ORIGIN OF AXON MEMBRANE HYPERPOLARIZATION UNDER SUCROSE-GAP

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ABSTRACT One of the disadvantages of the sucrose-gap method for measuring membrane potentials with extracellular electrodes is a membrane hyperpolarization of the order of 30 to 60 mv, as compared with the resting potential obtained with intracellular microelectrodes in the absence of a sucrose-gap. In the present study the contribution of the sucrose–sea water junction potential to this hyperpolarization effect has been evaluated by comparing the effects on the resting potential of several anion and cation substitutions in the sea water bathing the lobster giant axon under sucrose-gap. Measurements with microelectrodes demonstrate a significant liquid junction potential between sucrose and standard artificial sea water. The value of this liquid junction potential as well as the measured resting membrane potential varies as a function of the anions and cations substituted in the sea water. Both the liquid junction potential and the sucrose-gap–induced hyperpolarization can be eliminated by using a low mobility anion to replace most of the chloride in sea water while the normal cation content remains unchanged. These data provide evidence that loop currents at the sucrose–sea water-axon junctions are at least partly responsible for membrane hyperpolarization under a sucrose gap.

INTRODUCTION

The technique of utilizing a high resistance sucrose solution as an insulator to aid in the measurement of nerve membrane potentials with external electrodes was introduced by Stämpfli in 1954. Flowing sucrose solution streams serve to maintain a high external leak resistance between a segment of normal membrane (the so-called "node") bathed by flowing sea water and a depolarized region of the axon bathed by flowing isotonic KCl. Julian, Moore, and Goldman (1962*a*) adapted this technique for use with single, lobster giant axons. In particular, they found the so-called "sucrose-gap" method well suited to the study of current-voltage relations in the lobster axon under a voltage clamp (1962*b*). They, as well as other workers (Narahashi et al., 1964; Stämpfli, 1963), drew attention to the fact that a possible drawback to the method was the observation that under a sucrose-gap, resting membrane potentials, as well as action potentials, were always some 30 to 60 mv

greater than when intracellular microelectrodes were employed in the absence of the sucrose-gap. Furthermore, Julian et al. (1962*a*) also observed this hyperpolarization effect with microelectrodes when the sucrose-gap was applied. These observations lead to the conclusion that the membrane potential observed with external electrodes in the presence of a sucrose-gap is quite close to the potential existing across the "nodal" membrane, excepting for a slight leakage IR drop (see Julian et al., 1962*a*). Thus, the hyperpolarization appears to be a result of the sucrose-gap itself rather than of the electrodes or any other part of the recording system.

The problem, then, has been to provide an explanation for the hyperpolarization associated with the sucrose-gap technique. In this regard, Julian et al. (1962*a*) found that the replacement of chloride ion by the supposedly nonpenetrating methylsulfate ion in the sea water bathing the active membrane resulted in a 15 to 20 mv drop in the recorded "resting" membrane potential without a significant resistance change. On the other hand, when pyroglutamate, another supposedly nonpenetrating ion, was the anion in the solution bathing all three regions of the axon, and when the solution bathing the central node was then replaced by chloride sea water, the resting potentials were increased by some 20 to 30 mv. These results suggested that the chloride ion played a significant role in the resting potential as recorded with sucrose-gap methods.

In 1941, Steinbach reported that the chloride ion concentration of the axoplasm was markedly reduced as a consequence of bathing squid giant axon in hypertonic sucrose. Along the same line, Julian et al. (1962*a*) found that during the sucrose-gap experiment, the resistance of the axoplasm between the active node and the depolarized end increased with time. Thus, loss of internal chloride from the region of the axon directly under the sucrose, possibly leading to a diffusion potential, was considered to be another possible cause of the hyperpolarized resting potential.

Still another suggestion was that inward current flow through the node, possibly as a result of hyperpolarization of the membrane directly under the sucrose, might account for the observed hyperpolarization of the central node.

Recently Stämpfli (1963) demonstrated that the interposition of a vaseline seal between the sucrose and the saline eliminated the sucrose-gap hyperpolarization effect in frog myelinated fibers. He therefore suggested that the hyperpolarization was a result of local inward current driven through the node by the liquid junction potential of the sucrose-saline boundary.

The present study was undertaken in order to analyze the effect of the sucrose–sea water-axon junction on the observed membrane hyperpolarization in an effort to resolve the origin of this hyperpolarization.

METHODS

The sucrose-gap techniques for measuring membrane current and voltage have been described in detail by Julian et al. (1962*a*). The single giant axon from the circumesopha-

geal connective of the lobster, *Homarus americanus*, has been used for all of the studies to be described. In brief, the method consists of arbitrarily separating the isolated axon into three regions by the use of flowing, deionized isotonic sucrose (see Fig. 1). The central region, or artificial node, was made some 50 to 70 μ wide by adjusting the perfusion rates. The potential difference between this pool and one of the side pools, which was depolarized by isotonic potassium chloride (V-pool), was used as a measure of the membrane potential. Current for the elicitation of action potentials, or for voltage clamping was injected into the axon via the third pool (I-pool). The three regions of

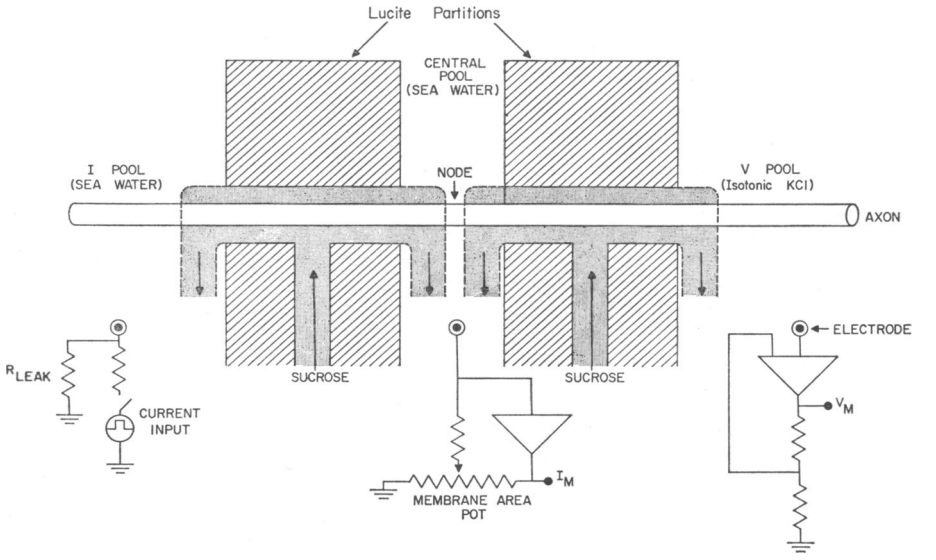

FIGURE 1 Diagram of arrangement (not to scale) used for recording membrane potentials of lobster giant axon with sucrose-gap methods (after Julian et al., 1962*a*). The I-pool is used for injecting current, the central pool for measuring current flow across the membrane in the gap, and the V-pool, for measuring the membrane potential. The gap or "node" is the exposed area of axon membrane between the two sucrose flows (hatched areas) in the central pool. Also shown are the circuit arrangements for the current and voltage measurements.

the axon were connected to the electronic equipment via agar KCl bridges leading to calomel half cells. With sucrose flows separating the sea water pools, and with the axon removed, the resistance between the sea water pools was of the order of 40 meg.

Microelectrodes for potential measurement were filled with 2.5 M KCl and had resistances of 5 to 10 megohms. In isotonic KCl tip potentials were about 3 to 6 mv and all measurements to be reported below were corrected for the tip potentials. The ionic compositions of the various sea waters tested are listed in Table I. All solutions were adjusted to pH 6.3 to 6.6 by addition of either HCl or tris(hydroxymethyl) aminomethane where necessary.

In order to measure the sucrose–sea water liquid junction potentials, both the agar KCl electrode and the microelectrode were first placed in flowing sea water in the nerve chamber, and the potential between the two electrodes was recorded. The microtip was

TABLE I

IONIC CONCENTRATIONS OF VARIOUS SEA WATERS TESTED*

	Cations‡				Anions			
Sea water§	Na^+	K^+	Other ion		Cl^-	$SO_4^=$	Other ion	
	meq/l	*meq/l*		*meq/l*	*meq/l*	*meq/l*		*meq/l*
NaCl (normal)	465	10	—	—	533	8	—	—
NaBr	465	10	—	—	68	8	Br^-	465
NaI	465	10	—	—	68	8	I^-	465
$NaNO_3$	465	10	—	—	0	0	NO_3^-	541
NaSCN	465	10	—	—	58	8	SCN^-	475
$NaBrO_3$	465	10	—	—	68	8	BrO_3^-	465
NaCl + NaAc	465	10	—	—	270	8	Ac^-	263
$NaMeSO_4$	465	10	—	—	68	8	$MeSO_4^-$	465
NaGlut	465	10	—	—	68	8	$Glut^-$	465
NaIseth	465	10	—	—	68	8	$Iseth^-$	465
NaAc	465	10	—	—	0	0	Ac^-	541
NH_4Cl	0	10	NH_4^+	465	533	8	—	—
TMA Br	0	10	TMA^+	465	68	8	Br^-	465
LiCl	0	10	Li^+	465	533	8	—	—
TEA Br	0	10	TEA^+	465	68	8	Br^-	465
TEA Cl	0	10	TEA^+	465	533	8	—	—
Iso KCl	0	541	—	—	541	0	—	—
Iso KNO_3	0	541	—	—	0	0	NO_3^-	541
Iso KAc	0	541	—	—	0	0	Ac^-	541
Iso K pyroglut	0	500	—	—	0	0	$Pyroglut^-$	500

* All sea waters adjusted to pH 6.3 to 6.6. HCl or tris(hydroxymethyl)aminomethane added where necessary to bring solutions into this pH range.

‡ In addition, all sea waters except the isotonic K sea waters contained 50 meq/l Ca^{++} and 16 meq/l Mg^{++}.

§ Abbreviations: Ac^- = acetate, $Glut^-$ = glutamate, $Iseth^-$ = isethionate, $MeSO_4^-$ = methylsulfate, $Pyroglut^-$ = pyroglutamate, TMA^+ = tetramethylammonium, TEA^+ = tetraethylammonium.

then moved into an adjacent region of flowing, deionized sucrose and the potential was again recorded. The difference between these two potentials (sucrose minus sea water) was taken as a measure of the sucrose–sea water liquid junction potential. From 5 to 40 measurements were made on each of the test sea waters, and for most of the test solutions, determinations were carried out with several different microelectrodes. With few exceptions, there was good agreement between consecutive measurements.

The following checks were used to test the validity of this method for the measurement of the liquid junction potentials:

(*a*) The sucrose-isotonic KCl liquid junction potential was of the order of −3 mv. This suggests that the sucrose itself had a negligible effect on the microelectrode tip potential.

(*b*) When the microtip was kept in the sucrose stream while the solution under the agar KCl electrode was changed, the relative changes in liquid junction potentials agreed with data obtained by the method described above.

(*c*) When two agar KCl electrodes were used with one electrode either in a sucrose or isotonic KCl pool, and the solutions under the second electrode (in a pool separated from the first by flowing sucrose) were changed, results similar to those from the microelectrode method were obtained. Thus, the measurements did not appear to be affected by the type of electrodes used.

The sucrose-gap voltage clamp methods of Julian, Moore, and Goldman (1962*b*) were employed for studies on the influence of hyperpolarization and of the sucrose–sea water liquid junction potentials on membrane current-voltage relations.

RESULTS

Resting Membrane Potential and Current Measurements. Fig. 2 shows the effect of various anions on the "resting" membrane potential and membrane current. The upper curve shows that in the presence of normal (chloride) sea water the resting potential is of the order of −120 mv. Following replacement of all anions by acetate (Section 2 of the diagram), the membrane potential is reduced by about 50 mv; and as seen in sections 3, 4, and 5, this is a reversible phenomenon. At the end of section 5 there was a sudden, temporary drop in the resting potential. While the axon was in normal sea water there was a spontaneous recovery and the experiment was therefore continued. As seen in sections 6, 7, and 8, replacement of chloride by bromide has no significant effect on the normal "resting" membrane potentials, as observed under the sucrose-gap.

The lower curve in Fig. 2 is a trace of "resting" membrane current measured with the circuit completed by a leak resistance to ground (R_{leak} of Fig. 1) of about 30 meg; inward current is in the downward direction. From the trace we can therefore

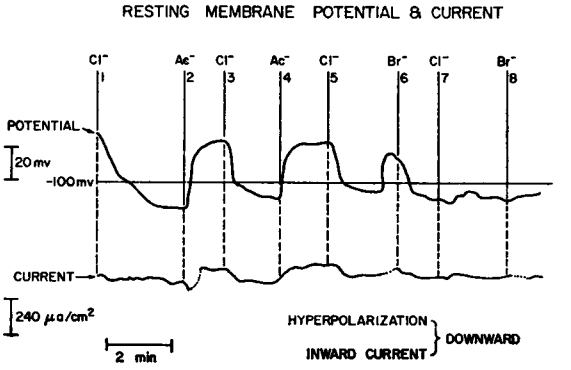

FIGURE 2 Influence of several anions on "resting" membrane potential (upper curve) and change in membrane current (lower curve). Horizontal line through the upper curve is at a level of −100 mv. For the lower curve, increasing inward current is in the downward direction. There was a large current flow (of the order of 3 ma/cm²) at all times, due to leakage (through the leak resistance shown in Fig. 1); consequently, only the changes in membrane current are seen in this curve. In sections 1, 3, 5, and 7, the central pool was bathed with normal (NaCl) sea water. Acetate sea water was used in sections 2 and 4, and bromide sea water was used in sections 6 and 8.

see that the hyperpolarization effect is associated with a significant increase in inward current through the central node of the axon as compared with the current obtained in the presence of acetate sea water. There is little difference between chloride and bromide ions with respect to this inward current.

The difference in current flow between sections 1 and 3, and 2 and 4 of Fig. 2 corresponds to an increased inward current of about 90 μ/cm^2 when normal sea water bathes the central pool as compared with acetate sea water. This is close to the expected current of about 120 μ/cm^2 calculated from Julian's gap membrane resistance value of about 1 meg (1962*b*) and a membrane hyperpolarization of 40 to 50 mv.

Fig. 3 shows the action potentials from the same experiment as that of Fig. 2 and it is readily apparent that the overshoot relative to zero poential (the upper horizontal line in each of the pictures) is about the same in all the sea waters tested.

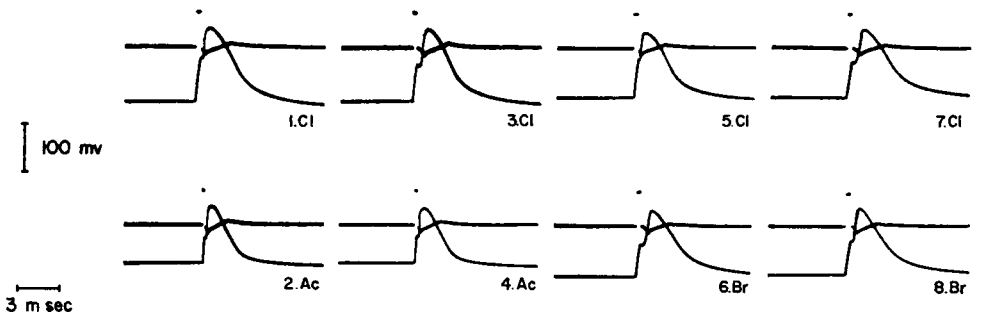

FIGURE 3 Action potentials for same experiment as in Fig. 2. Upper horizontal line in each frame is at "zero" potential. Numbers at lower right of each frame refer to the section numbers of Fig. 2.

This is to be expected if the sodium equilibrium potential is unchanged. The amplitude of the action potential is then determined primarily by the membrane resting potential and the action potential appears to be affected by the various anions only insofar as they affect the "resting" potential. Furthermore, the general shape of the action potential appears to be unaffected by the various ions tested in this particular experiment. None of the anions tested (see Table I) affected the shape of the action potential significantly.

Liquid Junction Potential Measurements. Fig. 4 shows the relationship between the measured sucrose–sea water liquid junction potential and the change in resting membrane potential for various anions and cations bathing the central node, as compared with our standard sea water (predominantly NaCl). In all cases where anions were tested, Na^+ was the predominant cation. In most experiments in which we varied the predominant cation, Cl^- was the predominant anion present. The

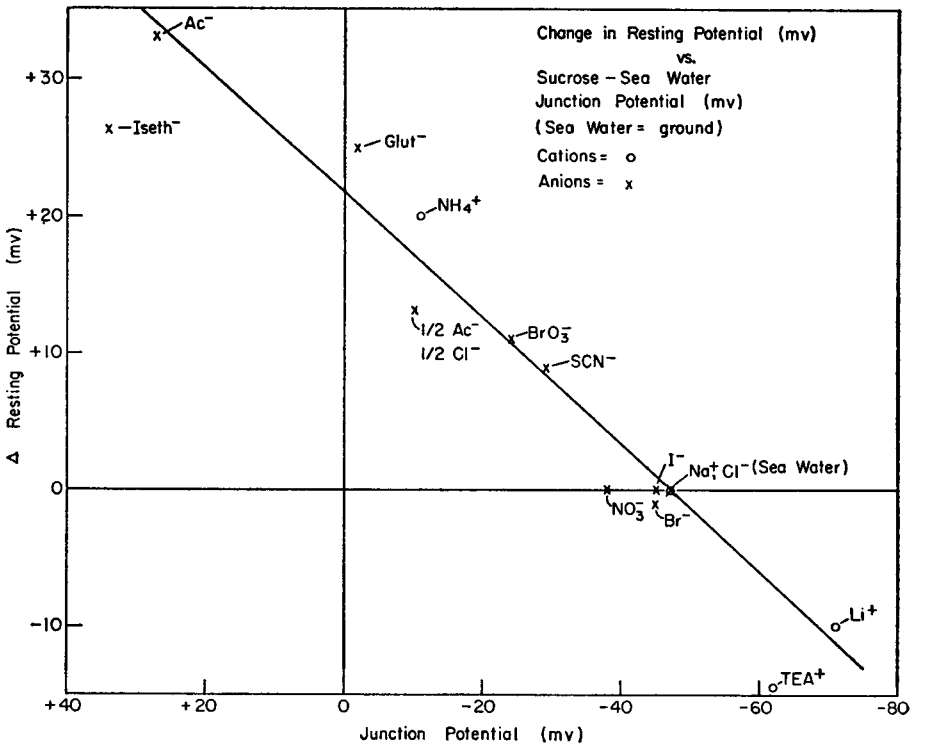

FIGURE 4 Graph of change in resting membrane potential versus sucrose–sea water liquid junction potential for several different sea water compositions, the predominant anion (x) or cation (o) of which is shown. A negative change in potential is in the hyperpolarizing direction. Note where not shown, the predominant cation was Na^+, or the predominant anion was Cl^-. (See Table I for complete ionic composition of these solutions.)

two exceptions in the case of the cation tests involved tetraethylammonium (TEA^+) and tetramethylammonium (TMA^+), when we used the bromide salts of these cations. We also tested TEA^+Cl^-, and found that there was a negligible difference between its effect and that of TEA^+Br^- on the sucrose–sea water junction potential (see Table II). Note that there is an approximate straight line relationship between the liquid junction potential and the change in resting membrane potential. If we assume a normal resting potential of −70 to −75 mv (see Dalton, 1958 and Julian et al., 1962*a*), the line of Fig. 4 would be expected to cross over the zero liquid junction potential axis at about +30 − +35 mv, since the zero "resting" potential of the graph refers to the hyperpolarized potential obtained in normal (NaCl) sea water. Thus, according to the graph, it would appear that a reversal of the normal sucrose–sea water junction potential rather than merely elimination of this potential is required in order to abolish the hyperpolarization effect.

TABLE II

EFFECT OF VARIOUS IONS ON RESTING V_m AND SUCROSE–SEA WATER JUNCTION POTENTIALS

Cation‡	Concentration	Anion‡	Concentration	Ion mobility* λ_+°	Ion mobility* λ_-°	ΔR.P.§	Sucrose–sea water junction pot.
	meq/l		*meq/l*			*−mv*	*mv*
Na^+	465			50.1			
						0	−47
		Cl^-	533		76.4		
Na^+	465	Br^-	465	50.1	78.2	−1	−45
Na^+	465	I^-	465	50.1	76.9	0	−45
Na^+	465	NO_3^-	541	50.1	71.4	0	−38
Na^+	465	SCN^-	475	50.1	67**	+9	−29
Na^+	465	BrO_3^-	465	50.1	55.8	+11	−24
		Ac^-	270		40.9		
Na^+	465			50.1		+13	−10
		Cl^-	263		76.4		
Na^+	465	$MeSO_4^-$	465	50.1	—	+17	—
Na^+	465	Glut.$^-$	465	50.1	29**	+25	−2
Na^+	465	Iseth.$^-$	465	50.1	—	+26	+34
Na^+	465	Ac^-	541	50.1	40.9	+33	+27
NH_4^+	465	Cl^-	533	73.4	76.4	+20	−11
Na^+	465	Cl^-	533	50.1	76.4	0	−47
TMA^+	465	Br^-	465	45.0	78.2	−9	—
Li^+	465	Cl^-	533	38.7	76.4	−10	−71
TEA^+	465	Br^-	465	32.7	78.2	−15	−62
TEA^+	465	Cl^-	533	32.7	76.4	−17	—
K^+‖	541	Cl^-	541	73.5	76.4	0	−3
K^+‖	541	NO_3^-	541	73.5	71.4	0	—
K^+‖	541	Ac^-	541	73.5	44.5	−22	+38
K^+‖¶	500	Pyroglut.$^-$	500	73.5	—	−20, −30	—

* Ion mobility data (at 25°C) from Harned and Owen, 1958, except those denoted by a double asterisk which were extrapolated from data in the International Critical Tables (Washburn, 1929).

‡ For complete composition of these solutions, see Table I.

§ Change in measured membrane "resting" potentials as compared with normal (NaCl) sea water. Negative values mean the resting membrane was hyperpolarized, compared with the membrane in normal sea water.

‖ Isotonic K sea waters were tested in the V-pool, and compared with isotonic KCl. Normal (NaCl) sea water was present in the other two pools for these experiments. Thus, for example, with KCl in the V-pool and normal sea water in the other pools, when KAc was switched into the V-pool, the membrane became further hyperpolarized by some 22 mv.

¶ Data from Julian et al., 1962*a*.

Fig. 5 provides a comparison between ion mobility and the change in resting membrane potential. Note that the use of either a high mobility cation or a low mobility anion, both of which decrease the liquid junction potential, results in a reduction of the hyperpolarization effect. A summary of the data comparing liquid

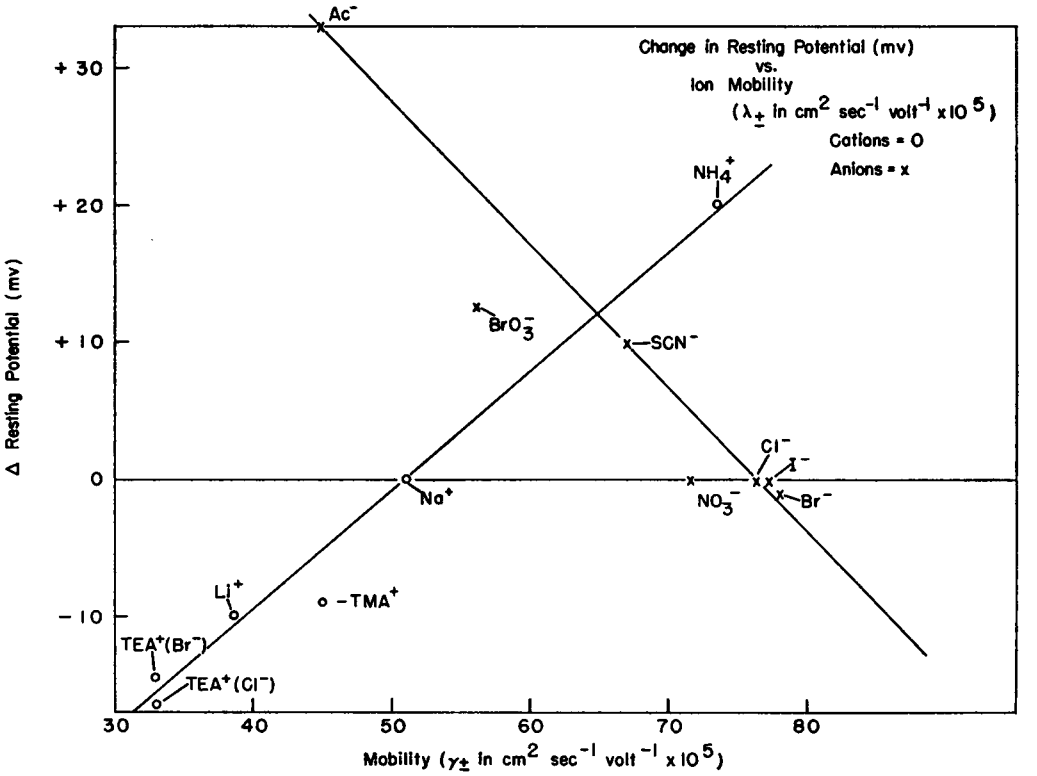

FIGURE 5 Graph of change in resting membrane potential versus ion mobility for several different sea water compositions. When the cation (o) was varied, the predominant anion was Cl^- except for the TEA^+Br^- (as shown). Conversely, when the anion (x) was changed, the predominant cation was always Na^+ (see Table I).

junction potential, change in "resting membrane" potential, and ionic mobility is presented in Table II.

If, in these experiments, chloride was the major anion present in all three pools, and if then a high mobility anion such as bromide or nitrate was substituted into either or both of the side pools, there was no significant change in the resting membrane potential. If, however, the substituted anion in either of the side pools was acetate, the observed resting potential increased. This observation suggested that the liquid junction potentials between the sucrose and the sea waters in the side pools also played a role in the hyperpolarization phenomenon and that the contribution toward the observed membrane potential was in the opposite direction from the contribution by the liquid junction potential between the sucrose and the central sea water pool.

Effect of Sucrose-Gap Width on Measured Membrane Potential. Julian et al. (1962*a*) found that when the sucrose flows were turned on at the start of an

experiment, with normal sea water flowing in all three pools, the side pool used to measure the membrane potential (V-pool) rapidly became 20 to 60 mv negative with respect to the central pool. This did not appear to result from any damage to the portion of axon in the side pool, and was believed to be a direct effect of the sucrose flows alone. When isotonic KCl was switched into the V-pool, there was a further fall in the potential of that pool, of as much as 75 mv.

We have had similar experience with the turning on of the sucrose flows. However, we have noted that when the sucrose flows are turned on with normal sea water in all three pools, potential differences of 40 to 60 mv between central pool and V-pool (V-pool negative) develop only if the sucrose-gap width (i.e., the width of the central node bathed by sea water) is of the order of the axon diameter or less.

The sucrose-gap width versus membrane potential data shown in Fig. 6 were obtained from one of our axons in which all three pools were bathed with normal sea water. With a very large gap width (of the order of 300 μ or more), there was a small asymmetry potential, possibly due to injury to the axon end, between the central pool and the V-pool. As the gap width was narrowed (curve 2*A*), the measured potential increased, reaching a plateau at a potential of the order of 60 to 70 mv, when the gap width was less than about 90 μ. Conversely, as the gap width was increased (curve 2*B*), the membrane potential fell. When acetate sea water was used in place of normal sea water for this experiment, the curves were much more variable and frequently there was only a small variation in membrane potential with large changes in gap width over the range of 45 to 450 μ. Furthermore, narrow-

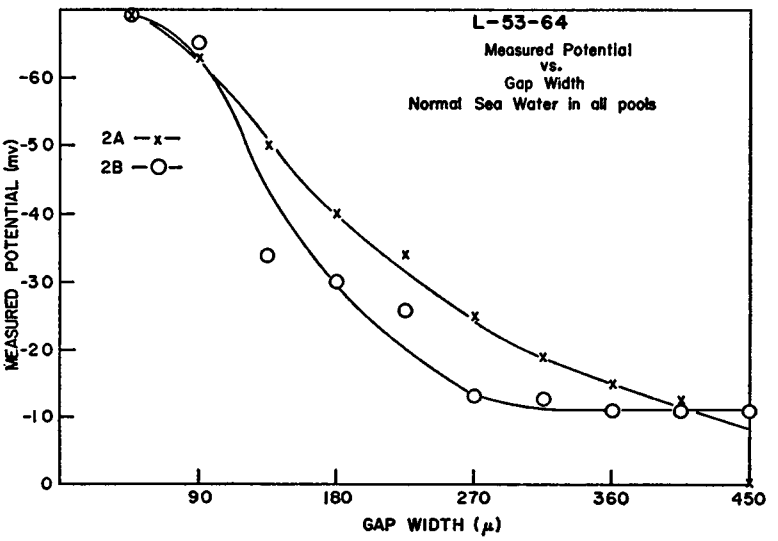

FIGURE 6 Effect of sucrose-gap width on potential measured between central and V-pools with normal sea water in all three pools. Curve 2*A* shows effect of narrowing gap width. Gap was then widened (curve 2*B*). Axon diameter = 100μ.

ing the gap frequently caused a decrease in the measured membrane potential when acetate sea water was used.

Note that if the asymmetry potential is substracted from all the points on the two curves of Fig. 6, the curves reach a plateau at a value of about −55 mv for very narrow gap widths. This value is fairly close to the measured sucrose–normal sea water liquid junction potential of −47 mv. Such effects provide further evidence that the sucrose–sea water liquid junction potential plays a significant role in the measured membrane potential.

Influence of Anions and of Liquid Junction Potential on Membrane Current-Voltage Relations. Fig. 7 compares the effects of acetate sea water and normal (chloride) sea water on current voltage relations in the lobster axon. As shown in

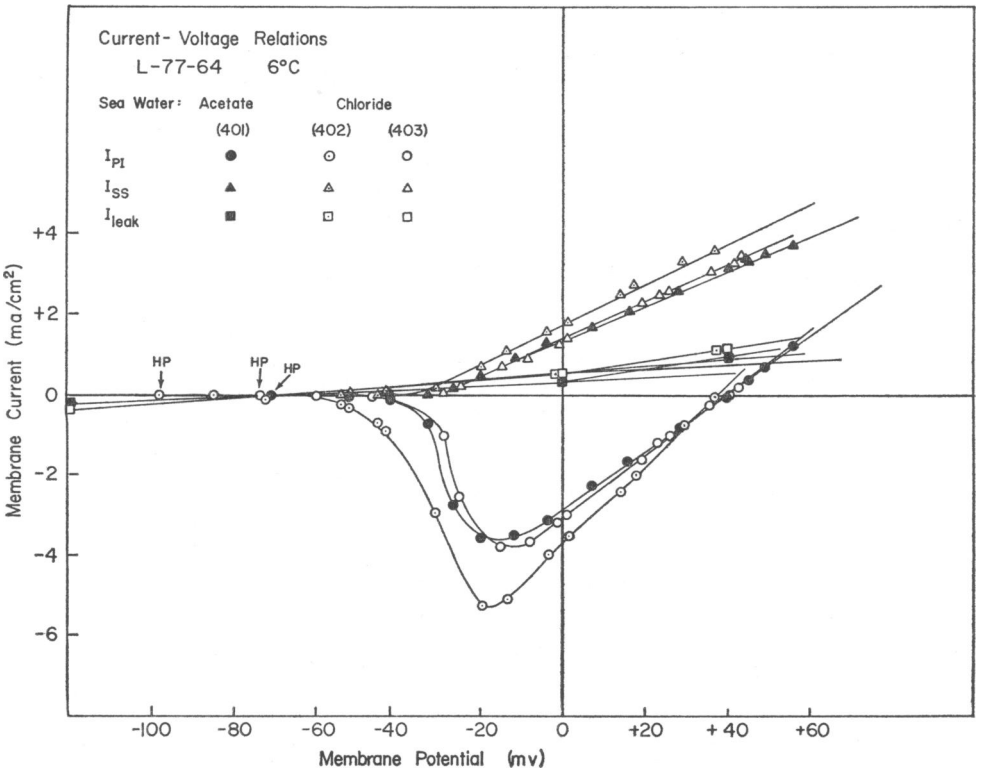

FIGURE 7 Current-voltage relations of lobster giant axon in normal (chloride) and in acetate sea water. The first run was made in acetate sea water (solid symbols) with the holding potential (H.P.) at about the resting potential in this solution. Normal sea water was then turned on and a run was made with the holding potential at the new resting potential (dotted open symbols). The holding potential was then reduced to about the same level as in the first (acetate) run, and another run in normal sea water was made (open symbols). Curves were corrected for leakage using the assumption that E_{leak} (the equilibrium potential for the leakage current) was unaffected by changes in the holding potential.

the figure, there is a negligible difference between the curves in acetate sea water and in normal sea water when the holding potential is the same (curves 401 and 403 respectively). The holding potential for the clamping sequences used to obtain these two curves was very close to the resting potential for the axon in acetate sea water. Curve 402 was obtained in normal sea water when the axon was clamped at a potential very close to the "resting" potential in this solution. It is obvious that the peak initial (sodium) currents are significantly larger at most depolarization steps in the latter case; thus, the membrane sodium conductance (calculated as: $g_{Na} = I_{Na}/(V_M - V_{Na})$ where I_{Na} is the peak sodium current at a step potential, V_M, and and V_{Na} is the potential at which the peak initial current reverses direction) is also larger. This is most likely attributable to the expected decrease in resting sodium current inactivation in a hyperpolarized axon (Hodgkin and Huxley, 1952). There also appears to be a small increase in the steady-state (potassium) current at large depolarization steps, but no significant change in the slope conductance ($g'_K = \Delta I_K/\Delta V_M$, where I_K is the steady-state current). Similar studies with various other anions such as bromide and nitrate demonstrate a negligible influence of these anions on current-voltage relations in lobster axon.

DISCUSSION

Effects of Anions and Cations on Axon Membrane. Before considering the hyperpolarization problem in the light of the experimental evidence presented above, it seems imperative to discuss briefly the influence of the various ions employed in the present study on the normal (unhyperpolarized) membrane. Horowicz (1964) has recently reviewed the effects of anions on excitable cells. Although there are some minor disagreements in the literature (cf. Wilbrandt, 1937; Straub, 1956; Hashimura and Osa, 1963; and Koppenhöfer, 1965), it appears, in general, that replacement of the chloride in the external fluid by nitrate, thiocyanate, pyroglutamate, lactate, or pyruvate may cause a slight hyperpolarization of the resting axon membrane potential usually of the order of 2 to 4 mv. Frankenhaeuser (1962) found that replacement of chloride by methylsulfate or isethionate did not affect the delayed (steady-state) currents of a myelinated axon under a voltage clamp. In muscle fibers, too, there is a slight hyperpolarization when certain anions are added, notably, thiocyanate, nitrate, iodide, and bromide (see Höber, 1945; and Horowicz, 1964).

Among the monovalent cations, lithium (see Shanes, 1958) and the quaternary ammonium ions, tetramethylammonium and tetraethylammonium appear to have a negligible effect on resting membrane potential in nerve (Lorente de Nó, 1949), while ammonium ion has a slight depolarizing action, at least at the motor end plate (Furukawa et al., 1956).

In a few studies of our own, we checked the effects of bromide, iodide, nitrate, acetate, and lithium on the resting membrane potential of the lobster giant axon with

microelectrode techniques in the absence of the sucrose-gap. Our results were in agreement with the findings of the studies just quoted in that there appeared to be no significant effect of these ions on the measured resting membrane potentials and action potentials. These studies therefore lend support to our contention, based on the data presented above, that in the presence of a sucrose-gap the effects of the various anions and cations tested on the measured resting membrane potential are not likely to be significantly influenced by the direct effects of these ions on the membrane since such effects have been shown to be quite small.

The Liquid Junction Potential and Hyperpolarization by the Sucrose-Gap. The data presented in Table II show that with large differences between the ionic mobilities of the predominant cation and anion in the test sea waters, a significant liquid junction potential develops. These liquid junction potentials thus appear to be accounted for by the relative ionic mobilities; the greater the disparity between the anionic and the cationic mobilities, the greater the liquid junction potential. Furthermore, there is a linear relationship between the sucrose–sea water liquid junction potential and the measured "resting" membrane potential. The fact that the slope in Fig. 4 is less than one (less than a 1 mv change in resting potential per millivolt change in liquid junction potential) could be due to a number of factors which ought to be considered; (*a*) The condition of the axon membrane lying immediately under the flowing sucrose is essentially unknown. Decalcification of the axon membrane and the leakage of ions from the axoplasm to the outside leads to an increase in the axoplasmic resistance (Steinbach, 1941 and Julian et al., 1962*a*). Since the ratio of internal to external potassium is increased, one might expect the "resting" membrane potential of the region of axon under the sucrose to be increased. Direct measurement of this potential has proven very difficult, but several attempts have given values of −90 to −120 mv across the membrane under the sucrose. Changes in ionic content of the axoplasm under the sucrose might give rise to diffusion potentials in the axoplasm which could also contribute to the hyperpolarization phenomenon. (*b*) Although there appears to be no reason to comment upon the relative potential values obtained in the different solutions tested, the absolute magnitudes of these potentials are open to question. All of the reported measurements were made in flowing solutions, and one of the two electrodes was a KCl-filled micropipette electrode. The validity of these microelectrode measurements has been discussed above (see section on Methods). Nevertheless, the possible unknown effects of streaming on potentials measured with microtips is another factor to be considered. (*c*) The ionic content of the side pools also plays a role in determining the potential across the membrane in the "gap." In particular asymmetries such as one side pool filled with KCl, and the other with normal (NaCl) sea water, may affect the "gap" membrane. An anion-cation combination which, when flowing in the central pool tends to hyperpolarize the membrane, will have a depolarizing effect on the "gap" membrane when used in one of

the side pools (see, Table II, bottom and Julian et al., 1962*a*). Since isotonic KCl is normally used in the V-pool, and since this solution does not give rise to a significant liquid junction potential with sucrose, no difficulties are ordinarily encountered from this source. (*d*) The direct effects of the various anions and cations on the axon membrane have already been discussed.

Origin of the Hyperpolarizing Potential under a Sucrose-Gap. In spite of the reservations just discussed, our data show that the sucrose–sea water liquid junction potential has a significant effect on the measured resting membrane potential under a sucrose-gap. The next question to be answered is: how does the liquid junction potential influence the resting potential, and how may the results of the effect of gap width (Fig. 6) be explained?

The electric circuit diagram seen in Fig. 8*b* is a simplified equivalent of the axon membrane under a sucrose-gap, in which all capacitances have been omitted since we are dealing only with a steady-state system. For the sake of simplicity, the very low resistances of the system (e.g. sea water resistance) have also been omitted. Fig. 8*c* shows an expanded circuit diagram of the region of membrane in the gap. Because the latter diagram is only concerned with a very short length of axon, the axoplasmic resistance has also been omitted here.

Since resistance is proportional to path length, the effective resistance through R_s' is very small compared with the R_s path (Fig. 8*b* and *c*), so that almost all of the current flow driven by the liquid junction potentials uses the R_s' path, giving rise to loop currents i_1 and i_2. Thus, as shown in Fig. 8*a*, we visualize these current loops as concentrated in the region of the sucrose where axon, sucrose, and sea water all meet.

Recently, Tomita and Wright (1965) described a modification of the sucrose-gap technique in which the sucrose was replaced by oil, leaving a thin film of sucrose around most of the axon. They reported action potentials with amplitudes of up to 150 mv and resting potentials of up to 86 mv. If the sucrose-gap effect is absent, these results appear to be at variance with the action potential data of Dalton (1958) and of Julian et al. (1962*a*), and our own data obtained with microelectrode methods. On the other hand, the results of Tomita and Wright could be considered a manifestation of sucrose-gap hyperpolarization, because (as just discussed) only a very small volume of sucrose at the sucrose–sea water-axon junction may be needed to produce significant hyperpolarization. Diffusion of ions into the thin sucrose layer might account for the relatively smaller hyperpolarization which they reported. Viewed in this light, the results of Tomita and Wright could be considered as evidence that only a thin layer of sucrose around the axon is required to produce the hyperpolarization effect.

Using the circuits of Fig. 8*b* and *c*, we have made some rough estimations of the contribution of the central pool sucrose–sea water liquid junction potential (V_{s_2}) to the measured membrane potential (V_0). From Fig. 8*b* and *c*,

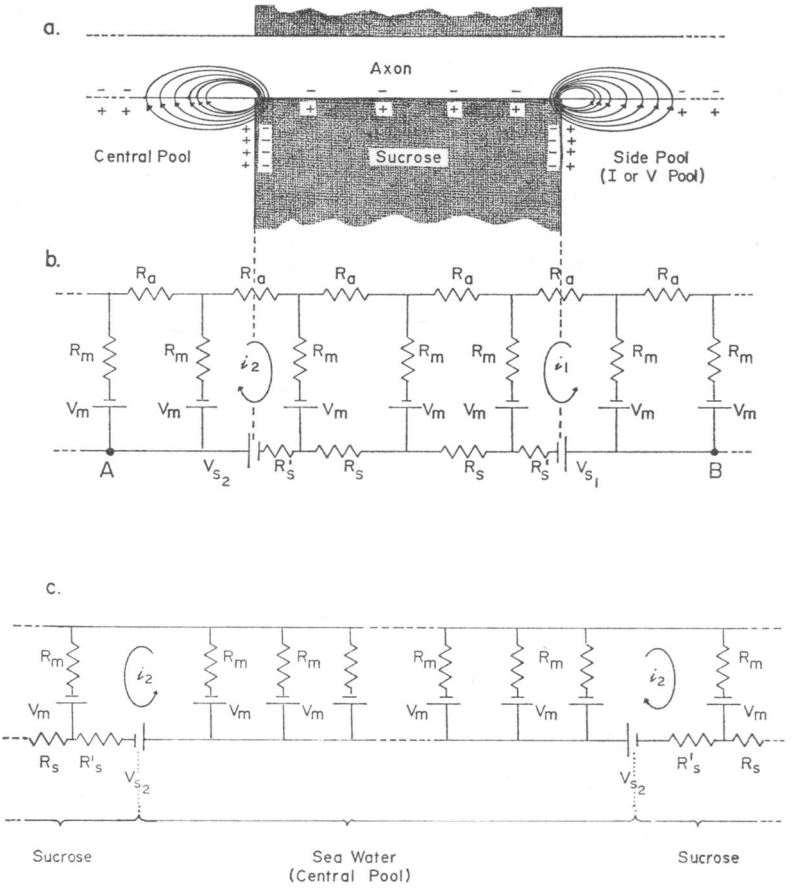

FIGURE 8 *a.* Diagram of one half of the sucrose-gap apparatus with the axon in place, showing liquid junction potentials and loop currents at the axon–sea water-sucrose junctions (see text). The "side pool" refers to either the I-pool or V-pool of Fig. 1. *b.* Electric circuit analogue of portion of apparatus shown in *a*. Potential normally measured between points *A* and *B*. R_a, axoplasmic resistance; R_m, membrane resistance; R_s, resistance through bulk of sucrose; R'_s, effective resistance of sucrose at axon sea water–sucrose corners; V_m, membrane potential; V_{s_1} and V_{s_2}, sucrose–sea water liquid junction potentials of side pool and central pool, respectively; i_1 and i_2, loop currents at axon-sea water–sucrose corners of side pool and central pool, respectively. *c.* Circuit analogue of central pool and the sucrose flows on both sides. Axoplasmic and sea water resistances omitted since only a very short length of axon is involved here. Note that in *b* and *c*, axoplasmic diffusion potentials, and differences in R_m and V_m for portions of axon membrane under sea water and under sucrose, have been omitted for simplicity.

$$R_{gap} = \frac{R_m}{n},$$

where R_{gap} is total resistance of the portion of membrane lying in the central sea water pool (the "gap") and n is the number of units of resistance R_m in parallel. As the gap width is narrowed, R_{gap} approaches the value of R_m. It can then be shown that:

$$V_0 = V_m + 2V_{s_2}\left[\frac{R_m}{2R_m + R_s'}\right], \tag{1}$$

where V_0 is the potential measured between points A and B of Fig. 8*b* when the side pool is depolarized by isotonic KCl.

If R_m is significantly greater than R_s' (as is likely to be true for regions in the sucrose lying very close to the gap margin), then:

$$V_0 \simeq V_m + V_{s_2}. \tag{2}$$

For experiments in which normal sea water is used, equation (2) states that the measured resting membrane potential (V_0) may be as much as 50 mv hyperpolarized, in comparison with the normal resting membrane potential (V_m), due to the effect of the liquid junction potential. Indeed, we frequently measure resting potentials as high as −130 to −140 mv in the lobster giant axon under sucrose-gap (Julian et al., 1962*a*; Narahashi et al., 1964). Since a much wider gap is employed when using sucrose-gap methods with the larger squid giant axon, this illustration may help to explain why membrane hyperpolarization is much less of a problem in the squid axon experiments (Moore et al., 1964).

Omitted from consideration in the simplified circuits of Fig. 8*b* and *c* are some of the ancillary factors which might be expected to contribute to the hyperpolarization effect. These include the results of mobile ion depletion from the region of the axon directly under the sucrose, and the direct effects of the various substituted ions on the "gap" membrane. Both of these factors would be expected to promote the hyperpolarization effect.

This problem may also be treated in terms of continuous cable theory. Using this approach, the equivalent statement to the electric circuit treatment above, is that when the sucrose-gap width is large, hyperpolarization is only observed close to the sucrose surface. On the other hand, when the gap width is small, the hyperpolarization potential is observed throughout the central pool.

During membrane depolarization (for example, during the transient depolarization phase of a voltage clamp experiment), when the membrane resistance is markedly reduced, we would expect that the hyperpolarization effect of the sucrose-gap would be minimal. As seen in Fig. 7, this expectation is borne out by experiment. There is a negligible difference between the curves in chloride and acetate sea water at the same low "holding" potential, while the differences between the

two chloride curves with the voltage clamped at different holding potentials may be explained on the basis of the known effects of membrane potential on sodium inactivation (Hodgkin and Huxley, 1952). In fact, because of this latter effect of membrane potential on sodium inactivation, it is standard procedure either to clamp at a hyperpolarized resting potential or to employ a prehyperpolarizing step immediately prior to a depolarizing step even when sucrose-gap methods are not employed.

CONCLUSIONS

On the basis of the data presented here, the hyperpolarized resting membrane potentials observed when sucrose-gap methods are employed appears to be largely accounted for by loop currents arising at the sucrose–sea water-axon junctions and driven by the sucrose–sea water liquid junction potentials although several other factors may also contribute to this effect. The hyperpolarization has a negligible effect on membrane current voltage relations, aside from the effect of the potential per se on sodium inactivation. Choice of an anion with appropriate mobility, to replace the chloride of normal sea water, may eliminate the hyperpolarization without introducing significant untoward effects. Although the hyperpolarization may be eliminated in this manner, current-voltage relations appear to be unaffected despite this superimposed potential. Sucrose-gap methods therefore appear capable of providing valid data when employed with voltage clamp procedures.

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