and may then apply the arguments given by Thomas.

It is worth noting that the condition given by equation (5) is the necessary and sufficient condition that the circulation be constant along the world lines of the fluid motion, cf. reference 2.

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FURTHER OBSERVATIONS ON RESTING AND ACTION POTENTIAL OF INTRACELLULARLY PERFUSED SQUID AXON

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The squid giant axon can still produce action potentials even when its interior is continuously perfused with artificial saline solutions.[1, 2] In a previous report[3] the electrophysiological properties of axons intracellularly perfused with solutions of potassium, ammonium, and cesium salts were described. The present report deals with perfusion with solutions of sodium chloride, sodium sulfate, tetramethylammonium, and other salts. The results have an important bearing on the possible mechanism of production of the resting and the action potentials.

Observations.—Experiments were carried out on the North Atlantic squid, *Loligo pealii*, which is available at the Marine Biological Laboratory at Woods Hole. The technique of intracellular perfusion was the same as described previously.[2, 3] The standard (or control) perfusing fluid was 0.4 *M* potassium sulfate solution mixed with an equal volume of 1.2 *M* sucrose solution. The pH was adjusted to 7.3 by adding a small amount of phosphate buffer. The perfusing fluid entered the axon through a small glass pipette (150μ to 250μ outside diameter) inserted longitudinally through a hole in the nerve membrane at one end. The fluid drained out through a large glass pipette (roughly 350μ) or through a large opening in the membrane at the other end of the axon. Stimuli were delivered through either a pair of electrically isolated platinum electrodes (*St* in Fig. 1, top) or an intracellular silver wire electrode (*St* in Fig. 3). Intracellular potentials were recorded either with a glass micropipette electrode (*M* in Fig. 1) or by means of an insulated silver wire with a small bare spot near its middle (*Re* in Fig. 3). A Tektronix oscilloscope and pulse generators, Grass stimulators and camera, and a unity-gain cathode-follower were used for stimulation and recording.

In giant axons immersed in natural sea water, perfusion with the standard fluid increases the amplitude of the action potential slightly. Nerve impulses are conducted across the perfused zone (8 to 12 mm) for 2 hours or more. The effect

of diluting this standard fluid with sucrose solution was discussed in the previous report.[3]

Perfusion of an axon with an isotonic NaCl solution abolishes its excitability within 1 minute. When the NaCl solution is more dilute, however, conduction continues across the perfused zone for a considerable length of time. The crucial factor, essential for maintenance of excitability under these conditions, is a proper balance between the divalent and the univalent ions in the surrounding medium. Figure 1 illustrates this point.

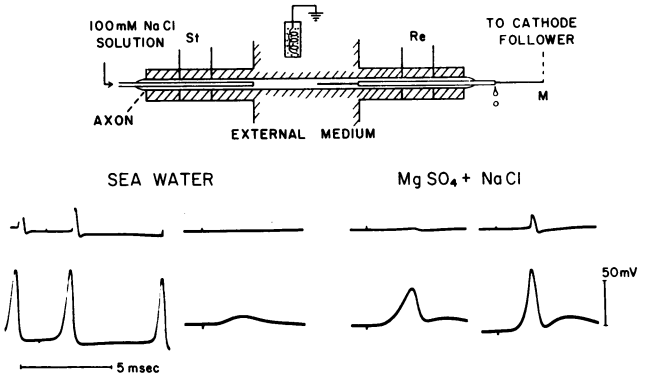

FIG. 1.—Top: Schematic illustration (not to scale) of the experimental arrangement employed for intra-cellular perfusion, and the recording of potentials of excised squid giant axons. *St*, stimulating electrodes; *Re*, extracellular recording electrodes; *M*, intracellular micropipette electrode. Upper oscillograph trace shows electric responses recorded from *Re* and lower trace responses obtained from *M*. External fluid medium was first (left) natural sea water and later (right) isotonic sodium chloride solution to which magnesium sulfate (200 mM) had been added. Room temperature was 23°C.

Using the experimental arrangement shown in Figure 1, a giant axon (approximately 500μ in diameter) was perfused with 100 mM NaCl (sucrose) solution with its pH adjusted to 7.3. About 15 seconds after the onset of perfusion, spontaneous firing of nerve impulses began. The amplitude of the action potential and the resting potential both declined rapidly (Fig. 1, bottom left). At approximately 40 seconds after the onset, conduction of nerve impulses across the perfused zone ceased. The magnesium concentration in the surrounding medium was then raised, keeping the sodium concentration at about the same level by simply replacing the surrounding sea water with a solution containing 200 mM $MgSO_4$ and 500 mM NaCl with its pH adjusted to 8.0. Both the resting potential and action potential recovered rapidly (Fig. 1, bottom right). Under these experimental conditions, conduction across the perfused zone could be maintained for 30 minutes or more.

The rate of flow of the perfusing fluid through the interior of the giant axon in these sodium perfusion experiments was of the order of 15 mm^3 per minute. 15 mm^3 is roughly 10 times the volume of the axoplasm that remains in the perfused zone. The recording micropipette electrode was filled, as a rule, with 1 *M* ammonium chloride solution. In most of the present experiments, the external medium

as well as the perfusate was potassium-free. It is therefore very unlikely that a significant amount of potassium ion remained in the layer of axoplasm.

When the perfusing fluid contained sodium sulfate instead of sodium chloride the results were essentially the same (see Figs. 2*B* and 3). Complete substitution of the chloride in the surrounding medium with sulfate does not alter the action potential of a sodium-perfused axon appreciably (Fig. 2*B*). Calcium chloride added to the surrounding (artificial or natural) sea water also effectively prevents depolarization by sodium-perfusion.

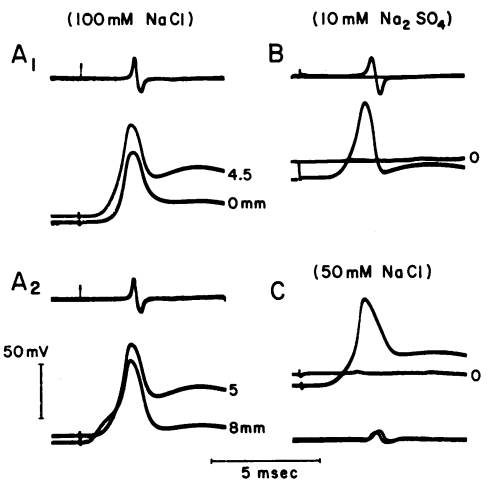

FIG. 2.—Records A_1 and A_2: Comparison of electric responses recorded at four different points in a sodium-perfused zone of squid giant axon (approximately 500μ in diameter). Distances are given from the distal end of the perfused zone. Records were taken about 18 minutes after onset of continuous perfusion with 100 mM NaCl solution. The outside medium contained 500 mM NaCl and 300 mM $MgSO_4$. Small responses in the upper oscillograph trace indicate conduction across the sodium-perfused zone. Record *B:* Resting and action potential of a giant axon perfused with 10 mM Na_2SO_4 and immersed in a medium containing 120 mM Na_2SO_4 and 480 mM $MgSO_3$. Record *C:* Similar to *A* except that the perfusing NaCl solution was more dilute. Oscillograph traces marked *"O"* were obtained with electrodes placed in the outside medium.

The electric responses recorded in the sodium-perfused zone do not represent mere spread of potential from the unperfused zones. They are actually developed by the membrane in the perfused zone. The responses recorded in the middle of the perfused zone are larger in amplitude and longer in duration than the responses at the boundaries between the perfused and unperfused zones. In records A_1 and A_2 of Fig. 2, the numerals indicate the distances between the tip of the recording micropipette and the end of the perfused zone, which was 8 mm long in this case. The action potential amplitude was largest in the middle of the perfused zone. This clearly indicates the existence of a real response accompanied by an inward-directed membrane current in this area. Furthermore, the responses developed in the perfused zone showed a long plateau while those derived from the unperfused zones had a normal, short duration (see Fig. 2).

The resting membrane potential in the sodium-perfused zone was, under the present experimental conditions, definitely smaller than that in the unperfused zone. Records *B* and *C* in Fig. 2 show the resting and action potentials in the perfused zone in relation to the potential level in the surrounding fluid medium. Although the resting membrane potential was found to vary with the intracellular cation concentration, this relation was often obscured by the dependence also of the potential upon the extracellular divalent ion concentration.

With the experimental arrangement of Figure 1, top, the perfused zone occupies a limited portion of the excised giant axon. It seemed worthwhile, therefore, to examine whether or not the presence of excitability could be demonstrated in axons in which much longer portions were perfused with solutions of sodium salts.

Perfusion of 16 to 22 mm long sections of an excised giant axon was accomplished

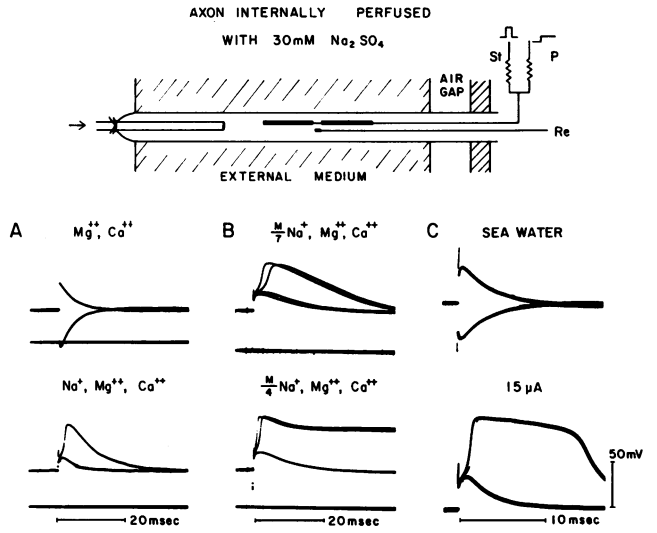

FIG. 3.—Top: Schematic illustration (not to scale) of the experimental arrangement used for internal stimulation and the recording of electric responses of a sodium-perfused squid giant axon. Exposed portions (11 mm in total length) of metal wire electrodes are shown by thick lines inside the axon. The external medium contained, in the experiment of column *A*, top, 120 mM $CaCl_2$ and 240 mM $MgCl_2$; in bottom of *A*, 250 mM NaCl, 100 mM $CaCl_2$ and 200 mM $MgCl_2$; in top of *B*, 143 mM ($1/7$ *M*), NaCl, 114 mM $CaCl_2$ and 228 mM $MgCl_2$; in bottom of *B*, same as in bottom of *A*, in *C*, potassium-free sea water. In column *C*, bottom, an anodal polarizing current of 15 μA was applied to the axon through terminal *P*. The voltage calibration (50 mV) applies to all records.

by introducing an inlet pipette at one end of the axon and slowly pushing it along the axis of the axon until the tip reached the other end. The perfusing fluid was then forced into the pipette by raising the pressure of the fluid. Next, while the fluid was continuously flowing out of the cut end of the axon, the pipette was gently withdrawn until its tip reached the final position shown in Fig. 3. The flow of fluid could be maintained under these conditions for 10 minutes or more until the channel in the axoplasm became clogged. When such clogging took place the pipette was advanced again toward the cut end of the axon. Contamination of the surrounding fluid medium by the flowing perfusing fluid was prevented by a narrow air gap between the open end of the axon and the main portion of the axon chamber.

The portion of the axon perfused with sodium salt solutions by this technique was actually capable of developing all-or-none action potentials in response to stimuli delivered through a long internal metal wire electrode. Record *A*, top, in Figure 3 was taken from an axon which was immersed in an external medium containing only magnesium chloride and calcium chloride. It was perfused with 30 mM sodium sulfate solution for 13 minutes. No action potential was evoked by short current pulses, 0.1 msec in duration and 0.1 to 0.5 mA/cm² in intensity. However, the addition of sodium ion (250 mEq/*l*) to the outside medium restored its excitability (Fig. 3*A*, bottom).

A slight change in the ratio of the external sodium concentration to the magnesium and calcium concentrations can markedly alter the configuration of the action potential of a sodium-perfused axon (Fig. 3*B*).

A sodium-perfused axon which has been rendered inexcitable by immersion in artificial sea water can develop a large all-or-none action potential when it is polarized with a constant current of the order of 50μA/cm^2 (Fig. 3*C*). The configuration of the action potential observed under such anodal polarization resembles in many instances that of the responses obtained by Mueller[4] from Ranvier nodes of a frog nerve immersed in sodium-free media.

An experiment is now in progress which is designed to determine whether or not there is a change in the membrane impedance during the action potential developed in a sodium-perfused axon. Since a high-frequency alternating current applied through a small extracellular metal electrode does not spread along the axon, such an impedance measurement should give direct information as to the excitability at any desired spot along the membrane. An all-or-none potential variation in the sodium-perfused zone should almost certainly be accompanied by a simultaneous reduction in the membrane impedance, as in the case of a normal axon.[5]

We have also tested the excitability of the axon membrane under perfusion with solutions of tetramethylammonium (TMA), choline, guanidine, and several other organic cations. When the levels of sodium, magnesium, and calcium ions in the outside medium were properly adjusted, conduction continued across a 10 mm zone perfused with these salt solutions for periods of as much as 20 to 60 minutes. The action potentials developed in the TMA-perfused zone were prolonged (Fig. 4*A*) but the configuration of the action potential of a TMA-perfused axon could be brought back to normal by perfusing with the standard perfusing fluid (Fig. 4*B*).

The resting potential of the interior of an axon perfused with 10 mM TMA

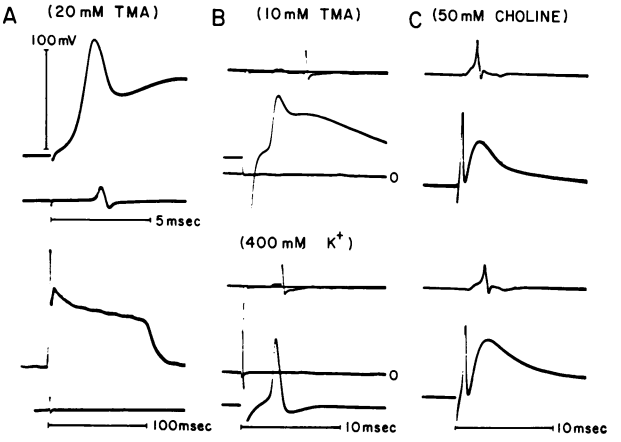

FIG. 4.—Action potentials of 3 squid giant axons (500 to 600 μ in diameter) intracellularly perfused with tetramethylammonium (TMA) chloride or with choline chloride. The concentrations of the salts in the perfusing fluid are given. The external medium was Mg-rich, K-free, sea water which contained 430 mM $MgSO_4$ and 140 mM NaCl in all cases.

chloride is above the potential of the surrounding fluid medium. Because of the uncertainty in the junction potentials of the recording electrodes and in the phase-boundary potentials at various interfaces in the membrane (see below), however, we do not attach much significance to the absolute values of the membrane potentials under these circumstances.

In the portion of an axon perfused with a dilute choline chloride solution the main action potential is followed by a slow potential change (Fig. 4*C*). This slow late potential change develops gradually, and an appropriate increase in the calcium concentration in the surrounding medium suppresses it without eliminating the main action potential. In the experiment of Figure 3*C*, conduction failed about 14 minutes after the onset of perfusion. At lower concentrations of choline chloride (10 to 20 mM), conduction across a choline-perfused zone could be maintained for 20 to 40 minutes.

Discussion.—It is already known that an unperfused squid giant axon retains its excitability in sodium-free media.[6, 7] Now we have demonstrated all-or-none action potentials in a squid axon membrane that separates two potassium-free solutions. Both univalent (Na) and divalent (Mg or Ca) cations must be present in the system, however, if it is to produce large discrete action potentials. Since the membrane potential can rise and fall in the absence of potassium on both sides of the axon membrane, we are unable to interpret the present findings in terms of any theory in which the fall in the membrane potential is attributed to an efflux of potassium (see Hodgkin and Huxley[8]). Furthermore, chloride ions are not essential on either side of the membrane for the production of all-or-none action potentials.

Our interpretation of the present experimental findings is as follows: We visualize a squid axon membrane as consisting of many chemically different layers, namely, axoplasm, axonal membrane, Schwann cell membranes, Schwann cell cytoplasm, connective tissue, etc.[3] The membrane as a whole is permselective in the sense that it is more permeable to cations than to anions.[9] This is evidence for the existence of negative fixed charges in at least one layer of the membrane, probably in the axonal membrane. We assume that in the resting state many of the negative sites are occupied by divalent cations. An outward-directed (stimulating) current through the membrane favors invasion of the intracellular univalent ions into the axonal membrane, thereby reducing the proportion of the negative sites occupied by divalent ions. We expect that this sudden change in the species of the predominant (positive) counter-ion in the membrane profoundly alters the membrane potential. In this model the configuration of the action potential is determined by the rate of exchange between univalent and divalent cations at the negative sites of the membrane. Similar views have been proposed in the past by several investigators who have emphasized the importance of calcium ions in excitation phenomena (see Brink[10]).

Summary.—The excitability of the squid giant axon membrane can be maintained under continuous intracellular perfusion with solutions of sodium chloride, sodium sulfate, tetramethylammonium chloride, guanidine sulfate, or choline chloride. The concentrations of sodium, magnesium, and calcium ions in the outside medium must be adjusted properly to maintain excitability under such intracellular perfusion. The resting membrane potential varies, depending upon

the extracellular concentration of divalent cations. Replacement of chloride on both sides of the membrane with sulfate has little effect on the membrane potentials. The experimental findings support our view that change of univalent for divalent positive counter-ions at negatively charged sites in the membrane is the electrochemical basis of nerve excitation.

Note added in proof: Measurements of the electric impedance of the squid axon membrane under sodium-perfusion are now completed, demonstrating that the action potential is accompanied by a simultaneous reduction in the membrane impedance under these conditions.

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*ON THE MECHANISM OF TISSUE RECONSTRUCTION BY DISSOCIATED CELLS, I. POPULATION KINETICS, DIFFERENTIAL ADHESIVENESS, AND THE ABSENCE OF DIRECTED MIGRATION**

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That the mutual architectural relationships assumed by cells in vertebrate tissues and organs are determined by intrinsic properties of the individual cells themselves has been demonstrated by experiments with dissociated embryonic cells. The diverse cells of an organ or a body-region, thoroughly intermixed and allowed to commingle in an appropriate culture medium, form aggregates in which the cells first appear to be distributed at random but then sort out from one another, taking up the mutual positions peculiar to them in the intact organism.[1–3]

The roughly concentric arrangement which such reconstructed tissues often tend to assume within a compound aggregate has led several authors to suggest possible mechanisms for the sorting-out process. Townes and Holtfreter[3] propose that the different kinds of cells first migrate either centripetally or centrifugally along a radial concentration gradient established within an aggregate, and then exercise a selectivity in their mutual adhesiveness by means of which like cells are bound together strongly while unlike cells adhere to greater or lesser degree. We have pointed out[4] that differences in mutual adhesiveness among cells can alone account for both sorting out and selective localization, since cells of a type which cohere strongly, when moving among and adhering to those of a more weakly cohesive type, could by their own progressive cohesion squeeze the other cells to the periphery and thereby as-