

Increased Chloride Conductance As the Proximate Cause of Hydrogen Ion Concentration Effects in *Aplysia* Neurons

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ABSTRACT A fall in extracellular pH increased membrane conductance of the giant cell in the abdominal ganglion of *Aplysia californica*. Chloride conductance was trebled whereas potassium conductance was increased by 50%. Half the giant cells were hyperpolarized (2–8 mv) and half were depolarized (3–10 mv) by lowering the pH. The hyperpolarizing response always became a depolarizing response in half-chloride solutions. When internal chloride was increased electrophoretically, the hyperpolarization was either decreased or changed to depolarization. The depolarizing response was reduced or became a hyperpolarizing response after soaking the cell in 10.0 mM chloride, artificial seawater solution for 1 hr. Depolarization was unaffected when either external sodium, calcium, or magnesium was omitted. A glass micropipette having an organic liquid chloride ion exchanger in its tip was used to measure intracellular chloride activity in 14 giant cells; 7 had values of 27.7 ± 1.8 mM (SEM) and 7 others 40.7 ± 1.5 mM. Three of the first group were hyperpolarized when pH was lowered and three of the second group were depolarized. In all six cells, these changes of membrane potential were in the direction of the chloride equilibrium potential. Intracellular potassium activity was measured by means of a potassium ion exchanger microelectrode.

INTRODUCTION

The preceding paper (Brown and Berman, 1970) showed that the effect of CO₂ on some *Aplysia* neurons was due to a fall in extracellular pH which in turn caused an increase in membrane conductance at or near the resting potential. The increased conductance led to depolarization and increased the rate of discharge in some cells although half the giant cells studied were hyperpolarized.

The ions involved in the increased conductance are unknown. DeMello

and Hutter (1966) showed that increased external hydrogen ion concentration (pH 8.5 to 4.5) increased anion conductance in crustacean (*Astacus*) muscle fibers and Reuben, Girardier, and Grundfest (1962) reported that a reduction of external hydrogen ion concentration (pH neutral to pH 10.0) reduced anion conductance in crayfish muscle fibers. Hagiwara, Gruener, Hayashi, Sabata, and Grinnell (1968) showed that increased external hydrogen ion increased chloride conductance in barnacle muscle. Finally, Strickholm, Wallin, and Shrager (1969) found that chloride conductance increased when pH was lowered from 7.5 to 6.0 outside the crayfish giant axon. On the other hand, Hutter and Warner (1967) reported that increased external hydrogen ion decreased anion conductance in frog skeletal muscle, a finding which has been confirmed by Mainwood and Lee (1968).

In the present experiments, the effect of decreased external pH on ionic conductances of the giant cell in the abdominal ganglion of *Aplysia californica* was studied. This cell was selected because of its size, its common occurrence in ganglia from different animals, and its lack of spontaneous discharge. Moreover, half the giant cells were hyperpolarized by CO₂ and decreased external pH whereas the other half were depolarized. An explanation of these two responses might account for the different effects of CO₂ on different nerve cells. Thus it produces hyperpolarization of cortical (Krjnević, Randić, and Siesjö, 1965) and phrenic neurons (Gill and Kuno, 1963) and depression of the monosynaptic reflex in lumbar motoneurons (Esplin and Rosenstein, 1963). By contrast, it causes depolarization and excitation of mammalian respiratory center neurons (von Euler and Söderberg, 1952) and neurons of *Aplysia* and *Helix* (Chalazonitis, 1963). Finally, in the carotid sinus of mammals, chemoreceptors are excited whereas adjacent baroreceptors are unaffected and at very low pH, depressed (Heymans and Neil, 1958; Eyzaguirre and Zapata, 1968).

The results of our experiments show that chloride conductance was trebled and potassium conductance increased by 50% when external pH was lowered. In addition, the intracellular activity of chloride was measured and was found to be either in the range of 27.7 ± 1.8 mM (SEM) or 40.7 ± 1.5 mM, giving calculated chloride equilibrium potentials of -63.6 ± 1.7 mv and -53.3 ± 0.9 mv, respectively. Intracellular potassium activity was 168 ± 5 mM and the calculated potassium equilibrium potential was -80 ± 0.78 mv. Decreased external pH caused the resting membrane potential to move towards the chloride equilibrium potential producing hyperpolarization in some giant cells and depolarization in others. A preliminary report of some of the results has been given (Walker and Brown, 1970).

METHODS

The experimental setup was described in the preceding paper (Brown and Berman, 1970). In order to study the depolarization produced by changes in pH without the

complication of action potentials, the ganglion was soaked in artificial seawater (ASW) containing zero Ca and tetrodotoxin 10^{-8} g/ml (TTX) (Sankyo Chemical Co., Tokyo, Japan). After it was found that spike discharge was largely prevented even when $(Ca)_o$ was normal (10 mM), control ASW with TTX was used. In experiments using TTX, the flow of ASW was not continuous. When the pH of the bathing fluid was changed, the chamber was washed out with 5–10 times its volume of the test solution (10–15 ml).

Membrane resistance was measured using two intracellular microelectrodes, one for passing current, the other for recording voltage. Constant current pulses of 1.0–2.0 sec duration were delivered every 10 sec (Fig. 2). The current used ($1-10 \times 10^{-9}$ amp) caused voltage deflections of 5–10 mv and current was adjusted so that voltage deflections fell well within the linear part of the current-voltage curve near the resting potential.

In other experiments, the membrane potential was set at various levels by passing constant current through one micropipette across a $5 \times 10^8 \Omega$ series resistor. Current flow was monitored continuously.

The solutions used are listed in Table I. Ionic substitutions were made using conversion tables (Handbook of Chemistry and Physics, 1967; Robinson and Stokes, 1968) to maintain activities and osmotic pressure as close as possible to those of the control solution. The osmolality of all solutions was within 5% of control (950 milliosmols/kg). pH was adjusted using NaOH, $NaHCO_3$, HCl, or H_2SO_4 as previously described (Brown and Berman, 1970). Such small amounts were needed to adjust pH that no change in osmolality was involved. Liquid junction potentials were less than 1 mv when pH fell from 8.0 to 5.0, for any of the solutions listed in Table I. The temperature was adjusted to any desired level between 5 and 20°C by passing solutions through a chamber cooled by Peltier cells. Temperature in the bath was measured with a thermistor.

TABLE I
SOLUTIONS

	NaCl	KCl	CaCl ₂	MgCl ₂	MgSO ₄	Tris- maleate	Na ₂ SO ₄	Choline Cl	Ca prop- ionate
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
Control	494	10	10	20	30	10	—	—	—
10 mM Cl	—	10	—	—	—	10	486	—	10
$\frac{1}{4}$ Cl	124	10	—	—	50	10	330	—	10
$\frac{1}{2}$ Cl	247	10	10	—	50	10	215	—	—
$\frac{3}{4}$ Cl	371	10	10	—	50	10	104	—	—
Zero Na*	—	10	10	20	30	10	—	494	—
Zero Ca	494	10	—	20	30	10	—	—	—
Zero Mg	494	10	10	—	—	10	—	—	—
$\frac{1}{10}$ K	503	1	10	20	30	10	—	—	—
5 × K	454	50	10	20	30	10	—	—	—
10 × K	404	100	10	20	30	10	—	—	—

* In three experiments atropine $2-4 \times 10^{-4}$ M was added to inhibit the parasymphomimetic effect of choline ion.

Measurement of Intracellular Chloride and Potassium Activity

A micropipette is pulled in the usual manner using a vertical pipette puller and Pyrex (Corning code 7750) tubing. Immediately after it has been removed from the puller the tip of the pipette is dipped in a siliconizing solution composed of 2% (v/v) Siliclad (Clay-Adams, Inc., Parsippany, N. J.) in 1-chloronaphthalene for 10–15 sec. The exact length of time that the pipette is in the siliconizing solution is determined by the amount of siliconizing solution that is taken into the tip. Results have been best when there is a column 150–200 μ long of the siliconizing solution inside the tip.

After being dipped the pipette is placed tip up in a drill hole in a metal block and as soon as the desired number of pipettes, usually 1–2 dozen, have been pulled and dipped, they are placed in a 250°C oven for 1 hr. Upon removal from the oven the pipettes are covered with an inverted beaker and left standing, tip up, until they are to be used.

In order to convert a siliconized pipette into an ion-specific electrode the first step is to dip the tip of the pipette into the appropriate liquid ion exchanger for 30–45 sec. The exact length of time is determined by measuring the length of the column of exchanger in the tip under magnification of 100 times. This should be 150–200 μ . In order to make chloride electrodes, Corning code 477315 chloride liquid ion exchanger is used and for potassium electrodes Corning code 477317 potassium liquid ion exchanger is used. For both potassium and chloride electrodes the pipette above the ion exchanger is filled with 0.5 M KCl. This is accomplished by first filling as far as possible using a syringe with a No. 30 needle and then while observing the pipette under a magnification of 100 times, advancing a fine glass needle down the inside of the pipette until it just touches the surface of the oil. When the needle touches the ion exchanger the KCl flows down and displaces the air far enough up so that it can be removed with the No. 30 needle.

Filled electrodes are stored with the tip immersed in 0.5 M KCl until they are used, a period of at least 2 hr. After the electrodes have equilibrated and are ready to use, they have a resistance of 10^{10} – 10^{11} ohms, a time constant of 0.5–1.0 sec, and a DC drift of less than 1 mv per hour when immersed in any given solution.

Equation (1), which is an empirical equation, can be used to describe the potential of the liquid ion exchanger microelectrodes (Walker, 1970).

$$E = E_o + (nRT/z_i F) \log_e (a_i + \sum_j K_{i,j} a_j^{z_i/z_j}) \quad (1)$$

E is the electric potential (v), E_o is a constant (v), R the gas constant (8.2 joules deg⁻¹ mole⁻¹), T the temperature (°K), F the Faraday (96,500 coulomb equivalent⁻¹), n is an empirical constant chosen so that $nRT/z_i F$ is the slope of the line when E is plotted as a function of $\log_e a_i$ with $\sum_j K_{i,j} a_j = 0$, z_i and z_j are the valences of the i th and j th ions, respectively, a_i is the activity of the ion the electrode is expected to measure (the principal ion), a_j are interfering ions whose valence sign is the same as that of the principal ion, and $K_{i,j}$ is the selectivity constant for the j th ion with respect to the i th ion. When $K_{i,j} < 1$ the electrode has a higher selectivity for the principal ion than for the competing ion.

K_{ij} was measured in mixtures of one interfering ion and the principal ion at constant ionic strength. Equation (1) is rearranged to the form shown in equation (2) and when the left side is plotted as a function of $a_j^{z_i/z_j}$, the resulting straight line has a slope of K_{ij} .

$$\exp(E - E_0)z_iF/nRT - a_i = K_{ij}a_j^{z_i/z_j} \quad (2)$$

Tables II and III present the values of K_{ij} for a number of interfering ions for the chloride and potassium electrodes, respectively. Measurements were made in solutions whose ionic strengths were 1 M ($\mu = 1.0$) and 0.1 M ($\mu = 0.1$).

Electric potential measurements were made with the ion-specific electrode connected to the input of a vibrating reed electrometer (Cary model 401, Cary Instruments, Monrovia, Calif.) with an input impedance of 10^{16} ohms. The reference electrode was a saturated KCl-filled pipette the tip of which was broken. The output of the electrometer was read on a digital voltmeter.

Just before being used to measure intracellular potassium activity the potassium electrodes were calibrated in KCl solutions varying in concentration from 1.0×10^{-3} M to 1.0 M. Immediately after making the intracellular measurement the calibration was repeated. Although there was sometimes a DC shift of 1–2 mv in the calibration curve, the slope of the curve remained constant at 57–58 mv per decade change in activity over the entire range of concentrations of the calibrating solutions at 20°C. The response of the electrodes is independent of whether the anion is chloride or sulfate.

Chloride electrodes are calibrated in the same way and have a slope slightly less than that predicted by equation (1), being 56 ± 1 mv for a 10-fold change in activity over the concentration range of 1.0×10^{-3} M to 1.0 M at 20°C. The slope of the chloride response is independent of the cation in hydrogen, sodium, and potassium chlorides (Fig. 1).

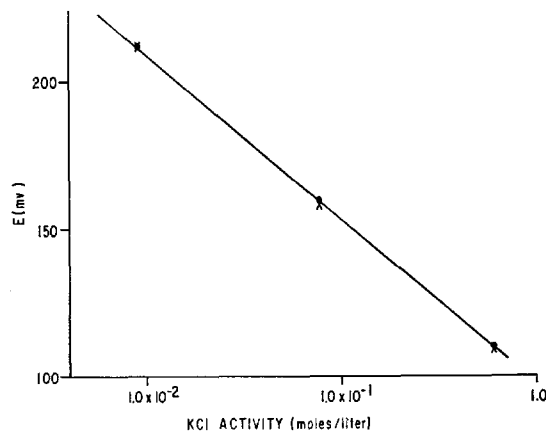


FIGURE 1. Calibration curve for a chloride microelectrode before and after being used to measure intracellular chloride activity in the giant cell of the abdominal ganglion of *Aplysia californica*. The solid dots are the “before” calibration; the crosses are the “after” calibration.

TABLE II
CHLORIDE ELECTRODE SELECTIVITY CONSTANTS, K_{ij}

	$\mu = 0.1$	$\mu = 1.0$
Bicarbonate	0.05	0.05
Isethionate	0.2	0.2
Propionate	0.5	0.7
Sulfate	0.03	0.02

TABLE III
POTASSIUM ELECTRODE SELECTIVITY CONSTANTS, K_{ij}

	$\mu = 0.1$	$\mu = 1.0$
Calcium	0.002	0.03
Hydrogen	0.02	0.03
Magnesium	0.001	0.001
Sodium	0.02	0.02

In order to measure the intracellular ionic activity of either potassium or chloride the same procedure is used. The potential of the electrode in the perfusing solution with respect to the extracellular reference electrode is noted, the electrode is then advanced into the cell, and the potential is allowed to stabilize at which time that potential is recorded. The electrode is then withdrawn from the cell and the potential in the perfusing solution is again noted. If the potential in the perfusing solution after withdrawal is more than ± 1 mv different from the reading before the penetration the measurement is rejected. While the ion-specific electrode is inside the cell the membrane potential of the same cell is measured with a 3 M KCl-filled micropipette. The membrane potential is subtracted from the difference in potential between the inside and the outside of the cell as measured with the ion-specific electrode, and then knowing the slope of the response of the ion-specific electrode and the activity of that ion in the perfusing solution, the intracellular activity of the ion is calculated.

For a more complete discussion of liquid ion exchanger microelectrodes see Walker (1970).

RESULTS

Effect of Decreased External pH on Membrane Resistance Initial penetration of a giant cell was accompanied by a burst of action potentials which subsided within a few minutes. Rarely, these cells were spontaneously active. Insertion of a second electrode produced a temporary burst of spikes and a decrease in membrane potential, followed by complete recovery.

The membrane resistance fell to 54% of control upon lowering pH from 8.0 to 5.0 (Table IV). The fall in resistance was accompanied by hyperpolarization in half of the cells studied (Fig. 2) and by depolarization in the others (Fig. 4, Brown and Berman, 1970; Fig. 3 B, Walker and Brown, 1970).

These differences occurred from resting potentials of -55 ± 4.0 mv for cells having hyperpolarizing responses, and -58 ± 3.0 mv for cells showing depolarizing responses (Table IV) (see also Brown, Sutton, and Berman, 1969).

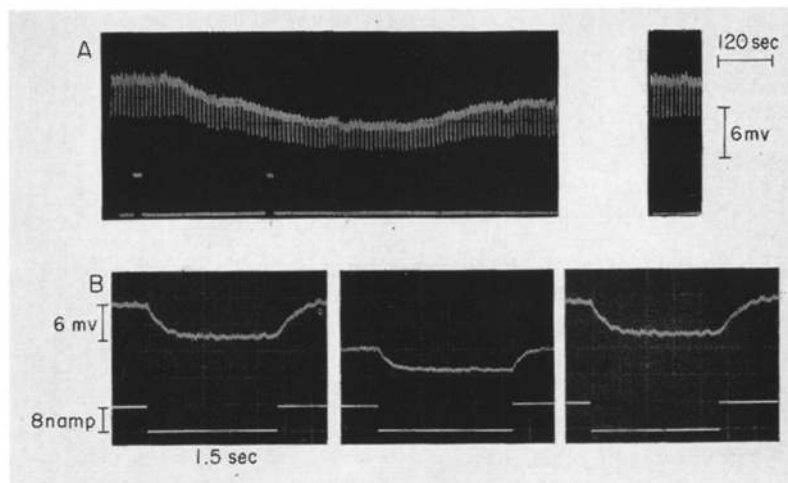


FIGURE 2 A. Effect of decreasing pH from 8.0 to 5.0 (between signals on bottom trace) on the membrane potential and resistance of the giant cell. After a short latency, the membrane potential hyperpolarized 6 mv and the resistance fell to 58% of control. Far right trace was taken 30 min after return to control solution. B. Individual voltage responses to constant current pulses during control, peak effect, and recovery, using a fast time base.

TABLE IV
COMPARISON OF GIANT CELLS HAVING
HYPER- OR DEPolarizing RESPONSES TO A
FALL IN pH FROM 8.0 TO 5.0

<i>n</i>	Hyperpolarizing response 17	Depolarizing response 17
$E_M, pH\ 8.0$	-55 ± 4.0	-58 ± 3.0
$E_M, pH\ 5.0$	-60 ± 3.0	-50 ± 5.0
$R_M, \% \text{ control}$	55 ± 4.0	53 ± 7.0

All values are mean \pm SEM.

The control membrane resistance (R_M) at the resting potential was 1.1 ± 0.4 M Ω (mean \pm SEM, 10 experiments) and the membrane capacitance was 0.2 ± 0.04 μ F (8 experiments). The resistance fell from 690 K Ω to 400 K Ω while the capacitance remained unchanged (Fig. 2). Recovery was complete about 30 min after return to the control solution. Occasionally, the membrane resistance rose above control levels after the control solution was first reintroduced.

The fall in membrane resistance was related to the change in pH according to the graph shown in Fig. 3A. The largest falls occurred when pH was dropped from 8.0 to 6.0 or to 5.0 and these were the changes generally used. In this particular giant cell, hyperpolarization was produced as the pH fell, and greater decreases in pH caused more hyperpolarization (Fig. 3 B). In other cells, a fall in pH evoked depolarization which was also more pronounced at lower pH. Increasing pH above 8.5 produced hyperpolarization

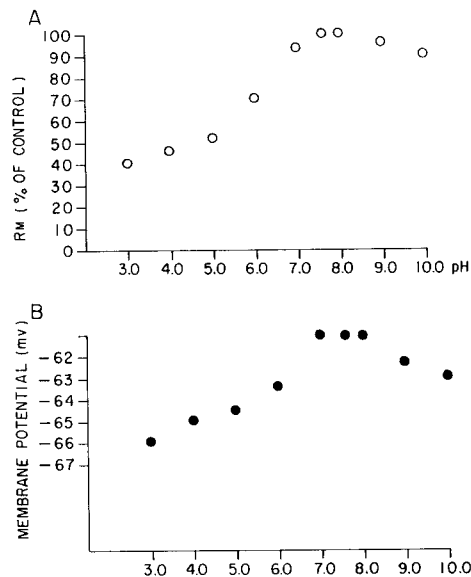


FIGURE 3 A. Effect of changes in pH on membrane resistance plotted as per cent of control. B. Effect of pH on membrane potential in the same giant cell. This cell showed a hyperpolarizing response when pH was lowered below 7.5 or increased above 8.5.

and a fall in membrane resistance as well, although the mechanism involved is different (Brown and Walker, unpublished data, 1970). In three giant cells, increasing pH above 8.5 always produced hyperpolarization.

The effect of lowering pH on the current-voltage curve of the giant cell was reported (Fig. 10, Brown and Berman, 1970). When the cell was soaked in TTX 10^{-5} g/ml for 10–30 min, very few spikes occurred even during strong depolarization. Addition of TTX did not alter the shape of the current-voltage curve and the response to increased $(H)_o$ did not change, the slope conductance at the origin rising from 1.5 to 2.8 μ mhos. Similar results were obtained in four other experiments. The effect of TTX on the action potential appeared completely reversible within 10–30 min after return to the control solution.

Effects of Altering Cl on the Hyperpolarizing Response to a Fall in pH

As we will demonstrate subsequently, the equilibrium potential for potassium, E_K , was always less than the membrane potential, E_M , of the giant cell (where $-80 \text{ mv} < -60 \text{ mv} < -40 \text{ mv}$). However, the chloride equilibrium poten-

tial, E_{Cl} , could be greater or less than E_M . Thus, the membrane hyperpolarization elicited by a fall in pH from 8.0 to 5.0 in half the giant cells could have been due to an increase in chloride conductance, g_{Cl} , or potassium conductance, g_K . On the other hand, the depolarization which occurred in the other half of the giant cells could not be accounted for by an increase in g_K and it seemed likely that changes in g_{Cl} were more important. Therefore, experiments were done in $\frac{1}{2}$ Cl ASW to test whether the changes in membrane potential elicited by increased $(H)_o$ could be altered. When low $(Cl)_o$ was introduced, the cell depolarized, transiently, then returned to control as might be expected since K leaves the cell (Hodgkin and Horowitz, 1959). However, for reasons that are unclear, the cell next became hyperpolarized by some 5–10 mv. The nature of this hyperpolarization has not been determined, but it was responsible for the lower membrane resistance in $\frac{1}{2}$ Cl ASW, pH 8.0, compared with control ASW, pH 8.0, since the current-voltage curve is steeper over this range of membrane potential. A cell that was hyperpolarized 5 mv when the pH of control ASW was reduced from 8.0 to 5.0, was depolarized 17 mv when the pH of $\frac{1}{2}$ Cl ASW was lowered from 8.0 to 5.0 (Fig. 4). The control resistance was $0.7\text{ M}\Omega$ in half $(Cl)_o$ and $0.8\text{ M}\Omega$ at normal $(Cl)_o$ and the fall in resistance was 62% in the $\frac{1}{2}$ Cl, pH 5.0 solution, compared to 61% in ASW with normal $(Cl)_o$, pH 5.0 solution.

When internal chloride was increased by passing a hyperpolarizing current

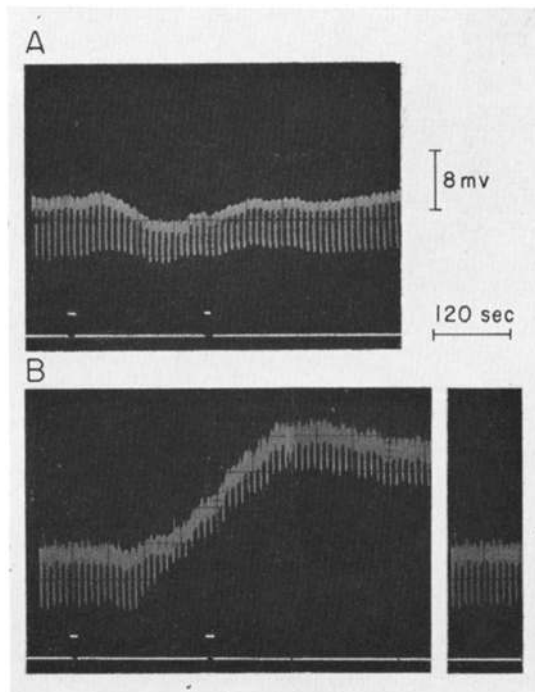


FIGURE 4 A. Effect of a fall in pH from 8.0 to 5.0 (between signal marks on bottom trace) using control ASW on membrane potential and resistance. Control resting potential -55 mv. B. Effect of pH 5.0 using $\frac{1}{2}$ Cl ASW. Control resting potential -60 mv.

of 0.8–1.0 μ amp for 1–3 hr through an intracellular 3 M KCl pipette, the hyperpolarization (induced by low extracellular pH) which initially was 5.0 mv (Fig. 5 A), was reduced or even reversed (Fig. 5 B). Again, the fall in membrane resistance (61%) related to a change from pH 8.0 to pH 5.0 in the extracellular fluid, was similar to that seen prior to the injection of Cl. In two experiments, the same hyperpolarizing current was passed for 2 hr through an 0.6 M K_2SO_4 pipette. The hyperpolarization provoked by a fall in pH was increased by 2.0 and 3.0 mv, respectively, rather than decreased.

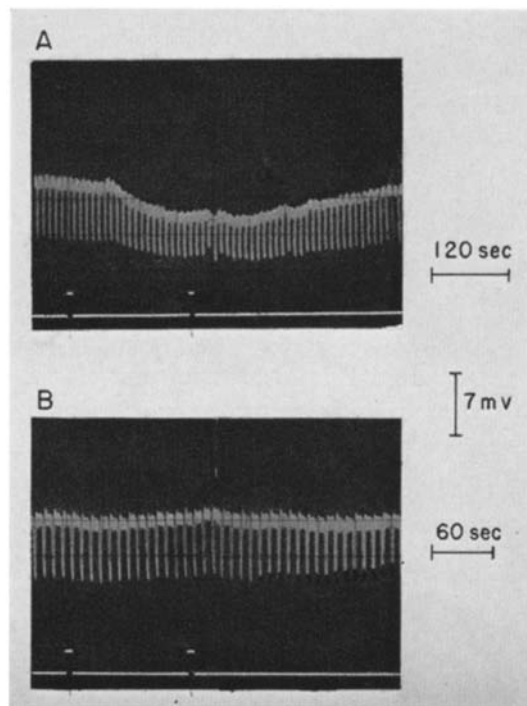


FIGURE 5 A. Effect of a fall in pH from 8.0 to 5.0, control ASW. B. Effect of same solution after electrophoresis, using 3 M KCl electrode and 1.0×10^{-6} amp hyperpolarizing current for 2 hr. Increasing internal chloride decreased the hyperpolarization evoked at pH 5.0. Note the faster time base in B.

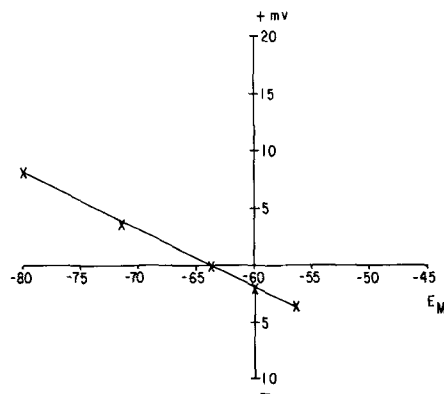


FIGURE 6. Reversal potential for the hyperpolarizing response evoked at pH 5.0. The change in millivolts from the membrane potential (E_M) when the pH was lowered from 8.0 to 5.0 was plotted against the various pre-set levels. At pH 5.0 the cell was hyperpolarized from -60 to -62 mv and the reversal potential for the hyperpolarizing response was -64 mv.

The reversal point of the hyperpolarization elicited by a fall in pH was about 4 mv below the resting potential of -60 mv (Fig. 6). This reversal point was in turn 4–8 mv above the null potential for the undershoot of the action potential which in turn is well above the E_K of -80 mv calculated from the measured internal K activity using the Nernst equation.

Nature of the Depolarization Provoked by Increased $(H)_o$. In half the giant cells studied, increased $(H)_o$ caused depolarization rather than hyperpolarization of the membrane (Fig. 4, Brown and Berman, 1970; Fig. 3 B, Walker and Brown, 1970). The depolarizing responses occurred mainly in experiments done in the spring and fall of 1969, whereas the hyperpolarizing responses were observed throughout the year. The changes in membrane resistance were very similar in either case (Table IV). Omission of Ca or Mg from the perfusate had no effect. When $(Na)_o$ was replaced by choline at pH 8.0, the membrane potential increased by about 10 mv indicating that Na conductance at rest was considerable as has been reported by Carpenter and Alving (1968). The effect was the same when the parasympathomimetic effect of choline was blocked by atropine. The depolarization provoked at pH 5.0 was unaltered when Na was omitted. These results are consistent with the fact that neither the rate of rise nor the overshoot of the action potential was different at lower pH.

These findings suggest that the depolarizing current is carried by Cl ions which infers that E_{Cl} is greater than E_M , the resting membrane potential. If $(Cl)_i$ is reduced in these cells, the depolarization should be reduced or even reversed. Thus, two cells giving depolarizing responses were soaked for 2 hr in 10 mM $(Cl)_o$ ASW. The depolarization of 3 mv (Fig. 7 A) was changed to

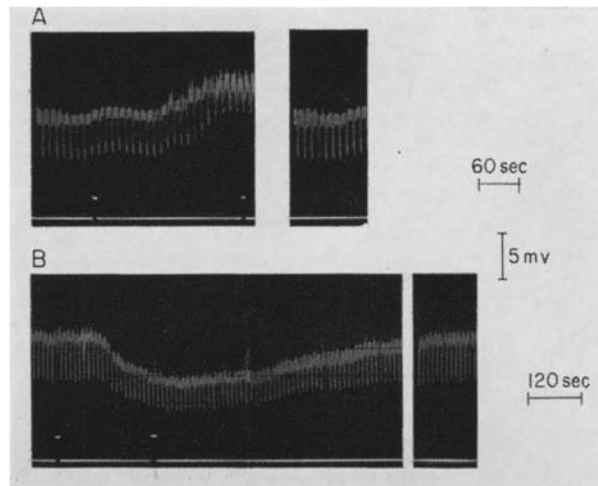


FIGURE 7. Effect of soaking giant cell in 10 mM $(Cl)_o$ ASW on the response to a fall in pH from 8.0 to 5.0. A. Before soak. B. After soak.

a hyperpolarization of 5 mv (Fig. 7 B). The decreases in membrane resistance were similar in both cases.

The reversal point for the depolarizing response was between -55 and -50 mv. The resting potential of the two cells shown in Fig. 8 was in each case -60 mv. The open circles represent the amount of depolarization from the different preset membrane potentials when pH fell from 8.0 to 6.0, while the solid circles were obtained when the pH fell from 8.0 to 5.0. The cells were soaked in TTX to avoid action potentials. The extrapolated null potentials were similar at the different pH's but the slope at pH 6.0 was smaller.

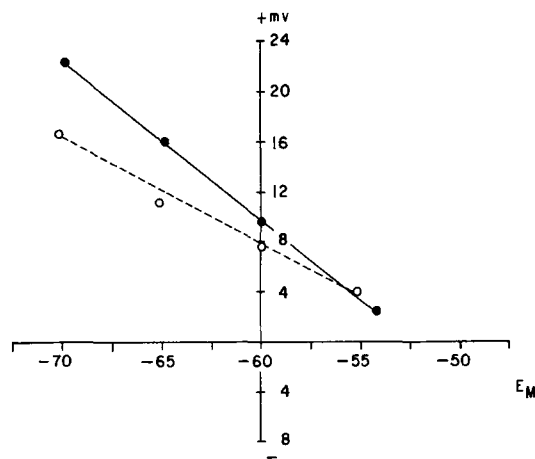


FIGURE 8. Graph of the null potential for depolarizing response elicited in one cell at pH 5.0 (solid circles) and in another at pH 6.0 (open circles). Resting potential, -60 mv in each case, was set at the origin. The ordinate was the amount of depolarization from each preset membrane potential provoked by the fall in pH.

Validation of Measurements Made with Cl and K Ion Exchanger Microelectrodes

The chloride microelectrode system gave measurements identical to those of an $\text{Ag}:\text{AgCl}_2$ electrode, calomel half-cell reference electrode system, using ASW and turtle serum as test solutions. Thus, proteins do not seem to affect the performance of this electrode. The potassium microelectrode gave measurements for K activity in ASW identical to those calculated from Davies' modification of the extended Debye-Hückel equation (Robinson and Stokes, 1968) taking into account the selectivity of 50:1 over Na.

A comparison of internal Cl and K activities measured by these electrodes with internal K and Cl measurements made with other methods is shown in Table V. Internal chloride and potassium activities are represented as a_{Cl}^i and a_{K}^i and internal concentrations as $(\text{Cl})_i$ and $(\text{K})_i$.

Complete accounts of the data in Table V will be presented elsewhere. Briefly in *Nitella*, the K and Cl activities agree quite well with the concentrations in extruded vacuolar sap measured by means of a flame photometer (K) or chloridometer. Our data are also in good agreement with those of Kurella (1969) who measured K activity with a K-sensitive glass microelectrode and Cl activity with a Ag wire coated with AgCl. The K activity in

crayfish giant axon agrees well with the concentration in extruded axoplasm measured by Wallin. However, the Cl activity we measured is half that measured by Strickholm and Wallin (1965), who used a Ag wire coated with AgCl, but it is more than would be expected based on the Cl concentration in extruded axoplasm subsequently measured by Wallin (1967).

A good correlation between Cl activity and concentration in the lumen of turtle thyroid follicle also exists.

TABLE V
COMPARISON OF Cl AND K ACTIVITIES MEASURED
BY MEANS OF ION EXCHANGER MICROELECTRODES WITH
ACTIVITIES OR CONCENTRATIONS
MEASURED BY OTHER METHODS

Data are mean \pm SEM

	E_M	a^i_{Cl}	$(Cl)_i$	a^i_K	$(K)_i$	n
	mv	mM	mM	mM	mM	
<i>Nitella</i>						
Our data	-110 \pm 3.3	96.6 \pm 1.1	130.8 \pm 1.0	60.6 \pm 1.0	67.6 \pm 2.0	15
Data of Kurella (1969)	-150 \pm 3.2	125.6 \pm 3.0		70.1 \pm 3.9		
<i>Crayfish giant axon</i>						
Our data	-79 \pm 2.4	13.73 \pm 0.4		209.9 \pm 0		14
Strickholm and Wallin (1965)	-80.3 \pm 0.8	25.2 \pm 2.9				
Wallin (1967)			12.7 \pm 1.4		265 \pm 1.8	
<i>Lumen of turtle thyroid follicle</i>						
Our data	-10.2 \pm 1.0	37.8 \pm 2.0				30
Woodbury and Chow (unpublished data, 1970)	-11.2 \pm 1.0		41.7 \pm 4.0			

Activities of Chloride, Potassium, and Sodium in the Giant Cell

Chloride activities of ASW, aquarium seawater, and Pacific Ocean seawater in which the animals were delivered were similar. The values ranged from 330 to 350 mM. Intracellular chloride activity was measured in 14 giant cells (Table VI). The cells fell into two groups according to whether the calculated E_{Cl} 's were greater or less than the E_M 's. In seven cells the intracellular chloride activity, a^i_{Cl} , was 27.7 ± 1.8 mM and ranged from 21 to 31 mM. Calculated E_{Cl} 's were -63.6 ± 1.7 mv compared to E_M 's of -56.4 ± 1.1 mv and ranged from -57 to -70 mv. A fall in pH from 8.0 to 5.0 provoked hyperpolarization in the three cells tested. In seven other cells a^i_{Cl} was 40.7 ± 1.5 mM and ranged from 37 to 47 mM. Calculated E_{Cl} 's were -53.3 ± 0.9 mv compared to E_M 's of -58 ± 0.8 mv, and ranged from -49 to -56 mv. A fall in pH from 8.0 to 5.0 produced depolarization in the three cells tested. Internal activities were unchanged during 2-3 min exposure to pH 5.0 ASW.

In 19 giant cells, the internal K activity was 168 ± 5.5 mM, giving a cal-

TABLE VI
INTRACELLULAR CHLORIDE ACTIVITIES
IN 14 *Aplysia* GIANT CELLS

Cell No.	a^o_{Cl} *	a^i_{Cl}	Calculated E_{Cl} †	E_M pH 8.0	E_M , pH 5.0
	mM	mM	mv	mv	mv
1	350	26	-65	-58	
2	350	31	-61	-53	-60
3	340	31	-60	-56	
4	340	25	-66	-59	-63
5	340	21	-70	-60	
6	340	25	-66	-57	
7	330	35	-57	-52	-56
Mean ± SEM	341.1±3.6	27.7±1.8	-63.6±1.7	-56.4±1.1	-59.7±2.0
8	330	37	-55	-60	
9	330	40	-53	-55	
10	330	47	-49	-60	-50
11	340	45	-51	-58	-52
12	340	40	-54	-56	
13	340	39	-55	-57	
14	340	37	-56	-60	-57
Mean ± SEM	335.7±3.0	40.7±1.5	-53.3±0.9	-58.0±0.8	-53.0±2.1
	$p > 0.1$	$p < 0.005$	$p < 0.005$	$p > 0.1$	

p values in each column are between cells 1 through 7 as one group and cells 8 through 14 as the other.

* Chloride activity of ASW.

† Chloride equilibrium potential calculated as, $E_{Cl} = \frac{RT}{F} \ln \frac{a^i_{Cl}}{a^o_{Cl}}$ where R , T , and F have their usual meaning.

culated E_K of -80 ± 0.78 mv. External K activity was registered by the ion electrode as 14 mM because of Na interference; correcting for this yields a value of 6 mM which agrees with the predicted value of 6 mM. This value was used in the calculation of E_K using the Nernst equation. E_M in these cells was -50 ± 1.1 mv. A full account of a^i_K in different *Aplysia* neurons will be presented (Kunze, Peterson, Combes, Brown, and Walker, manuscript in preparation).

Assuming that the overshoot of the action potential ($+48 \pm 1.2$ mv) equals E_{Na} , the equilibrium potential for Na (Geduldig and Junge, 1968), then

$$a^i_{Na} = a^o_{Na} \exp(-E_{Na}F/RT) \quad (3)$$

where a^o_{Na} was 337 mM (calculated from Davies' modification of the extended Debye-Hückel equation [Robinson and Stokes, 1968]). This gave a value of

50 mM for internal sodium activity in the giant cells studied in these experiments.

Effect of Decreased External pH on Cl and K Conductances A plot of E_M vs. $\log (Cl)_o$ at pH 8.0 and 5.0 is shown in Fig. 9. The membrane potential at each $(Cl)_o$ was taken as the point of maximum depolarization following exposure to that $(Cl)_o$. A straight line was fitted to the experimental points using the method of least squares. There is a clear increase in slope at pH 5.0 and the increase is in the range of two- to threefold.

The relation between membrane potential and $(K)_o$ for the same cell is shown in Fig. 10. Lowering $(K)_o$ to 1.0 mM caused depolarization of 1-3 mv

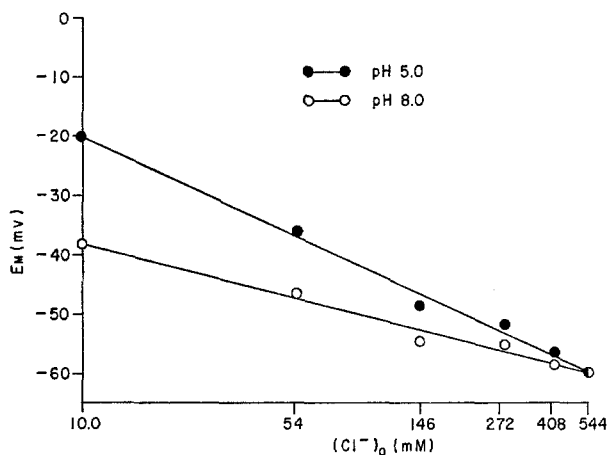


FIGURE 9. Effect of lowering pH from 8.0 to 5.0 on the relation of E_M and $(Cl)_o$.

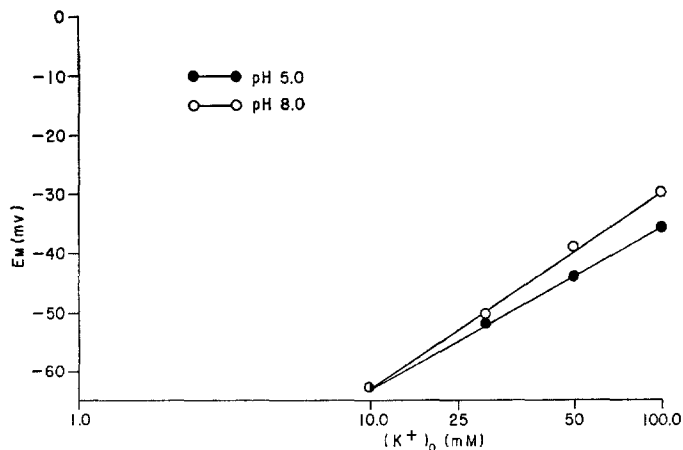


FIGURE 10. Effect of lowering pH from 8.0 to 5.0 on relation between E_M and $(K)_o$. Figure reprinted by permission from *Science*, 1970, 167:1502. Copyright 1970 by the American Association for the Advancement of Science.

which has not been shown on this graph. This was probably due to a decrease in activity of the sodium-potassium exchange pump and has been discussed by Carpenter and Alving (1968). The slope for a 10-fold change in $(K)_o$ ranged from 23 to 40 mv (mean, 33 mv in six experiments) at pH 8.0 and agreed with that reported by Carpenter and Alving (1968). When the pH was 5.0 the slope was reduced by 25%. Results similar to those shown in Figs. 9 and 10 were obtained in 11 experiments (Table VIII). TTX-containing solutions in which spike and synaptic activities were abolished did not affect the results in three experiments.

In order to determine the changes in g_K and g_{Cl} at pH 8.0 and 5.0 from the graphs shown in Figs. 9 and 10, we have followed the line used by Hodgkin and Horowitz (1959) with some modifications. We did not ignore the leakage current probably carried by Na (Kunze and Brown, unpublished observations), and in the giant cell of *Aplysia*

$$E_K \neq E_{Cl} . \quad (4)$$

In the absence of net membrane current,

$$E_M = E_K \cdot T_K + E_J \cdot T_J \quad (5)$$

where

$$E_J = \frac{g_{Cl}}{g_{Cl} + g_{Na}} E_{Cl} + \frac{g_{Na}}{g_{Cl} + g_{Na}} E_{Na} \quad (6)$$

and

$$T_J = T_{Cl} + T_{Na} \quad (7)$$

This assumes that Cl^- (see Fig. 9) and Na^+ (Kunze and Brown, unpublished observations) are the only other current-carrying species

$$T_K = \frac{g_K}{g_K + g_J} \quad (8)$$

where g_J is the conductance of the ions other than K contributing to membrane current and

$$T_J = 1 - T_K \quad (9)$$

T_K and T_J are the transport numbers of K and the other ions, respectively. From equations (5) and (9)

$$(\partial E_M / \partial E_K)_{E_J} = T_K + (E_K - E_J)(\partial T_K / \partial E_K)_{E_J} \quad (10)$$

We now assume T_K to be a function of $(E_K - E_J)$ and represent T_K by a polynomial in powers of $(E_K - E_J)$ as shown in equation 11.

$$T_K = a_0 + a_1(E_K - E_J) + a_2(E_K - E_J)^2 \dots \quad (11)$$

From which it follows that

$$\left(\frac{\partial T_K}{\partial E_K}\right)_{E_J} = a_1 + 2a_2(E_K - E_J) + 3a_3(E_K - E_J)^2 \dots \quad (12)$$

When T_K and $\left(\frac{\partial T_K}{\partial E_K}\right)_{E_J}$ in equation 10 are replaced by equations 11 and 12, one obtains

$$\left(\frac{\partial E_M}{\partial E_K}\right)_{E_J} = a_0 + 2a_1(E_K - E_J) + 3a_2(E_K - E_J)^2 \dots \quad (13)$$

Since $\left(\frac{\partial E_M}{\partial E_K}\right)_{E_J}$ was found experimentally to be constant for a wide range of values of $(E_K - E_J)$ (see Figs. 9 and 10), the implication is that

$$a_1 \cong a_2 \cong \dots \cong 0 \quad (14)$$

Hence

$$\left(\frac{\partial E_M}{\partial E_K}\right)_{E_J} \cong a_0 \cong T_K \quad (15)$$

This implies that whatever the changes, g_K and g_M both change by about the same amount as K_o increases. Recalling

$$E_K = 58 \log \frac{K_o}{K_i} \quad (16)$$

it follows that

$$\left(\frac{\partial E_M}{\partial \log K_o}\right)_{(J)_o} \cong 58T_K \quad (17)$$

Following an analogous line of reasoning it can be shown that

$$-\left(\frac{\partial E_M}{\partial \log Cl_o}\right)_{(J')_o} \cong 58T_{Cl} \quad (18)$$

where $(J')_o$ is K^+ and Na^+ . From the results provided in Figs. 9 and 10, and Table IV, we present Table VII. The conclusion from this table is that under acid conditions, potassium conductance increases to 150% of control, chloride conductance increases to 340%, and sodium conductance increases to 110%.

Values for T_K (pH 8.0) and T'_K (pH 5.0) were obtained in six cells and for T_{Cl} and T'_{Cl} in five other cells (Table VIII). These data gave mean

TABLE VII
POTASSIUM AND CHLORIDE CONDUCTANCES
AT pH 8.0 AND 5.0

At pH = 8.0	At pH = 5.0
(a) R_M and g_M are taken as 1.0	(a) $R'_M = 0.54$ and $g'_M = \frac{1}{0.54} = 1.85$
(b) $[\partial E_M / \partial \log (K)_o]_{(J)_o} = 33.0 \text{ mv} = (58 \text{ mv}) \cdot T_K$ $-\partial E_M / \partial \log (Cl)_o]_{(J)_o} = 12.5 \text{ mv} = (58 \text{ mv}) \cdot T_{Cl}$	(b) $[\partial E'_M / \partial \log (K)_o]_{(J)_o} = 27.0 \text{ mv} = (58 \text{ mv}) \cdot T'_K$; $-\partial E'_M / \partial \log (Cl)_o]_{(J)_o} = 23.0 \text{ mv} = (58 \text{ mv}) \cdot T'_{Cl}$
Hence $T_K = 0.57$; $T_{Cl} = 0.22$; and Na^+ gives a leakage current with $T_{Na} = 1 - T_K - T_{Cl} = 0.21$.	$T'_K = 0.47$; $T'_{Cl} = 0.40$; $T'_{Na} = 0.13$.
(c) g_K (10^{-6} mhos) = $T_K \cdot g_M = (0.57) \cdot (1.0) = 0.57$ $g_{Cl} = 0.22$ $g_{Na} = 0.21$	(c) $g'_K = 0.87$ $g'_{Cl} = 0.74$ $g'_{Na} = 0.24$

TABLE VIII
TRANSPORT NUMBERS FOR K AND
Cl AT pH 8.0 AND 5.0

	T_K	T'_K	T_{Cl}	T'_{Cl}
	0.57	0.47	0.22	0.40
	0.57	0.45	0.22	0.39
	0.52	0.37	0.17	0.31
	0.62	0.46	0.26	0.49
	0.40	0.27	0.28	0.69
	0.69	0.45		
$\bar{X} \pm \text{SEM}$	0.56 ± 0.03	0.41 ± 0.03	0.23 ± 0.01	0.46 ± 0.02
	$T_{Na} = 1 - (0.56 + 0.23) = 0.21$			
	$T'_{Na} = 1 - (0.41 + 0.46) = 0.13$			

T_K and T'_K were obtained in six cells; T_{Cl} and T'_{Cl} were obtained from five different cells.

values very similar to those for the giant cell in which all four values were obtained (Table VII; Figs. 9 and 10).

Effects of Increased $(H)_o$ on E_M Calculated from the Constant-Field Equation

The results to this point indicate that increased $(H)_o$ provokes a large increase in g_{Cl} and a smaller increase in g_K . Consequently, the change in E_M should depend upon the relation of E_{Cl} to E_M . If E_{Cl} is less than E_M , the cell should hyperpolarize and vice versa. If the permeabilities to Cl, K, and Na (P_{Cl} , P_K , and P_{Na}) can be calculated at pH's 8.0 and 5.0, then E_M can be calculated from the constant-field equation at both pH's and for cells having different internal Cl. In this way the relation of the predicted E_M at pH's 8.0 and 5.0 to the actual E_M can be determined.

It is necessary in solving for P_K , etc., to express g_M per unit surface area.

Assuming that the giant cell is a sphere of 0.3 mm diameter, g_M at pH 8.0 is calculated to be 40×10^{-6} mho cm^{-2} and at pH 5.0, g'_M is 75×10^{-6} mho cm^{-2} (Table VI).

From the constant-field equation (Hodgkin and Horowicz, 1959), at pH 8.0,

$$\begin{aligned} P_K &= g_K(E_M - E_K) \cdot RT/E_M F^2 \cdot \frac{\exp(E_M F/RT) - 1}{a_K^i \exp(E_M F/RT) - a_K^o} \\ &= 0.21 \times 10^{-6} \text{ cm sec}^{-1} \end{aligned} \quad (19)$$

From Table VI, at pH 5.0,

$$\begin{aligned} P'_K &= g'_K(E'_M - E_K) \cdot RT/E'_M F^2 \cdot \frac{\exp(E'_M F/RT) - 1}{a_K^i \exp(E'_M F/RT) - a_K^o} \\ &= 0.32 \times 10^{-6} \text{ cm sec}^{-1} \end{aligned} \quad (20)$$

At pH 8.0,

$$\begin{aligned} P_{Cl} &= g_{Cl}(E_M - E_{Cl}) \cdot RT/E_M F^2 \cdot \frac{1 - \exp(-E_M F/RT)}{a_{Cl}^o - a_{Cl}^i \exp(-E_M F/RT)} \\ &= 0.08 \times 10^{-6} \text{ cm sec}^{-1} \end{aligned} \quad (21)$$

$$\begin{aligned} P'_{Cl} &= g'_{Cl}(E'_M - E_{Cl}) \cdot RT/E'_M F^2 \cdot \frac{1 - \exp(-E'_M F/RT)}{a_{Cl}^o - a_{Cl}^i \exp(-E'_M F/RT)} \\ &= 0.27 \times 10^{-6} \text{ cm sec}^{-1}, \end{aligned} \quad (22)$$

and the constant-field equation can be solved for P_{Na} .

$$E_M = 58 \log \frac{P_K a_K^o + P_{Na} a_{Na}^o + P_{Cl} a_{Cl}^i}{P_K a_K^i + P_{Na} a_{Na}^i + P_{Cl} a_{Cl}^o} \quad (23)$$

and $P_{Na} = 0.004 \times 10^{-6} \text{ cm sec}^{-1}$.

Since we have assumed that Na is the main ion carrying leakage current,

$$P'_{Na} = 0.0055 \times 10^{-6} \text{ cm sec}^{-1}.$$

Since we know the permeabilities, we can now predict E_M at pH's 8.0 and 5.0 for cells having the two different values of a_{Cl}^i . At pH 8.0, a theoretical cell having a_{Cl}^i 41 mM has an E_M of -59 mv. At pH 5.0, E_M becomes E'_M and the cell is depolarized to -57 mv. A theoretical cell with a_{Cl}^i 28 mM has an E_M of -62 mv and an E'_M of -64 mv; i.e., was hyperpolarized by the fall in pH. Equivalent increases or decreases in a_K^i or a_{Na}^i change E_M but always result in depolarization when the pH falls from 8.0 to 5.0. This supports the conclusion that the depolarizing or hyperpolarizing effect of a fall in pH depends mainly upon an increase in g_{Cl} and the relation of E_{Cl} to E_M . When values of T_K , T'_K , T_{Cl} , and T'_{Cl} taken from Table VIII are used to solve for g_K , etc., and P_K etc. (Table VII, equations 19–22), E_M and E'_M

show changes similar to those calculated for the theoretical cell described above.

A plot of E_M vs. $\log (K)_o$ or $\log (Cl)_o$ at pH's 8.0 and 5.0 for a cell with the permeability values we have derived is shown in Fig. 11. The actual experimental points for the cell (Figs. 9 and 10) from which we have derived the permeability constants are also shown. The ratio of actual to derived T_{Cl} 's (pH 8.0) is 1.07 and for T'_{Cl} 's (pH 5.0) it is 1.04. For potassium, the T_K ratio (actual to derived) is 1.03 and the T'_K ratio is 1.13; the agreement between the actual and derived curves is very good.

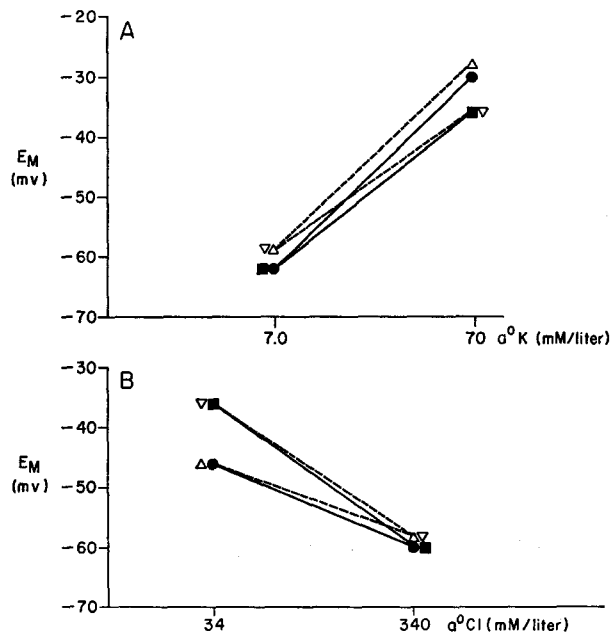


FIGURE 11 A. Membrane potentials of a giant cell measured at pH 8.0 (●) and pH 5.0 (■) for two different $a^{\circ}K$'s (solid lines). Calculated membrane potentials for the same cell (see text) at pH 8.0 (Δ) and pH 5.0 (▽) are represented by dashed lines. B. Measured and calculated membrane potentials for the same giant cell shown above at two different $a^{\circ}Cl$'s and pH's 8.0 and 5.0. Δ, ●, pH 8.0, and ▽, ■, pH 5.0.

Effects of Temperature Part of the response elicited by lowering pH may have been due to interference with an energy-requiring system. In order to test this, experiments were done over a temperature range from 5° to 20°C. When the temperature was lowered from 18–20°C to 10–11°C or 5–6°C, the cell depolarized 5 or 10 mv, respectively. This has been attributed to a reduction in the rate at which the Na-K pump is working in the giant cell (Carpenter and Alving, 1968). The changes in membrane resistance provoked by pH 5.0 were the same at the lower temperatures, but hyperpolarizing responses only, occurred (six experiments).

DISCUSSION

It appears from the data presented that a fall in pH elicits a large increase in chloride conductance and a smaller increase in potassium conductance in the giant cell of the abdominal ganglion of *Aplysia californica*. The ensuing change in membrane potential can be predicted from the relation of the chloride equilibrium potential to the membrane potential. When $E_{Cl} < E_M$, hyperpolarization results. When $E_{Cl} > E_M$, depolarization follows. Maneuvers by which E_{Cl} is changed (Figs. 4, 5, and 7) substantiate this conclusion. Moreover, the reversal potential for either the depolarizing or the hyperpolarizing response is very similar to the appropriate E_{Cl} (Figs. 6 and 8; Tables IV and VI). While E_{Cl} differs between giant cells and amongst *Aplysia* neurons, being either greater or lesser than E_M , E_K is always less than E_M (Kunze and Brown, unpublished observations). Thus the increased potassium conductance produced by a fall in pH would always have a hyperpolarizing effect. The variability of response therefore depends upon the variable relation of E_{Cl} to E_M .

If the same cell from different animals can show these two responses, it seems likely that the differences between several types of *Aplysia* neurons (Brown and Berman, 1970) in response to extracellular acidification have similar explanations.

We may reconsider the many vertebrate neurons, cited earlier, which become hyperpolarized by exposure to CO_2 or decreased pH. If their normal E_M 's depended primarily on potassium permeability and if their E_{Cl} 's were smaller than their E_M 's, an increase in chloride permeability would produce hyperpolarization in those cells. The difference between such cells and arterial chemoreceptors or *Aplysia* visceromotor cells which are depolarized by CO_2 and decreased external pH, is then reduced simply to the relative values of E_{Cl} and E_M of each cell type.

In some cells, a'_{Cl} was greater than that expected from a simple passive distribution between intracellular and extracellular fluids. We may speculate that chloride ion is actively transported into such cells. Similar results have been obtained by Keynes (1963) in squid giant axon and by Strickholm and Wallin (1965) in crayfish giant axon. In other cells a'_{Cl} was less than expected. We may, therefore, speculate that chloride ion is actively transported out of such cells as has been suggested for other molluscan neurons (Chiarandini and Gerschenfeld, 1967).

The two different levels of intracellular chloride recorded in these experiments may not be too surprising since the giant cell shows quite marked variations in color and size under the dissecting microscope from animal to animal and this may reflect different metabolic functions at different times. Furthermore, we have noted seasonal variations in the response. Strumwasser, Jack-

lett, and Alvarez (1969) have reported a similar seasonal rhythm in the neural extract induction of behavioral egg laying in *Aplysia*.

A somewhat parallel situation exists in the D (depolarizing) or H (hyperpolarizing) responses to acetylcholine shown by neurons of *Aplysia depilans* or *Helix pomatia* (Tauc and Gerschenfeld, 1960; Tauc, 1967). In both cases Cl conductance is increased and the directional change of E_M is determined by E_{Cl} (Frank and Tauc, 1964; Kerkut and Meech, 1966). In D cells of *Helix aspersa* (Cl), was 27.5 mM and in H cells, it was 8.7 mM (Kerkut and Meech, 1966).

The nature of a membrane which shows an increase in both anion and cation conductance at lower pH is unclear. Such a result may not be unique for the giant cell of *Aplysia* however. For example, calculation of the data presented by Hagiwara et al. (1968, Fig. 4) according to the method of Table VII, shows a 25% increase in potassium conductance associated with a 64-fold increase in chloride conductance in the membrane of barnacle muscle when pH fell from 7.7 to 4.0. It has also been reported that potassium conductance of some *Aplysia* neurons increases at pH greater than 8.0 (Sato, Austin, and Yai, 1968); this could explain the fall in membrane resistance at higher pH shown in Fig. 3.

It was shown in the preceding paper (Brown and Berman, 1970) that decreasing internal pH with CO₂ had no effect on cells, including the giant cell, which were responsive to decreased external pH. That result differs from the report of Hagiwara et al. (1968) that in barnacle muscle, changes of internal pH had effects similar to those found for changes in external pH. It seems that in the *Aplysia* giant cell, hydrogen ion exerts its effect on the outer but not on the inner surface of the membrane.

The fact that the response to changes in external pH was qualitatively unaltered over a range of temperatures from 5° to 20°C indicates that the membrane changes are passive and not due to interference with metabolically dependent systems.

Finally, in the giant cell of *Aplysia*, E_{Cl} , which is thought to determine the response to acetylcholine (Tauc, 1967), was calculated from the internal chloride activity and equaled the equilibrium potential for acetylcholine, E_{ACh} (Kunze, Combes, Brown, and Walker, manuscript in preparation, 1970). In the left upper quadrant cells, (L₁-L₅), E_K , which is thought to determine the later hyperpolarization produced by acetylcholine in the presence of *d*-tubocurarine (Kehoe and Ascher, 1970), was calculated from internal K activity and also equaled E_{ACh} (Kehoe and Ascher, 1970; Kunze, Combes, Brown, and Walker, manuscript in preparation, 1970). This is, therefore, strong evidence that the ion exchanger electrodes are measuring the true internal K and Cl activities in these neurons.

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