 shock/sec. Transient restoration of the action potential occurred at 12 and 16 minutes (when KBr was switched to K propionate, and K aspartate to KF, respectively); there was no restoration of the action potential when the perfusion solution was changed from K propionate to K aspartate (at 13.5 minutes). With each of the foregoing changes of perfusion solution there was a decrease in the relative conductance of the membrane.

fusion fluid (KF) present (see Discussion). Thus, the action potential amplitude does not completely describe the physicochemical state of the axon membrane.

The diagram on the right in Fig. 3 shows that membrane conductance increases and decreases at the same time as the action potential amplitude. When perfused with a 400 mM KBr solution, the action potential gradually

declined; there was a concomitant rise in membrane conductance. When conductance across the perfusion zone was suspended, 12.5 minutes after the onset of perfusion, the perfusion solution was switched to K propionate. A transient restoration of conduction was accompanied by a definite reduction in the membrane conductance. Immediately after the second conduction block, the axon was perfused with K aspartate. This procedure reduced the membrane conductance, but there was no restoration of conduction. When subsequently perfused with KF, both the action potential amplitude and the membrane conductance approached their respective normal values.

Conductance measurements of this type were carried out on five axons using 400 mM K aspartate, KCl, KBr, and KI perfusion solutions. Increase in the action potential amplitude was always accompanied by significant reduction of membrane conductance.

3. *Anion Sequence Determined by the Restoration Technique.* The complete anion sequence was determined as follows. An axon was perfused intracellularly with a solution of one K salt; at the moment when neural conduction was blocked, the perfusion fluid was quickly switched to one with a different K salt. If this procedure promptly restored conduction, the anion in the second perfusion solution was designated as being "more favorable" than the anion in the first solution. If, on the contrary, switching from one solution to another increased the depolarization of the membrane, the second anion was regarded as "less favorable" than the first. In this study, the common cation concentration in the perfusion fluid was held constant at 400 mequivalents/liter. The results obtained with common cation concentrations of 500 or 600 mequivalents/liter were entirely consistent with the results obtained at 400 mequivalents/liter. The results obtained when Cs, Rb, or NH_4 were common cations, were consistent with those obtained with K. Note that it was necessary to maintain the phosphate buffer concentration below 5 mM to prevent phosphate from acting as a bulk anion (see Methods).

These data are summarized in Table I for K salts. (Similar data for Rb salts are presented in Table II A.) The anions examined are arranged in order from the most favorable (F) down to the least favorable (SCN). The anion in the first perfusion fluid is listed in a vertical column (labeled 1) on the left side of the table; the anion in the second perfusion fluid is shown in a horizontal row at the top (labeled 2). The symbols ++ or + indicate that there was restoration of conduction with a distinct or slight increase, respectively, in the action potential amplitude when the first anion was replaced with the second anion. For example, when the first anion was Cl (horizontal row marked Cl) and the second anion was F (vertical column marked F), a distinct increase in the action potential amplitude was observed on eleven

TABLE I

Anion sequence determined by the restoration technique for various potassium salts. The anionic member of the first (starting) perfusion solution is listed vertically at the left (under heading 1). The anionic member of the second (restoring) perfusion solution is listed horizontally at the top (to the right of heading 2). The results of switching from solution 1 to solution 2 are indicated in the square represented by the intersection between the corresponding row and column. The numbers before each symbol refer to the number of trials with a particular outcome; all trials are included in the table. The possible outcomes are: ++, action potential amplitude restored to within 25 mv of the amplitude of the action potential at the start of the trial, and lasting for at least 5 minutes; +, action potential restored with an amplitude or duration less than the preceding case, or restoration of the normal resting potential after depolarization; ±, equivocal changes, *e.g.* spontaneous repetitive discharge; *, no discernible changes; −, significant decrease in the amplitude of the action potential, gradual depolarization of the resting membrane, or most frequently, total or intermittent conduction block. The parentheses refer to those trials performed with butyrate (BUTY). The restoration technique did not permit separation of butyrate from propionate (PROP), or the separation of glutamate (GLUT) from aspartate (ASP). Other abbreviations include: citrate (CIT), tartrate (TAR), and acetate (ACET).

1 \ 2	F	HPO_4	GLUT ASP	CIT	TAR	PROP	SO_4	ACET	Cl	NO_3	Br	I	SCN
F		1± 1−	2± 2* 1−		1−	6−	4−	3−	2* 12−	4−	1* 19−	8−	
HPO_4	2++ 6+ 1± 3* 1−		1+ 1± 1*								2−		
GLUT ASP	11++ 7+ 5*	3+ 2*		1*				1* 5−					
CIT	2++ 2+		1+ 2*										
TAR	2+		1*	1+				1−					
PROP (BUTY)	6++ 2+ (1++) (2+)	1+	1+ 1*	2+	2+		1+ 1*		1± 1−				
SO_4	9++ 3+ 2*	1+ 1*	2+	1+	2+				1−				
ACET	2++		1+		1++ 1*	1+	2+ 1*		1−				
Cl	11++ 9+ 2*	1++ 3+	1++ 4+ 3*			2+ 1±	2+	2+		1−		1*	
NO_3	2++ 5+			1±		2*		1±	1+ 1±			3*	1−
Br	22++ 7+	2++ 3+	1+			1+	2+ 1± 2*	1+ 1*	3+	2+ 1± 4*			
I	3++ 3+	4+	2+			1+ 1*	1+ 2±		1+ 1*	1+	2+		
SCN	1++ 1+										1+		

occasions, a slight increase on nine occasions, and a questionable improvement in two cases.

This table demonstrates the following rule. When anion A (*e.g.*, chloride) is found to be more favorable than anion B (*e.g.*, bromide) and B is more favorable than anion C (*e.g.*, iodide), direct comparison of A with C always indicates that A is more favorable than C; the anions thus form a self-consistent sequence, A > B > C. The relative anion position in the sequence gives a rough estimate of the relative magnitude of favorability; *i.e.*, if A > B and B > C, then generally A ≫ C.

The anion sequence shown in this table does not follow the order of the ion radii. For example, the atomic or molecular weights of F, glutamate, and Cl are in the following increasing order: F < Cl < glutamate. The equivalent conductivities at infinite dilution, which reflect the radii of the hydrated ions, are in the increasing order: Cl < F < glutamate. Therefore, it does not appear possible to interpret the anion sequence in terms of any relationship between membrane "pore" size and ion radii.

The anion sequence obtained above closely follows the lyotropic series of classical protein chemistry (Hofmeister, 1888; see also Voët, 1937). However, this table includes a number of anions whose positions in the lyotropic series were not known. The positions of these anions were determined by the technique described in the Appendix. The order found was fluoride (4.9), phosphate (5.0), aspartate and glutamate (5.7), sulfate (6.6), citrate (7.0), tartrate (7.1), acetate (8.4), chloride (10.0), nitrate (10.7), bromide (11.2), iodide (12.1), and thiocyanate (13.3). The numerals in the parentheses indicate the lyotropic numbers of these anions. Comparison of multivalent anions with univalent anions was made by expressing concentrations in "equivalents per liter," instead of the "moles per liter" adopted by previous investigators (see Appendix).

The position of isethionate is probably between SO_4 and propionate (six out of ten trials support this position; the remaining four trials were equivocal). Since some uncertainty is introduced by the presence of a small amount of Na (estimated to be about 30 mequivalents/liter) persisting through the ion exchange procedure used to prepare the K salt, isethionate is not included in the table.

There is a surprisingly good parallel between the anion sequence for the squid giant axon and the lyotropic series. Except for a slight irregularity in the relative positions of the sulfate and propionate anions, the agreement in the anion order is perfect. This striking agreement suggests that the electrochemical properties determining the classic lyotropic series of anions also determine the anion sequence in the squid giant axon, although the precise mechanisms of the two processes involved might be very different.

4. *Cation Sequence.* The cations examined in the present study include the alkali metal ions, NH_4, and several organic ions. The method used to determine the cation sequence was similar to that for anions. An axon was perfused with a 400 mequivalents/liter solution of one salt until neural conduction across the perfusion zone was blocked. Then the perfusion fluid was

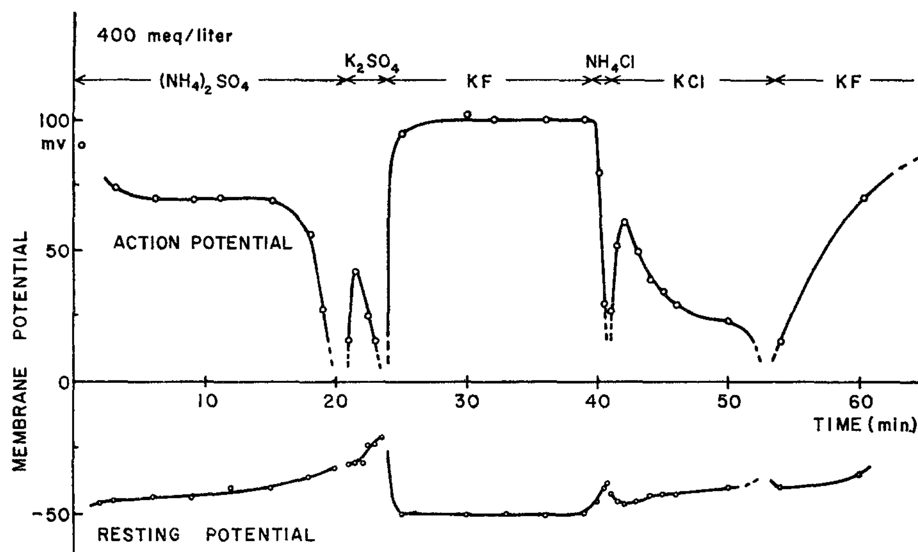

FIGURE 4. Cation effect demonstrated by the restoration technique. As in previous figures, the resting and action potentials (obtained with 1 shock/sec. stimuli) are indicated by the lower and upper curves, respectively. Note that the restoration of both the resting and action potentials produced by changing from $(NH_4)_2SO_4$ to K_2SO_4 (at 21 minutes) was transient, as was the restoration produced by changing from NH_4Cl to KCl (at 41 minutes). Both recoveries obtained with KF (from K_2SO_4 at 23 minutes, and from KCl at 53 minutes) are representative of the anion effect. Also note the sudden fall in both resting and action potentials produced when the perfusion solution was changed from KF to NH_4Cl (at 39 minutes, before the break in the curves).

quickly switched to a solution of a salt of a different cation, but of the same anion and of the same concentration. Restoration of conduction by this procedure was taken as an indication that the second cation is more favorable than the first.

An example obtained by switching from NH_4 ion to K ion is presented in Fig. 4. Immediately after the onset of perfusion there was a short (approximately 1 minute) period during which the action potential amplitude increased rapidly; this period corresponds to the time required for ion diffusion across the thin layer of axoplasm between the perfusion fluid and the axon

membrane. The action potential amplitude remained at an approximately constant level during the following 16 minute period, and then decreased gradually. In this example, neural conduction was blocked about 20 minutes after the onset of perfusion with $(NH_4)_2SO_4$. When the perfusion fluid was changed to a 400 mequivalents/liter solution of K_2SO_4, the action potential transiently, but definitely, recovered. After conduction was blocked by perfusion with K_2SO_4 solution, conduction was restored by perfusion with KF solution (which would be expected from the anion sequence determined

TABLES IIA AND B

TABLE II A. Anion sequence determined by the restoration technique for several rubidium salts. (See Table I for explanation of symbols.)

TABLE II B. Cation sequence determined by the restoration technique for the common anions F, SO_4, Cl, NO_3. Format and symbols are the same as for Table I. Parentheses have been placed around Cs to indicate some uncertainty introduced by the use of artificial sea water externally to prevent spontaneous repetitive discharges (see text).

A

1 \ 2	F	Cl	NO_3
F	\	1-	
Cl	2++	\	
NO_3		1+	\

[Rb^+ SALTS]

B

1 \ 2	(Cs)	Rb	K	NH_4	Na
(Cs)	\	1+ 1± 1* 1-			
Rb		\	1± 2* 3-		
K	1++ 1+ 4±	15+ 3±	\		
NH_4			7+ 1*	\	
Na			1+		\
Li		1±	1+		

[F^-, Cl^-, NO_3^-, SO_4^{--} SALTS]

above). At about 40 minutes, the KF solution was switched to an NH_4Cl solution; this procedure blocked conduction within 1 minute (which would be expected if the effects of the first perfusion fluid persist to this time). Conduction was then restored transiently by perfusing with KCl solution, demonstrating again that the K ion (in KCl solution) is more favorable than the NH_4 ion (in NH_4Cl solution).

These results are summarized in Table IIB. Use of a different common anion does not alter the cation sequence, in so far as comparison of two cations is made with salts of the same anion. In this table, therefore, the results obtained with F, Cl, NO_3, and SO_4 salts are combined. When Cs was substituted for

K, spontaneous repetitive firing of impulses was encountered frequently; this occurred less frequently with Rb. Repetitive firing could be suppressed by raising the divalent cation concentration in the medium surrounding the axon. As previously reported, it was not possible to maintain excitability with internal Cs when natural sea water was used externally (Baker *et al.*, 1962 *b*; Tasaki *et al.*, 1962). For this reason the Mg-rich external medium was used frequently in this series of experiments, instead of the natural sea water used elsewhere. Persistent repetitive firing rapidly reduced the action potential amplitude and blocked conduction.

Table II shows that the univalent cations examined are in the order: Rb > K > NH_4 > Na > Li. Rb is the most favorable, and Li the least favorable cation. Cs appeared to be slightly more favorable than Rb, but the difference was small, and to some extent appeared to depend on the external divalent cation concentration.

Divalent cations applied intracellularly (Ba, Sr, Ca, and Mg) were less favorable than the univalent cations presented in the table. It has been shown previously that Ca ion is less favorable than Mg ion (Tasaki, Watanabe, and Takenaka, 1962).

Several organic cations were also studied. Comparisons were made by dissolving the organic salts in a 400 mM K aspartate solution, and determining the lowest concentration required to block conduction. The intracellular concentration required to block conduction was roughly 1.0 mM for strychnine sulfate, 5.0 mM for tetracaine, and 5.0 mM for D-tubocurarine. It has been shown previously that $N(CH_3)_4$ ion is more favorable than the divalent cations above, but less favorable than Na (Tasaki and Shimamura, 1962). Then, the order for the organic cations is: $N(CH_3)_4$ > tetracaine, D-tubocurarine > strychnine.

To interpret these results, it must be noted that reduction of the action potential amplitude is observed only after a considerable delay following perfusion with unfavorable salt solutions (see Figs. 2 and 3). If a more favorable solution is substituted while the action potential is apparently normal, the effects of the less favorable salt are not observed. Diffusion across the thin layer of axoplasm takes place in roughly 1 minute, and is much shorter than the delay described. Therefore, the observed effects cannot be explained simply in terms of differences in the ionic mobilities within the membrane.

An alternative explanation is provided by the work of Bungenberg de Jong and others (Kruyt, 1949, pp. 283–285; Eisenman, 1961), who have shown that the cation order based on the relative cation affinities to negative sites ("ion fixation effect") varies with the chemical species of the negatively charged groups. When the cation sequence obtained for squid giant axons is compared to the cation sequences for various negatively charged colloids, it is

found that the cation sequence for intracellularly perfused squid giant axons is similar to the sequence found for phosphate colloids, but is quite different from the sequence observed for carboxyl or sulfate colloids. (The cation sequence observed for external media suggests that a carboxyl colloid is more likely at the external membrane surface, whereas a phosphate colloid is more likely at the internal surface.) In "dicomplex" formation by mixtures of phosphate colloids and proteins, the cation order for Cl salts established for their ability to suppress dicomplex formation is: Li > Na > K; and the anion order for K salts is: SCN > NO_3 and I > Br > Cl. The salt on the left of each inequality sign has the greater suppressing ability (Kruyt, 1949, p. 377; see also Bank and Hoskam, 1940). This order is the same as that found for intracellular perfusion of the squid giant axon. It appears reasonable, therefore, to propose that both the anion and cation sequences observed in squid axons are directly related to the effects of the ions on the charged protein and phospholipid groups in the membrane. Since procaine, strychnine, etc. have strong affinities to negatively charged colloids (Kruyt, 1949, p. 301), the effects of organic cations on neural excitability may be readily explained.

The axon membrane may be visualized as a macromolecular structure, primarily bound together by salt linkages. Such linkages are weakened, and can be disrupted, by neutral salts of sufficient concentration (see Kauzmann, 1959, p. 49). Disruption of these linkages would both loosen the tightly packed membrane structure and increase the density of ionized groups in the membrane. This would alter the permselectivity of the membrane, increase the membrane conductance, and abolish excitability. When cation affinity to fixed anionic sites in the axon membrane is great, the tendency to disrupt linkages between membrane macromolecules will be great, the survival time will be short, and the restoring ability of the cation will be small.

5. *Effect of Mixing Two Anions.* On a number of occasions a mixture of two K salts was used as the perfusion solution. The ratio of the more favorable to the less favorable anion (see section 3) was varied from 1:1 to 1:20. Aspartate, phosphate, and all the halides were used in various combinations, in a total of twenty axons. The total K ion concentration was maintained at 400 mequivalents/liter. Both survival time and restoring ability were used as criteria for evaluation of the effects of these mixtures.

In general, mixtures of a more favorable anion with a less favorable anion up to a ratio of 1:5 behaved as if the more favorable component alone were present. When the less favorable component was increased until the ratio was 1:20, the mixture behaved as if the less favorable component alone were present. For example, mixtures of KF (more favorable) and KBr (less favorable) in ratios up to 1:5 behaved as if the KF alone were present; mixtures

in a ratio of 1:20 behaved as if KBr alone were present. In axons in which conduction block occurred while being perfused with a mixture ratio of 1:20 or more, conduction could be restored by mixtures of 1:10 or less. There may be mixtures that behave in an intermediate fashion, but such behavior was not detected by the criteria used in the present study.

An apparent exception to the general behavior described above occurred with mixtures of 1:1 KF and K phosphate. In seven cases the mixture clearly was more favorable than either component alone, and could restore excitability after conduction was blocked during perfusion with either component alone. This synergism was also observed with mixtures of K phosphate with RbF. Perhaps related to this unexpected finding is the previous report of phosphate binding within the axon demonstrated by tracer studies (Tasaki, Teorell, and Spyropoulos, 1961).

It is not easy to explain why a 1:5 mixture of favorable and unfavorable salts in the perfusion solution behaves like a solution of the favorable salt alone. To explain behavior determined by the minor (favorable) component of the mixture, long-range forces need to be invoked. It is possible that such effects are derived from modifications of the "ice-like" structure of water (Frank and Evans, 1945; Frank and Wen, 1957), produced by (favorable) anions; *e.g.*, F ion is a potent modifier of water structure. Such modifications could affect intra- and intermolecular bonds extensively. Alternatively, the more favorable anions may stabilize the membrane structure by forming coordination compounds with intramembrane cations. Such coordination compounds normally present would not only provide a high degree of selectivity, but would also enable the rapid, reversible alterations of macromolecular structure necessary to the process of excitation.

6. *Effects of Maintained High Frequency Stimulation on the Perfused Axon.* The effects of high frequency stimulation on axon excitability were studied in axons perfused with various solutions of K salts. In all cases high frequency stimulation accerated the rate at which the action potential amplitude declined, and decreased the time required to block conduction. For a given high frequency, the time required by a perfused axon to develop intermittent conduction block varied with the lyotropic number of the anion used. Perfusion with the more favorable salts of the series permitted longer periods of stimulation before intermittent conduction block occurred. Total conduction block brought about by perfusion with one salt solution could be removed by switching to a more favorable perfusion solution (Fig. 5, left).

With no perfusion, at a stimulus frequency of 1 shock/sec., axons produced action potentials of roughly 120 mv for several hours. However, at a frequency of 200 shocks/sec. the action potential amplitude decreased to roughly 75 mv and intermittent conduction block occurred in about 30 minutes.

When the frequency was reduced at the time intermittent conduction block occurred, conduction was restored with a slight increase in amplitude (see Fig. 5, right, at 20 and 50 minutes). However, the increase in amplitude was transient, and shortly after reduction of the frequency (to as low as 10 shocks/sec.) conduction ceased. Finally, a stage was reached when small action potentials could be produced only at 1 shock/sec. (In other cases total con-

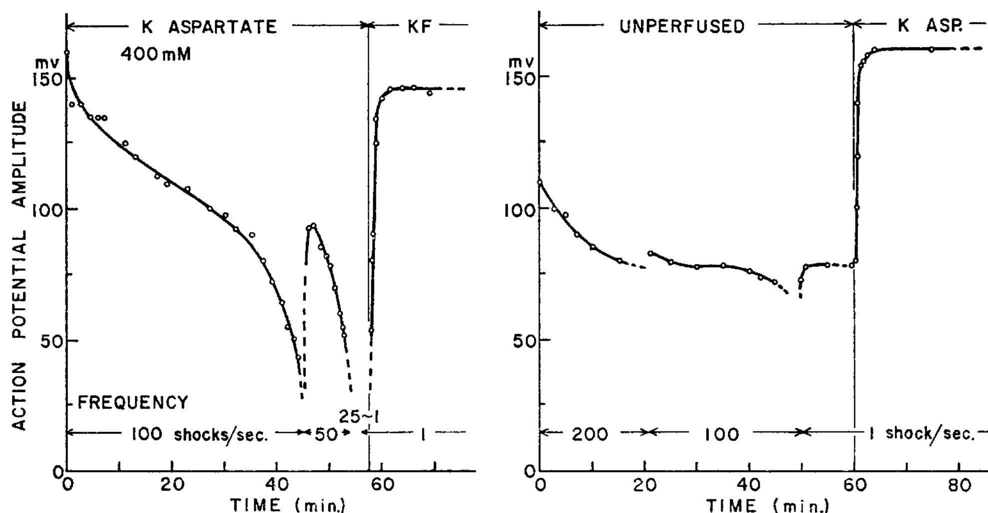

FIGURE 5. *Left.* Effect of high frequency stimulation on the perfused axon. The amplitude of the action potential is plotted as a function of time. The durations of the various frequencies of stimulation used are indicated above the abscissa. The interval between the 50 shocks/sec. period and the 1 shock/sec. period represents frequencies of 25, 10, and finally, 1 shock/sec., during which time no discernible potential changes were noted. The breaks in the curve represent those times when the frequency was changed and when the perfusion solution was changed from K aspartate to KF. Note the restoration of the action potential when perfusion solutions were changed. *Right.* Effect of high frequency stimulation on the unperfused axon. The first two breaks in the curve represent times during which the frequency was changed. The third break in the curve (at 60 minutes) represents the time when perfusion was instituted with K aspartate (with the stimulus frequency maintained at 1 shock/sec.).

duction block occurred.) Even at this stage, conduction of action potentials of at least normal amplitude could be restored by instituting perfusion with more favorable salt solutions (see Fig. 5, left).

It might be argued that institution of perfusion carries away some toxic metabolic products or accumulated unfavorable ions, or that the perfusion fluid provides some essential substance that has been exhausted. But, since the same high frequency effects are observed in continuously perfused axons as well as in unperfused axons (compare Fig. 5, left and right), some other explanation is needed. It is also unlikely that any possible K ion accumu-

lation externally (Frankenhaeuser and Hodgkin, 1956) can explain restoration of conduction by a perfusion solution with a different anion, but with the same K ion concentration, without additional assumptions. The major anionic components of the axoplasm are known to include isethionate, aspartate, glutamate, phosphate, and Cl (Deffner, 1961). Therefore, it is possible that replacement of the less favorable anions of this group (*e.g.*, Cl, isethionate) with the more favorable aspartate or F anions produced the recovery.

7. *Effects of the Removal of Axoplasm by Enzymes.* The effects of enzymes on the action potential and survival time of perfused axons have been described in some detail previously (Tasaki and Takenaka, 1964 *a*). In this study enzymes were employed to remove the axoplasm remaining in the perfusion zone. The purpose was to obtain axons that were free of axoplasm, with normal action potentials and survival times.

Perfusion was initiated with the enzyme-containing solution, and after a given time, changed to the same perfusion solution with no enzyme. The effectiveness of axoplasm removal was observed directly by adding talc or kaolin to the second perfusion solution, and by observing particle movement within the axon under the dissecting microscope. The type and concentration of the enzyme, the duration of exposure to enzyme, and the composition of the perfusion solution were varied. Most experiments were carried out with the enzyme added to 400 mM K aspartate or KF perfusion solutions. Other anions were clearly inferior, as might be expected from the anion sequence. The most effective enzymes were found to be trypsin, papain, and ficin. Trypsin is known to attack only proteins containing positively charged lysine or arginine side chains. Papain and ficin are endopeptidases of plant origin which attack a wide variety of proteins.

Axons were obtained with no visible axoplasm in the perfusion zone, and still capable of producing normal action potentials. Such axons were extremely sensitive to variation in the transmembrane hydrostatic pressure; large action potentials could be maintained only within a narrow pressure range. This finding is consistent with the observations of Baker *et al.* (1962 *a* and *b*), although no significant pressure effects were observed in axons not treated with enzymes (see Methods). Frequently action potentials of rectangular configuration, with a duration of greater than 500 milliseconds, were observed when excessive peptidase was used. (Action potentials of this type have been described previously (Tasaki and Takenaka, 1964 *a*).) The appearance of such action potentials can be taken to indicate that protease effects on the axon membrane are not merely non-specific injuries.

The conditions required to obtain axons without axoplasm were quite variable and the results were not easily reproducible. In general, excitability could not be maintained with enzyme concentrations greater than 1 mg/ml

perfusion fluid, even with duration of exposure less than 30 seconds. With lower concentrations of enzyme, increased duration of exposure was necessary to remove the axoplasm effectively. However, increase in the duration of exposure to more than 1 minute, even with enzyme concentrations decreased to 20 μg/ml, blocked conduction rapidly. The most effective combination, in terms of removing the axoplasm while retaining excitability, was 20 μg ficin/ml perfusion solution, for a duration of 1 minute. To a great extent the variability of results was related to axon diameter. Large axons (greater than 500 μ), which required either a greater concentration or longer exposure to remove the axoplasm, nevertheless remained excitable, whereas small axons (less than 400 μ) rapidly became inexcitable under the same conditions.

Moderate concentrations of enzyme, even for short periods of time, often produced such rapid dissolution of the axoplasm that the drainage cannula was blocked, and flow could not be resumed. This difficulty was overcome to some extent by initiating perfusion with the enzyme-free solution, changing to the enzyme-containing solution for 1 minute, and then returning to the enzyme-free perfusion solution. This procedure reduced the chance of blocking the drainage cannula with the large chunks of axoplasm removed by the enzyme, and also allowed the enzyme to work more uniformly within the perfused zone.

All the results described in previous sections could be reproduced in those axons in which the excitability remained after axoplasm removal.

8. *Possible Interactions between Favorable Anions and Divalent Cations.* Since several of the most favorable anions have appreciable divalent cation-combining properties, it was possible that reduction of the intracellular concentration of ionized divalent cations (*e.g.*, Ca ion) might be responsible for the restoring ability of the more favorable anions. Both F and phosphate ions strongly precipitate divalent cations, and glutamate, aspartate, citrate, and tartrate chelate with a variety of divalent cations. Since there is a continuous influx of Ca ions in perfused axons (Tasaki, 1963), perfusion with any of these more favorable salts would tend to lower the Ca ion concentration at and near the inner membrane surface. The pK values of the respective solubility product and chelation equilibrium constants for the Ca salts are, for pK_{sp}: fluoride = 10.31; phosphate = 7.0 (Meites, 1963); and for pK_{ch}: glutamate and aspartate = 1.6; tartrate = 1.8; citrate = 3.3 (Martell and Calvin, 1952, Appendix I). Although differences in anion valence make direct comparisons difficult, there is no simple correlation between the equilibrium constants and the anion sequence. Moreover, there are significant differences in the physiological effects among anions which have no appreciable Ca-combining properties whatever (*e.g.*, Cl, Br, SCN, etc.).

A more direct evaluation of reduced Ca ion concentrations was also under-

taken. Na-EDTA (sodium salt of ethylenediaminetetraacetic acid) was used as the Ca-combining agent. EDTA acts primarily as a Ca-chelating agent with a pK_{ch} greater than any anion used in perfusion. Concentrations of 5 mM EDTA (20 mM Na) were used in perfusion solutions containing 400 mM KBr or K aspartate, with glycerol carrier. This concentration of Na has no effect on squid axons (Tasaki and Takenaka, 1964 *b*), and Ca (pK_{ch} = 10.6) is significantly preferred by EDTA over Na (pK_{ch} = 1.6). In five axons EDTA had no effect on the action potential amplitude, survival time, etc. Perfusion fluid containing EDTA had no restoring ability other than that of the salt-glycerol carrier used. EDTA dissolved in unfavorable perfusion solutions had no effect on the restoring ability of favorable perfusion solutions. Since all these observations indicate that EDTA has no direct effect on excitability in the concentrations used, and since significant differences have been observed between several anions, none of which has appreciable Ca-combining properties, divalent cation binding does not seem to play a determinant role in establishing the anion sequence.

9. *Effects of Metabolic Inhibitors.* Since F is a well known inhibitor of glycolysis, a limited investigation of other metabolic inhibitors was undertaken. Iodoacetate, another inhibitor of glycolysis, had no effect on either survival time or on restoring ability of the favorable anions. This inhibitor of sulfhydryl enzymes was used in concentrations of 10 mM in a 400 mM KBr glycerol carrier in three axons. Other sulfhydryl inhibitors, iodoacetamide (1 mM) and *N*-ethyl maleimide (1 mM), had no demonstrable effects when included in a 400 mM KF perfusion solution.

A free amino group inhibitor, 5-dimethylamino-1-naphthalene sulfonyl chloride (1 mM), in a perfusion solution of 400 mM KF, had no significant effect. Neuraminidase, an enzyme which attacks neuraminic acid, in a concentration of 1 mg/ml perfusion solution (400 mM K aspartate, glycerol), had no effect on the perfused axon.

The effect of adenosine triphosphate (ATP) in the perfusion fluid was investigated in two axons. In concentrations of 1 mg ATP/ml perfusion fluid (400 mM KCl, glycerol), ATP had no effect on conduction block produced by the same perfusion solution without ATP. After ATP had no effect, conduction was restored in these same axons by the more favorable fluoride or phosphate perfusion solutions.

DISCUSSION

Various electrophysiological effects of neutral salts are described for intracellularly perfused squid giant axons. Under continuous intracellular perfusion, the chemical compositions of the solutions on both sides of the membrane are well defined. Since diffusion through the remaining axoplasm takes

place in less than 1 minute, the observed time-dependent changes in electrophysiological properties are regarded as signs of slow alterations of the physicochemical state of membrane macromolecules. The effects of neutral salts on macromolecules in general have been investigated extensively by Bungenberg de Jong (1949, p. 226).

Neutral salt effects on macromolecular biocolloids can be divided into three categories: (*a*) screening effect; (*b*) ion fixation effect; and (*c*) salting-out effect. The simple screening effect (*a*) arises from the formation of a Debye-Hückel atmosphere by the added ions around charged groups of macromolecules. This effect depends on the concentration and valence of the ions in the medium, and is insensitive to differences in the specific chemical properties of ions. The ion fixation effect (*b*) refers to binding of the ions studied to various oppositely charged groups on the macromolecules, and is dependent on the specific chemical nature of both the ions and the macromolecules. The salting-out effect (*c*) is attributed to alterations of water structure by various ions. This effect is independent of the charged groups on the macromolecules, since salting-out can occur in the absence of charged groups. (In the actual process of precipitation of macromolecules, effects (*a*) and (*b*) may be significant.)

In the reversal-of-charge experiments of Bungenberg de Jong (Kruyt, 1949, pp. 227 and 299), the anion affinities to positively charged groups of various proteins (ion fixation effects) decrease in the order: $\mathrm{SCN} > \mathrm{I} > \mathrm{Br} > \cdots > \mathrm{F}$, with SCN having the greatest affinity. In the macromolecular picture of the axon membrane, the inter- and intramolecular linkages between membrane macromolecules are required to maintain normal excitability. It is assumed that ions with great affinity to oppositely charged groups within membrane macromolecules strongly tend to disrupt such linkages. Then, SCN would be most effective, and F least effective in suppressing normal excitability.

The salting-out effect (*c*) of anions increases in the order: $\mathrm{SCN} < \mathrm{I} < \mathrm{Br} < \cdots < \mathrm{F}$, where SCN is least effective (Hofmeister, 1888; von Hippel and Wong, 1964). If it is assumed that hydrophobic bonds are important in stabilizing the membrane structure, then this effect (*c*) would be cooperative with effect (*b*) above. The simple screening effect (*a*) cannot explain the observed electrophysiological differences between different ions of the same valence and concentration. Hydrogen bonds present within the membrane would also be disturbed indirectly by the added salts (Hamaguchi and Geiduschek, 1962).

In contrast to the anions, cation affinities for negatively charged groups of protein are largely determined by the nature of the group concerned (Bungenberg de Jong, in Kruyt, 1949, pp. 284–289; Eisenman, 1961). For example, the order of affinity for protein carboxyl colloids is $\mathrm{Li} < \mathrm{Na} < \mathrm{K}$

< Rb, with Li having the weakest affinity; generally, the reverse order has been found for phospholipid colloids. In salting-out experiments, the behavior of cations is rather complex. Many examples may be found (Gortner *et al.*, 1928; Frey and Landis, 1932; Eckfeldt and Lucasse, 1943; Höber 1928; Haldi *et al.*, 1927), where the cation order changes with concentration, pH, and temperature, as well as with the nature of the colloid concerned.

There is good reason to suppose that ion fixation effects on salt linkages play the determinant role in ion sequences of the squid axon. With perfusion solutions of various salts (KI, KCl, NaCl, Na glutamate, etc.), the survival time decreases with increasing salt concentrations. It would be difficult to interpret this fact if membrane macromolecules are held together primarily by hydrophobic bonds, because more concentrated solutions of these salts are expected to strengthen the attraction between non-polar portions of macromolecules (*cf.* Kauzmann, 1959, p. 49). But the effects of neutral salts on salt linkages between macromolecules are very different. The rate of disruption of salt linkages increases with concentration for a given salt and, at a given concentration, it should increase with ion affinity to oppositely charged groups of the macromolecule. The results indicate that the cation sequence for the axon interior coincides with the order of cation affinity to phosphate groups.

In the macromolecular hypothesis amphoteric membrane macromolecules are assumed to possess significant numbers of charged groups which do not participate directly in intra- or intermolecular salt linkages. Since the isoelectric points of most biocolloids are on the acid side of neutrality, such excess charges are largely negative. These negative charge sites can be occupied by mobile cations from the surrounding medium and can be regarded as the sites of "fixed charges" in Teorell's theory of the ionic membrane (see Teorell, 1953). The membrane potential is then composed of at least three components: a diffusion potential within the matrix of the membrane, and phase-boundary potentials at each of the membrane interfaces. In a membrane with a high negative charge density, the phase-boundary potential varies with the logarithm of the activity of the mobile cation in the medium and is insensitive to large changes in anions (Teorell, 1953).

In the theory of nerve excitation proposed by one of us (Tasaki, 1963), the excitation process is considered to represent transitions between two stable states of the membrane. In the "resting" state the negative charge sites near the external membrane surface are occupied predominantly by external divalent cations. In the "active" state, these sites are occupied mainly by univalent cations. Between the two stable states, there is an "unstable state," *i.e.* within a range of concentration ratios uni- and divalent cations cannot coexist within the membrane. The potential variation associated with transition from the resting to the active state (*i.e.*, the action

potential) primarily represents variation in the phase-boundary potential at the outer interface. Following such a transition, the membrane potential gradually changes as the result of gradual alteration of the ionic composition in and near the membrane.

The concept of two stable states of the membrane also may be interpreted from a macromolecular point of view. According to Bungenberg de Jong *et al.* (see Kruyt, 1949, p. 416), a biocolloidal amphoion, a biocolloidal anion, and a suitable inorganic multivalent cation can form a "tricomplex system." In this process of complex formation, the inorganic multivalent cation plays an essential role in holding the two macromolecules together, probably by chelation. (The chelation process would provide an excellent means for achieving high ionic selectivity (Martell and Calvin, 1952, p. 204).) When various neutral salts of univalent cations are added to the solution, complex formation is suppressed. In a tricomplex consisting of phospholipid, protein, and divalent cations, protein molecules with carboxyl side chains can act as the colloidal anions. Since the affinity of such carboxyl groups to alkali metal ions increases in the order: Li $<$ Na $<$ K (see p. 284 in Kruyt, 1949; Bank and Hoskam, 1940), K salts should be more effective in disrupting the complex than Na or Li salts. This order corresponds exactly to the cation sequence determined by their ability to suppress excitability when applied to the squid axon externally.

The neural membrane becomes inexcitable without external divalent cations (Mg, Ca, or Sr). Metal ions which form very stable complexes (Ni, Co, etc.) alter the electric activity of axons profoundly (Spyropoulos, 1961). The macromolecular hypothesis qualitatively explains the ion sequence, classical univalent-divalent ion antagonism, abolition of action potentials by divalent cations, the effect of transition metals, etc., by treating the external membrane surface as a tricomplex.

It does not appear possible to interpret the major experimental findings (dilution effect, delayed and irreversible anion effects, etc.) simply in terms of differences in intramembrane ion mobilities. One early attempt to explain excitability phenomena in colloid chemical terms was made by Höber (1926, 1945). The present macromolecular hypothesis offers one reasonable interpretation of most of these phenomena, and future investigations are expected to produce more advanced macromolecular approaches to excitability.

Appendix

The lyotropic numbers for the anions studied were determined by a method similar to that discussed by Büchner and Postma (1931), Bruins (1932), and by Voët (1937). Gelatin was the colloidal material used to compare precipitating abilities of various salts. Both Na_2SO_4 and Na glutamate were used as standard salts, with consistent

results. The critical concentrations, expressed in equivalents per liter necessary to precipitate a 1.0 per cent gelatin sol at 39° C, were 1.2 for Na_2SO_4 and 1.05 for Na-glutamate. When a test salt was added to the gelatin solution, the critical concentration of standard salt was raised or lowered, depending on the nature of the test salt.

The results obtained with Na glutamate standard are shown in Fig. 6. With 1.0 equivalent/liter NaCl, the critical concentration of standard salt was 0.75 equivalent/liter; with the concentration of NaCl raised to 1.5, 2.0, and 3.0 equivalents/liter, the

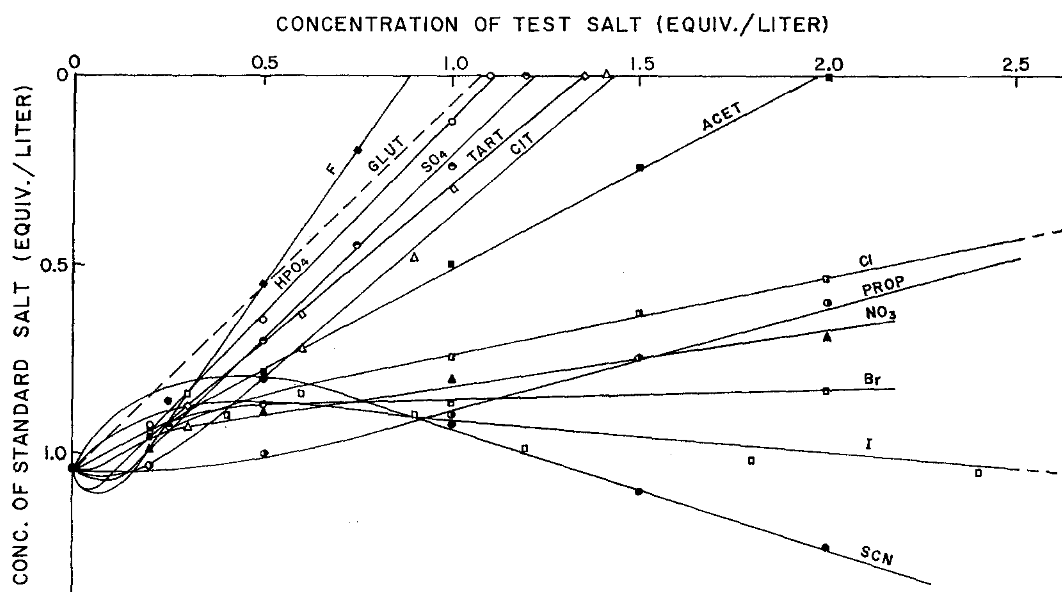

FIGURE 6. Precipitation of 1.0 per cent gelatin solutions by sodium glutamate in the presence of various other sodium salts. All determinations were made at a pH of 6.5–6.9 and a temperature of 37–39°C. The concentration of the standard salt (sodium glutamate, ordinate), necessary to produce visible precipitation of the mixture, in the presence of a given concentration of the test salt (abscissa), is plotted. A concentration of 1.05 equivalents/liter sodium glutamate alone will produce barely visible precipitation of the gelatin; this is represented by the dashed line (labeled GLUT), where sodium glutamate is both the test and standard salt, and the strictly additive concentrations produce a slope of 45°. (See text for details.)

respective critical concentrations were 0.63, 0.54, and 0.33 equivalent/liter of the standard salt. The relationship between the concentration of NaCl and the critical concentration of Na glutamate can be expressed by a straight line. The tangent of the angle θ between the straight line for Cl and the horizontal axis is +0.21. Among the salts tested, F had the greatest positive slope (tan θ being +1.45), and SCN had the greatest negative slope (−0.32). These anions are arranged according to the slopes of their respective straight lines.

To determine the lyotropic number $N(x)$ for the anion species x, only the slopes of the straight lines were considered (Bruins, 1932). An equation of the form

$$N(x) = A \tan \theta(x) + B$$

was employed, where $\theta(x)$ is the angle of the straight line for anion species x; A and B are constants which depend on the colloidal material (*e.g.*, agar or gelatin), but do not depend on anion species x. These constants were determined by selecting arbitrary numbers for $N(x)$ of two anions. Bruins adopted $N = 2$ for SO_4 and $N = 10$ for Cl, and Voët (1937) estimated that the lyotropic number for F was 4.8. In this study, A and B were determined for gelatin by adopting the numbers for Cl and F given by Voët. When the values $N(F) = 4.8$ and $N(Cl) = 10$ are adopted, together with the experimental values $\tan \theta(F) = 4.5$ and $\tan \theta(Cl) = 0.21$, it is found that $A = 4.3$ and $B = 10.9$ for the experiments with glutamate standard. The lyotropic numbers thus determined were: F, 4.8; phosphate, 5.9; glutamate and aspartate, 6.6; citrate, 7.2; tartrate, 7.4; propionate, 9.8; SO_4, 6.8; acetate, 8.7; Cl, 10.0; NO_3, 10.3; Br, 10.8; I, 11.2; SCN, 12.3. The lyotropic numbers determined with Na_2SO_4 standards are given under Results.

Deviations from straight line behavior at very low test salt concentrations are generally attributed to electrostatic effects (Hamaguchi and Geiduschek, 1962; von Hippel and Wong, 1964).

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