Active Transport of Chloride by the Giant Neuron of the Aplysia Abdominal Ganglion

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ABSTRACT Internal chloride activity, $a_{CI}^i$, and membrane potential, $E_m$, were measured simultaneously in 120 R2 giant neurons of Aplysia californica. $a_{CI}^i$ was $37.0 \pm 0.8$ mM, $E_m$ was $-49.3 \pm 0.4$ mV, and $E_{CI}$ calculated using the Nernst equation was $-56.2 \pm 0.5$ mV. Such values were maintained for as long as 6 hr of continuous recording in untreated neurons. Cooling to 1°-4°C caused $a_{CI}^i$ to increase at such a rate that 30-80 min after cooling began, $E_{CI}$ equalled $E_m$. The two then remained equal for as long as 6 hr. Rewarming to 20°C caused $a_{CI}^i$ to decline, and $E_{CI}$ became more negative than $E_m$ once again. Exposure to 100 mM K+-artificial seawater caused a rapid increase of $a_{CI}^i$. Upon return to control seawater, $a_{CI}^i$ declined despite an unfavorable electrochemical gradient and returned to its control values. Therefore, we conclude that chloride is actively transported out of this neuron. The effects of ouabain and 2,4-dinitrophenol were consistent with a partial inhibitory effect. Chloride permeability calculated from net chloride flux using the constant field equation ranged from 4.0 to $36 \times 10^{-8}$ cm/sec.

INTRODUCTION

Proof of an active transport system for chloride in neurons requires a demonstration of the following conditions (Brown, 1965): (a) an intracellular concentration, or more precisely activity, of chloride which is maintained at a level different from that predicted from passive gradients; (b) the dependence of this difference upon cellular metabolism and its suppression by cooling or metabolic inhibitors; and, (c) the maintenance of a net flux of chloride in the absence of favorable passive gradients.

Proof of such a system has not been forthcoming. However, there have been many reports which indicate its probable existence. For example, certain neurons have chloride-dependent reversal potentials which were elicited either by synaptic action, chemical agents, or spontaneous action potentials which differed from the corresponding resting membrane potentials.
(Kerkut and Thomas, 1964; Strumwasser, 1965; Keohoe, 1967; Chiarandini and Gerschenfeld, 1967; Eccles, Eccles, and Ito, 1964; Pinsker and Kandel, 1969; Lux, 1971; Pritchard, 1971). Transient measurements of intracellular chloride activity in the giant axon of squid and crayfish (Mauro, 1954; Keynes, 1963; Strickholm and Wallin, 1965; Wallin, 1967) and certain molluscan neurons (Kerkut and Meech, 1966; Brown, Walker, and Sutton, 1970; Kunze and Brown, 1971) provided more direct evidence but were still incomplete. Other evidence came from squid axon where chloride influx was suppressed by a metabolic inhibitor (Keynes, 1963).

Both direct and indirect measurements (Brown, Walker, and Sutton, 1970; Kunze and Brown, unpublished observations) of the types referred to above suggested that active transport of chloride takes place across the membrane of the giant neuron of the abdominal ganglion of Aplysia californica (R2, according to the nomenclature of Frazier, Kandel, Kupferman, Waziri and Coggeshall, 1967). Therefore, we measured intracellular chloride activity, \(a_{ci}\), in R2, calculated the chloride equilibrium potential \(E_{cl}\), and compared \(E_{cl}\) with the measured membrane potential \(E_m\) under a variety of conditions designed to test whether chloride is actively transported by the neuron. We found that \(E_{cl}\) was more negative than \(E_m\) throughout prolonged recording under control conditions, indicating that chloride was actively extruded. When the neuron was cooled to 1°-4°C, \(a_{ci}\) increased and \(E_{cl}\) became equal to \(E_m\), indicating that active chloride transport was abolished. When the neuron was rewarmed, \(a_{ci}\) decreased and \(E_{cl}\) once again became more negative than \(E_m\), indicating the re-establishment of active chloride transport. Exposure to 100 mM K+-artificial seawater resulted in an increase in \(a_{ci}\) which was reversible upon return to control fluids. Ouabain slowed the rate at which \(a_{ci}\) declined after treatment with high K+ solutions.

Thus, our results provide substantial evidence that chloride is actively transported out of the Aplysia giant neuron. A preliminary account of some of the results has been reported (Russell and Brown, 1972).

METHODS

Aplysia californica, obtained from Pacific Bio-Marine Supply, Venice, Calif., were kept at 14°C in a seawater aquarium. The abdominal ganglion was excised and pinned to a Sylgard (Dow Corning Corp., Midland, Mich.) resin in the bottom of an acrylic-plastic chamber having a volume of 2.0-2.5 ml. In most cases, the connective tissue capsule of the ganglion above R2 was removed.

The chamber containing the ganglion formed the roof of a constant temperature bath through which flowed a 40% v/v mixture of methanol and water. The temperature of this mixture was maintained at the desired level by means of a refrigeration unit and a combination heater-pump unit with an internal thermostat. The fluid bathing the ganglion passed through a series of coils immersed in this constant

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temperature bath and emerged at the rate of 1–3 ml/min into the chamber containing the ganglion. This system could cool the fluid bathing the ganglion from 20°C to 1°C within 3–5 min and rewarm it within 1–4 min.

The composition (millimoles per liter) of the artificial seawater (ASW) bathing the ganglion was as follows (Hayes and Pelluet, 1947): NaCl, 494; KCl, 10; CaCl₂, 10; MgCl₂, 20; MgSO₄, 30; tris(hydroxymethyl)aminomethane (Tris) buffer, 10 (made according to Gomori, 1948) or NaHCO₃, 2.0. In experiments using ASW containing 100 mM potassium, 100 mM K⁺-ASW, Na⁺, and K⁺ were exchanged on an equimolar basis. The solutions were made with analytical reagent grade chemicals.

The pH of the ASW at room temperature (20°C) was 7.45–7.55. Since Tris buffer has a significant temperature coefficient (0.03 pH U/degree C; Sigma Technical Bulletin No. 106B, August, 1967), it was necessary to lower the pH of those solutions which were to be cooled to compensate for the alkaline changes in pH due to cooling. The necessary acidification could be accomplished without a significant change in ASW composition. In later experiments, a bicarbonate buffer which has no significant temperature dependence was used. The nature of the buffer had no discernible effects on the results being reported.

The osmolality of the ASW, routinely measured by an osmometer was 950 ± 5 milliosmols/kg.

**Measurement of Membrane Potential**

Membrane potential ($E_m$) was measured with conventional glass micropipettes having tip diameters less than 1 μ and resistances of 5–12 megohm when filled with 3 M KCl. The micropipette was connected to an electrometer with negative capacitance compensation, and the output was displayed on both an oscilloscope and a penwriter from which it could be read with accuracy to within ±0.5 mv. The reference electrode was a low resistance 3 M KCl-filled micropipette connected to ground. Tip potentials were determined by measuring the potential shift caused by breaking the tip of the $E_m$-sensing micropipette after completion of the experiments. Results from micropipettes with tip potentials greater than 5 mv were rejected (Adrian, 1956). The potential outside the cell was measured at the beginning and end of each experiment. Any change in this value was defined as drift. This drift was minimized by using a Brush Mark 220 DC recorder (Brush Instruments Div., Clevite Corp., Cleveland, Ohio) which drifted only ±0.2 mv/8 hr. However, the drift in the electrometer or the micropipette was occasionally greater. Changes of 5 mv or less were assumed to have been linear with time and the appropriate corrections made since such changes were linear when the micropipettes were placed in ASW for several hours.

Membrane resistance and capacitance were measured either by passing a test pulse through the $E_m$-sensing micropipette in a Wheatstone bridge circuit (Martin and Pilar, 1963) or by using two intracellular micropipettes, one to pass constant current pulses across the cell membrane, and the other to measure the $E_m$. In some experiments, the membrane potential was set at certain levels by passing constant current across the membrane from one stimulator, and test pulses of current were
applied from a second stimulator resulting in a membrane potential change that was within the linear portion of the current-voltage relationship of the membrane.

Measurement of Intracellular Chloride Activity

The fabrication and testing of the chloride-sensitive microelectrodes has been previously reported (Brown, Walker, and Sutton, 1970; Walker, 1971). The selectivity constants for chloride over the following anions are: bicarbonate, 20:1; isethionate 5:1; propionate, 2:1; and glutamate, 200:1. Chloride microelectrodes were made daily and each was calibrated in a series of KCl solutions varying in activity from $6.05 \times 10^{-4}$ to $9 \times 10^{-3}$ M, and ASW before and after the experiment (Fig. 1). Activities in the calibrating solutions were determined from tables in the Handbook of Chemistry and Physics, by use of the extended Debye-Hückel equation (Robinson and Stokes, 1959), or by direct measurement of chloride activity using an Ag:AgCl electrode. There were no significant differences among the various methods. The calibrating solutions were maintained at the experimental temperatures being used by placing them in 0.5-ml chambers in the top of the constant temperature bath. It was necessary to cool the ion-sensitive electrodes slowly (over 1–3 min) to prevent their destruction which was apparently due to a differing coefficient of expansion of the exchanger oil and the glass pipette. By plotting the log10 of the activities against the voltage output at each activity, the slope of the microelectrode was obtained. At 20°C it was 53–56 mV per 10-fold change in chloride activity. At 1°C it was 50–53 mV, and the difference is attributable to the temperature difference (Walker, 1971).

Since the resistance of these microelectrodes, measured directly with a Keithley 600B electrometer (Keithley Instruments, Inc., Cleveland, Ohio), varied between $10^9$ and $10^{11}$ ohms, the output was connected to a high-input impedance vibrating reed electrometer. The voltage was read directly from a digital voltmeter with an accuracy of ±0.5 mV; it could also be recorded simultaneously on the penwriter (Fig. 1 B).

After calibration, the ion-sensitive microelectrode was positioned over R2, and the voltage reading in ASW ($E_o$) noted. The electrode was then advanced into the neuron using a micromanipulator (Fig. 1). If the electrode tip was fully inside the cell, current pulses passed across the cell membrane resulted in equal voltage deflections of the ion-sensitive microelectrode and $E_m$-sensing micropipette (Fig. 1 B). Such pulses were passed periodically throughout an experiment.

Intracellular chloride activity ($a_{ci}^{\prime}$) was then calculated as follows:

$$a_{ci}^{\prime} = a_{ci} / 10^{\frac{E_i - E_o - E_m}{b}}$$

where $a_{ci}^{\prime}$, $E_m$, and $E_o$ are as previously defined; $E_i$ is the voltage output of the microelectrode inside the neuron, $b$ is the slope of the electrode and equals $nRT/ZF$ as described in Brown, Walker, and Sutton (1970), and $a_{ci}$ is the chloride activity in the ASW measured at the same time the slope was determined.

Since three voltage measurements are required to calculate $a_{ci}^{\prime}$, and each can be read with an accuracy of ±0.5 mV the maximum possible error is 1.5 mV. For the range of $a_{ci}^{\prime}$ observed in the present work (30–60 mM), 1 mV is equivalent to 1.7
A

B

Figure 1 A. Plot of chloride-sensitive microelectrode voltage output against chloride activity for a microelectrode before (○) and after (●) it had been used to measure intracellular chloride activity. This microelectrode had an input resistance of $8 \times 10^9$ ohms, a slope of 55 mV/decade change in chloride activity, and gave an ASW chloride activity of 350 mM (denoted by the arrows) before and after impalement. A +2 mV DC shift occurred during the experiment.

Figure 1 B. Left-hand panel is a penwriter record of impalement of R2 by a chloride-sensitive microelectrode. Top trace is the ion electrode output, bottom trace is membrane potential; action potentials are greatly attenuated by the penwriter. The $E_i$ reading before impalement was +59 mV. Right-hand panel is a penwriter record taken 3 min after records in preceding panel. 5 nAmp of current was passed from a second 3 M KCl-filled micropipette resulting in equal deflection (7 mV) of the $E_m$ record and the $E_i$ record. At this time $E_i = +74$ mV. Calculated $a'c_l = 40$ mM. A 5 mV calibration signal was imposed on the $E_m$ trace between the two test pulses.

mm chloride (see Fig. 1 A). Thus, $a'c_l$ values calculated from single readings of $E_i$ and $E_m$ are accurate to within less than 3 mm. To further improve our accuracy for each data point measured, three readings of $E_i$ and $E_m$ were made 1 min apart and the values averaged.
It was noted during the first 1 or 2 hr of use that the voltage output of the chloride microelectrodes drifted; rapidly at first, then progressively more slowly. This problem was obviated by equilibrating the electrodes for 2 hr in ASW before calibration. Thereafter the voltage output was very steady for at least 10 hr. Results from electrodes whose \( E_{o} \) readings before and after impalement differed by more than 3 mv were rejected. Changes of 3 mv or less were assumed to have occurred linearly with time and corrections were made on this basis. However, changes in slope of the ion-sensitive electrodes which could not be accounted for by temperature changes always resulted in rejection of the experiment.

**Determination of Acetylcholine Reversal Potentials**

Application of acetylcholine (ACh) to R2 causes hyperpolarization (Kandel and Tauc, 1965). The reversal potential for this ACh response (\( E_{ACh} \)) was determined as follows. The membrane potential was preset by passing current through a second 3 m KCl-filled micropipette. Acetylcholine was then applied via the perfusate after a steady potential was reached. The change in potential elicited by ACh was plotted against the various preset membrane potentials. The membrane potential at which the sign of the potential change reversed was the reversal potential. At least three different membrane potentials were used for determining each \( E_{ACh} \). It was necessary to determine \( E_{ACh} \) at three different times in each cell and some desensitization to the effects of ACh always occurred. In order to elicit measurable responses for each determination, the concentrations of ACh in the perfusate were increased for each separate determination of \( E_{ACh} \). The first determination used \( 10^{-8} \) g/ml, the second \( 5 \times 10^{-8} \) g/ml, and the third \( 10^{-4} \) g/ml.

**RESULTS**

We measured intracellular chloride activity in 120 giant neurons between November 1969 and June 1972. The neurons were usually quiescent. For the first 40–80 min after impalement by the electrodes, membrane resting potential usually became more negative before reaching a steady-state value which was maintained for as long as 6 hr. Intracellular chloride activity showed little change during this initial change in \( E_{m} \) and increased only slightly during periods of continuous recording as long as 6 hr (Fig. 2; Table I). The average steady-state resting potential of these 120 neurons was \(-49.3 \pm 0.4\) mv, and the simultaneously measured average \( a_{\text{Cl}}^{\prime} \) was \( 36.7 \pm 0.8\) mM (mean \( \pm \text{sem} \)). The chloride equilibrium potential (\( E_{\text{Cl}} \)) calculated using the Nernst equation was \(-56.4 \pm 0.5\) mv, based on the measured external chloride activity (\( a_{\text{Cl}}^{\prime} \)) which averaged \( 341 \pm 3.5\) mM. The difference between \( E_{m} \) and \( E_{\text{Cl}} \) is statistically significant (\( P < 0.01 \)). Of the 120 individual neurons, \( E_{\text{Cl}} \) was more negative than \( E_{m} \) in 105, with 15 neurons having an \( E_{\text{Cl}} \) less negative than \( E_{m} \) (Brown, Walker, and Sutton, 1970). It may be of interest that of the 15 neurons where \( E_{\text{Cl}} \) was less negative than \( E_{m} \), 11 were studied during the months of September and October. However, because the seasonal incidence of neurons having \( E_{\text{Cl}} \) less negative
than $E_m$ was confirmed only in retrospect and so few were encountered, such neurons have not been further studied.

In nine neurons we recorded $E_m$ and $a_{Cl}^i$ continuously for 3–6 hr to see if $E_{Cl}$ would eventually equal $E_m$. Table I shows that even though the membrane potential usually became more negative and $a_{Cl}^i$ usually increased slightly, $E_{Cl}$ always remained definitely more negative than $E_m$.

Fig. 2 shows one experiment in its entirety and demonstrates clearly that

![Figure 2](image)

**Figure 2.** Intracellular chloride activity, $\square; E_m$, $\bullet; \text{and } E_{Cl}$, $\mathbf{I}$ during a 6 hr period of continuous impalement of R2 (temperature 20 ± 1°C). The symbols used in this figure are used in all subsequent figures. Each data point on this and all succeeding graphs represents the average of three values obtained 1 min apart.

**Table I**

|       | $a_{Cl}^i$ | $E_{Cl}$ | $E_m$  |
|-------|------------|----------|--------|
| **mm** | **mV**     | **mV**   | **mV** |
| Initial value | 36.4±2.0 | -56.2±1.4 | -40.0±1.6 |
| Final value   | 37.6±1.6  | -55.2±1.2 | -48.4±1.2 |

All values are mean ± standard error of the mean of nine neurons. Initial values were taken as soon as all electrodes were in the neuron. The final values were taken 3-6 hr later (average 4.6 hr).

$a_{Cl}^i$ does not change in response to the relatively large gradient ($E_m - E_{Cl}$) which existed for the first 60–70 min after impalement. It should be pointed out that any contribution by a competing, intracellular anion for which the electrode selectivity was poor would increase the calculated $a_{Cl}^i$ and bring the apparent $E_{Cl}$ closer to $E_m$.

To test the possible effects of leakage from the 3 M KCl-filled micropipettes, steady levels of $a_{Cl}^i$ and intracellular potassium activity ($a_{K}^i$) (see Russell and Brown, 1972 b) were measured in two neurons over a period of 2–3 hr. A second 3 M KCl-filled micropipette was then inserted. This resulted in a transient depolarization, and a slight increase in $a_{Cl}^i$ (2–4 mM)
which was reversed to near control levels after 120-180 min. Changes in $a_i^K$ were biphasic; first there was a decrease of about 4 mM followed by an increase to a level that was 1-3 mM greater than the control level. Part of the increase in $a_i^Cl$ and decrease in $a_i^K$ could be due to an entrance of extracellular fluid occurring during penetration of the neuron. Considering the small changes upon introduction of the second 3 M KCl-filled micropipette and the fact that continuous recording shows very slight changes in $a_i^Cl$ or $a_i^K$, we conclude that leakage from the micropipettes does not affect our results.

![Figure 3. Intracellular chloride activity, $E_{Cl}$, and $E_m$ before and during cooling to 3°C. The seemingly inequivalent changes in $E_{Cl}$ and $a_i^Cl$ after cooling are due to the fact that the calculation for $E_{Cl}$ contains a temperature factor; therefore, even without any change in $a_i^Cl$, $E_{Cl}$ would decline about 4 mv.](image)

**Effects of Cooling on Intracellular Chloride Activity**

Active transport of ions is generally thought to involve energy-dependent chemical processes and should, therefore, be inhibited by cooling. 15 giant neurons were cooled to 1-4°C. Within 40-80 min after such cooling, $E_{Cl}$ equaled $E_m$ (Table II) and remained equal for as long as 6½ hr (Fig. 3). The results were the same regardless of whether $E_m$ hyperpolarized (seven neurons) or depolarized (eight neurons) in response to the cooling. In six neurons the increase in $a_i^Cl$ toward an asymptote, $a_i'_{Cl}$, which is the equilibrium value for $a_i^Cl$, was plotted as $\log [(a_i'_{Cl} - a_i(t))]$ vs. time where $a_i(t)$ is the measured $a_i'_{Cl}$ value at each point after cooling (Fig. 4). Re-
TABLE II

|          | \(a'c_1\)   | \(E_{c1}\) | \(E_m\) | n  |
|----------|-------------|------------|---------|----|
| Control (20°C) | 34.2±1.4    | -58.5±1.0 | -48.6±1.3 | 17 |
| 40-80 min after cooling to 0.4-4°C | 42.9±2.3    | -48.6±1.2 | -48.2±1.3 | 15 |
| 40-80 min after cooling to 10°C | 39.9±1.1    | -51.5±0.7 | -40.3±0.6 | 2  |

All values are mean ± standard error of the mean. The 40-80 min post-treatment time was chosen as the period during which \(E_{c1}\) became equal to \(E_m\) after cooling to 4°C or below; thereafter \(E_{c1}\) remained equal to \(E_m\) in such cells. \(E_{c1}\) never became equal to \(E_m\) in cells cooled to 10°C.

Figure 4. Exponential decrease in the difference between \(a'c_1\), the value of \(a'c_1\) where \(E_{c1}\) first equaled \(E_m\), and \(a'c_1(t)\) the increasing values of \(a'c_1\) after cooling to 2°C. Fuller explanation is in the text. The data were obtained from the same experiment shown in Fig. 5. The last two points represent changes which are less than our estimates of measurement accuracy, but they did fall on the calculated regression line.

Regression lines determined by the method of least squares had correlation coefficients for linear regression greater than 0.95. Hence, the increase could be fit with a single exponential function after a variable latency of 5–20 min. The average rate constant calculated from the regression lines of these six neurons was \(7.2 ± 2.4 \times 10^{-4} \text{ sec}^{-1}\). In the remaining nine neurons, there
were too few measurements of $a_{ci}^*$ during its increase to characterize the way in which it increased, either because one of the electrodes slipped out of the cell or the increases were small and occurred too quickly.

During the 1st hr of cooling, membrane input resistance ($R_m$) was observed to decrease by 33% in five neurons cooled to 1°C-4°C. In these neurons, care was taken to measure $R_m$ with the membrane potential at its control resting value by applying the necessary current clamp. This simplified the interpretation since the current-voltage relation is nonlinear in this neuron (Brown and Berman, 1970). Thus, it might be argued that the relatively rapid equilibration of chloride across the cell membrane after cooling was attributable to an increase in chloride permeability. We investigated this by calculating chloride conductance, $g_{Cl}$, at 20°C and at 1°C-4°C. The $g_{Cl}$ was calculated from the equation, $g_{Cl} = T_{Cl} \cdot g_m$. The total membrane conductance, $g_m$, was measured directly, and the transport number, $T_{Cl}$, was determined by the change in membrane potential produced by a change in external chloride concentration. Sulfate was substituted for chloride (Brown, Walker, and Sutton, 1970) to yield $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{10}$ normal chloride concentrations. Neurons were in contact with the low-chloride solution for not more than 3 min and direct measurements revealed no change in $a_{ci}^*$ during such treatment. Thus, $\frac{\partial E_m}{\partial \log [Cl]}_o = 58 \ T_{Cl}$ (Brown, Walker, and Sutton, 1970). At 20°C, there was an 18 mv decrease in $E_m$ for a 10-fold decrease in [Cl]o ($n = 6$), giving a $g_{Cl}$ of $2.3 \times 10^{-7}$.

**Figure 5.** Effects of cooling to 2°C, then rewarming on $a_{ci}^*$, $E_{Cl}$, and $E_m$.
mhos. After cooling to 0.5°-2.5°C, \( g_{\text{Cl}} \) decreased to \( 1.7 \times 10^{-7} \) mhos. Therefore, it seems unlikely that the increase in \( a'_\text{Cl} \) was due to an increase in chloride conductance.

Three neurons in which \( E_{\text{Cl}} \) equaled \( E_m \) after cooling for 40-100 min were rewarmed to room temperature (Fig. 5). Within 5-10 min after rewarming, the passive driving force \( (E_m - E_{\text{Cl}}) \) favored an increase in \( a'_\text{Cl} \), yet a decrease was actually observed.

Cooling these neurons to 10°C only slightly increased \( a'_\text{Cl} \) while causing \( E_m \) to fall an average of 12 mv in two neurons (Table II). Thus, the passive gradient favoring an increase in \( a'_\text{Cl} \) was greater in these neurons than those cooled to 1°-4°C, yet the increase was smaller.

These results provide strong support for the view that chloride is actively extruded from the giant neuron. To obtain further evidence, the effects of several candidate inhibitors were studied.

**Effects of Ouabain on \( a'_\text{Cl} \)**

Ouabain has been reported to inhibit active chloride transport in certain epithelial tissues (Cooperstein, 1959; Kitahara, 1967; Finn, Handler, and Orloff, 1967). Therefore, \( a'_\text{Cl} \) was measured continuously in 13 neurons before and after applying ouabain in three different concentrations, \( 2 \times 10^{-3} \text{ M} (n = 2) \), \( 2 \times 10^{-4} \text{ M} (n = 9) \), and \( 2 \times 10^{-6} \text{ M} (n = 2) \) for varying periods of time. Table III presents data showing the slow increase in \( a'_\text{Cl} \) after exposure to ouabain. We have not found the effects of ouabain to be reversible. A further analysis of the action of ouabain on the proposed active extrusion of chloride was carried out by exposing neurons to 100 mM K+-ASW.

**Effects of Ouabain or Cooling on the Response to High External \([K^+]\)**

Application of 100 mM K+-ASW to this neuron caused a rapid increase in \( a'_\text{Cl} \). The effect was reversible when the neuron was returned to normal ASW at room temperature, (Fig. 6), \( a'_\text{Cl} \) returning to within 1 mM of its pretreatment value in seven out of eight neurons and to within 3 mM in the other. The recovery process could be fitted with an exponential function having a rate constant of \( 6.4 \pm 0.8 \times 10^{-4} \text{ sec}^{-1} \) (n = 8).

The effects of ouabain on the recovery process were tested as follows. After a suitable control period (40-70 min), 100 mM K+-ASW was applied for 15 min followed by a return to normal ASW. When \( a'_\text{Cl} \) had returned to control levels, ouabain was applied for 60 min, a ouabain 100 mM K+-ASW solution for 15 min, and then ouabain-ASW was reintroduced. The recovery process for \( a'_\text{Cl} \) in control ASW had an average rate constant of \( 6.7 \pm 0.6 \times 10^{-4} \text{ sec}^{-1} \) (n = 8). After the various concentrations of ouabain, the rate constants for recovery were: \( 2 \times 10^{-6} \text{ M}, \ 7.6 \pm 0.8 \times 10^{-4} \text{ sec}^{-1} \) (n = 2);
2 × 10^{-4} M, 3.7 ± 0.6 × 10^{-4} sec^{-1} (n = 4); 2 × 10^{-3} M, 1.8 ± 0.4 × 10^{-4} sec^{-1} (n = 2). The rate constants after 2 × 10^{-3} M and 2 × 10^{-4} M ouabain were significantly less than the control rate constant, but 2 × 10^{-6} M ouabain had no significant effect. This graded dose response differs from the effects of the same concentrations of ouabain on intracellular potassium activity where no difference was observed among the three concentrations (Russell and Brown, 1972b).

In marked contrast to the partial inhibition of \(a_{\text{Cl}}\) recovery caused by...
ouabain, cooling to 0.5°–2.5°C completely prevented any recovery of $a_{\text{cl}}$ towards control levels against its electrochemical gradient (Fig. 6). Rewarming to 20°C produced a return of $a_{\text{cl}}$ to precooling levels, and now treatment with 100 mM K+-ASW was followed by a complete recovery of $a_{\text{cl}}$ upon return to control ASW even though the passive gradient became unfavorable.

**Effects of 2,4-Dinitrophenol (DNP)**

The effects of this uncoupler of oxidative phosphorylation were of interest because of its inhibition of the proposed active uptake of chloride in squid axon (Keynes, 1963). However, DNP, as well as sodium cyanide and sodium azide, reduced the slope relating chloride activity to voltage output of the chloride-sensitive electrode in an inconsistent manner, particularly at chloride activities less than 100 mm. However, the effect was immediately reversible after removal of the inhibitor. Thus, $a_{\text{cl}}$ could be measured before and after but not during DNP treatment.

A way of testing the effects of DNP on chloride transport was based upon the observation that the equilibrium potential for the acetylcholine response in this neuron was very near $E_{\text{cl}}$ (12 neurons; $E_{m} = -47.6 \pm 1.3$ mv; $E_{\text{cl}} = -54.2 \pm 2$ mv; $E_{\text{ACH}} = -53.0 \pm 2.0$ mv; $P > 0.05$). In three neurons 110–150 min after treatment with DNP (0.2 mM), $E_{m}$ decreased to $-37.9 \pm 2.2$ mv, while $E_{\text{ACH}} = -46.7 \pm 5.2$ mv. 90–120 min after the wash-out of DNP, $E_{m} = -48.6 \pm 1.2$ mv, $E_{\text{ACH}} = -52.7 \pm 5.0$ mv, and $E_{\text{cl}} = -52.4 \pm 4.5$ mv ($P > 0.05$). For comparison, ouabain (2 X $10^{-4}$ m) applied to one neuron for 2 hr caused $E_{m}$ to decrease from $-50$ mv to $-34$ mv, $E_{\text{ACH}}$ from $-53$ mv to $-50.5$ mv, and $E_{\text{cl}}$ from $-53$ mv to $-52.6$ mv. When this particular neuron was cooled to 1°C, $E_{m}$ increased 1 mv to $-35$ mv, but $E_{\text{cl}}$ declined to $-37$ mv and $E_{\text{ACH}}$ to $-37.7$ mv. Thus, the effects of ouabain and cooling indicate that $E_{\text{ACH}}$ does follow $E_{\text{cl}}$ quite well in this neuron. $E_{\text{ACH}}$ was always more negative than $E_{m}$, even after 2 1/2 hr of DNP treatment, whereas after cooling they were nearly equal within 50 min. Thus, at most, only partial inhibition of the chloride transport process may have been achieved by DNP treatment. It might be that the depolarization caused by DNP increased the electrochemical gradient to a level which the pump could not sustain. Then the increase in $a_{\text{cl}}$ might represent the attainment of a new steady state rather than inhibition of a chloride pump. The data do not permit a choice between the two interpretations.

**Results of Treatment with Acetazolamide or Ammonium Salts**

The carbonic anhydrase inhibitor, acetazolamide, has been suggested as an inhibitor of active chloride transport in certain epithelial tissues (Woodbury,
Sodium acetazolamide in concentrations of 1 or 10 mM was applied to three neurons. The 10 mM concentration produced a rapid membrane depolarization of about 6 mV whereas 1 mM acetazolamide produced a slow, gradual depolarization. Neither concentration caused any significant change in $a_{Cl}^-$.

It has recently been proposed that the ammonium ion inhibits active extrusion of chloride from cat spinal motoneurons (Lux, 1971). In R2, neither ammonium acetate nor ammonium chloride had any significant effects on $a_{Cl}^-$ in four neurons when applied in concentrations of 10–100 mM.

**Calculations of Chloride Permeability**

The rate at which an ion passively crosses a biological membrane is a function of its electrochemical gradient and the membrane permeability. In the present experiments, the electrochemical gradient was determined as the difference between the measured $E_m$ and the calculated $E_{Cl}$ derived from direct and continuous measurements of $a_{Cl}^-$. Net fluxes were calculated assuming that changes in $a_{Cl}^-$ reflected only net movements of $Cl^-$ across the neuronal membrane. We recognize that increases in $a_{Cl}^-$ may be due either to a decrease in efflux, an increase in influx, or some combination of the two. In the case of cooling, we found that chloride conductance was slightly reduced, and we interpret the increase in $a_{Cl}^-$ to be the result of decrease in efflux. We attribute the increase in $a_{Cl}^-$ during treatment with 100 mM K⁺-ASW to a rapid influx of chloride which greatly exceeded chloride efflux. It was, therefore, possible to calculate chloride permeability during cooling to 1°–4°C and during treatment with 100 mM K⁺. We also assume that during cooling, active efflux had ceased and that during exposure to 100 mM K⁺, it was small compared to the passive influx of chloride. Hence, the permeabilities we estimated are minimum values.

6 cooled neurons and 14 neurons treated with 100 mM K⁺ had increases in intracellular chloride activity which could be fitted by means of regression analysis with a single exponential function. Using these exponential increases in $a_{Cl}^-$, net fluxes were calculated according to the relation:

$$M_{Cl} = \frac{C_{Cl1}^i - C_{Cl2}^i \cdot V}{t^A}$$

where $M_{Cl}$ is net chloride flux in moles/sec·cm², $(C_{Cl1}^i - C_{Cl2}^i)$ is the change in intracellular chloride concentration in moles/liter, concentration being obtained by dividing the measured activity by 0.7, the activity coefficient calculated for ASW from Davies' modification of the extended Debye-Hückel equation. The assumptions made are that intracellular and extra-
cellular ionic strengths are similar and that Cl\textsuperscript{-} is free in solution inside the neuron. \( t \) is time in seconds; \( V \) is cell volume in liters, and \( A \) is the cell surface area in square centimeters. Cellular volume was calculated as the volume of a sphere, the diameter being directly measured for each neuron in which a permeability was calculated with an eyepiece reticle accurate to within 10 \( \mu \). In 50 neurons, the average cell diameter was 519 \( \pm \) 19 \( \mu \), giving a cell body volume of 7.4 \( \times \) 10\textsuperscript{-8} liters. If one assumes a cylinder-shaped axon, 50 \( \mu \) in diameter and 2 mm long (Coggeshall, 1967), a volume of less than 4 \( \times \) 10\textsuperscript{-9} liters is obtained. This amounts to less than 6\% of the average somal volume so the contribution of the axon to the total volume may be ignored. Since electron microscopic studies have shown that this neuron is highly invaginated, the surface area of the cell body is greater than that of a smooth sphere (Coggeshall, 1967) and has been estimated to be about ten times greater (Mirolli, quoted by Carpenter, 1970).

To obtain further information regarding the cellular surface area, we studied membrane capacitance in eight neurons using two 3 M KCl-filled micropipettes as described in Methods. Six of the eight neurons exhibited a biphasic charging process which, when plotted as \( \ln [(V_o - V)V_o^{-1}] \) vs. \( t \) could be fit with two exponential functions having time constants of 175 \( \pm \) 30 and 1035 \( \pm \) 200 msec, respectively. The remaining two neurons had only one time constant, 200 and 210 msec, respectively.

The average value for the faster time constant of all eight neurons was 185 \( \pm \) 26 msec while the average membrane input resistance of these neurons was 1.5 \( \pm \) 0.2 \( \times \) 10\textsuperscript{4} ohms. This gave an input capacitance of 1.2 \( \times \) 10\textsuperscript{-1} \( \mu \)F. If one assumes a specific capacitance of 1 \( \mu \)F/cm\textsuperscript{2} (Katz, 1965; Marmor, 1971), a cellular surface area of 1.2 \( \times \) 10\textsuperscript{-1} cm\textsuperscript{2} is obtained. This represents a 13-fold increase over the surface area calculated for these neurons on the basis of a smooth sphere and is in good agreement with the estimate of Mirolli. The slower time constant gave a 75-fold increase in surface area and may include axonal membrane. Therefore, the factors 13 and 75 were both used to estimate cellular surface area for the calculation of net chloride flux (equation 1).

Average values for \( M_{cl}(M \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}) \) ranged from (a) 0.1 to 0.6 \( \times \) 10\textsuperscript{-11} in six neurons cooled to 1\textdegree-4\textdegree C; (b) 0.5 to 2.6 \( \times \) 10\textsuperscript{-11} in four neurons treated with 100 mM K\textsuperscript{+}-ASW, and (c) 0.7 to 3.7 \( \times \) 10\textsuperscript{-11} in eight neurons treated with ouabain and 100-K\textsuperscript{+} ASW. There was no difference in these results among the three ouabain concentrations, and the difference between 100 mM K\textsuperscript{+}-ASW-treated neurons and ouabain-100 mM K\textsuperscript{+}-ASW-treated neurons was not statistically significant.

Using the directly measured \( E_a, a_{\text{cl}}, a_{c1} \), and the calculated net flux, \( M_{cl} \), chloride permeability (\( P_{cl} \)) in centimeters per second was calculated.
from the constant field equation (Hodgkin and Horowicz, 1959). This equation predicts with accuracy the $E_m$ of R2 (Russell and Brown, unpublished observations). Hence,

$$P_{cl} = M_{cl} \frac{RT}{E_m F} \frac{1 - \exp(-E_m F/RT)}{a_{cl} - a_{cl}' \exp(-E_m F/RT)}$$

where $R$, $T$, and $F$ have their usual meanings.

The $P_{cl}$ was (a) $4.7 - 27.0 \times 10^{-8}$ cm/sec for six neurons cooled to $1^\circ-4^\circ$C; (b) $8.7 - 36 \times 10^{-8}$ cm/sec for four neurons treated with 100 mM $K^+$, and (c) for eight neurons treated with ouabain-100 mM $K^+$-ASW, it was $4.0 - 23 \times 10^{-8}$ cm/sec, the difference between the treatments was not statistically significant.

To test the contribution of the axon to the observed changes in $a_{cl}'$, two neurons were ligated as closely as possible to the soma by means of fine silk thread. At the end of each experiment, the neuron was fixed in a phosphate-buffered mixture of glutaraldehyde and formaldehyde, then postfixed in osmic acid. Serial sections, prepared and kindly examined for us by Dr. L. J. Stensaas, revealed no visible axon between the ligature and the axon hillock. Within 1-2 hr of ligation, the neuron had average resting potentials of $-40$ mv, $a_{cl}'$'s of 48 mM, and $E_{cl}$'s of $-48$ mv. When ouabain and 100 mM $K^+$ were applied to these neurons, $a_{cl}'$ increased with a rate constant of $2.4 \times 10^{-3}$ sec$^{-1}$ which compares very well with the rate constants obtained in intact neurons. The $P_{cl}$ values for these two neurons ranged from $4.7$ to $27 \times 10^{-8}$ cm/sec, which are quite similar to the $P_{cl}$ values of intact neurons treated similarly.

**DISCUSSION**

The evidence presented shows that chloride is actively transported out of R2, the giant neuron of the abdominal ganglion of *Aplysia californica*. Thus, during continuous recording $E_{cl}$ never equaled $E_m$ for as long as 6 hr, whereas $E_{cl}$ equaled $E_m$ within 30-80 min after cooling to $1^\circ-4^\circ$C. Rewarming a cell in which $E_{cl}$ equaled $E_m$ caused a net movement of chloride out of the cell against the electrochemical gradient. Loading a neuron with chloride by exposing it to 100 mM $K^+$-ASW, then washing with normal ASW again showed that the neuron could extrude chloride against an electrochemical gradient.

The effects of several possible chemical inhibitors were also examined. Ouabain caused a slow net increase in $a_{cl}'$ and a dose-dependent decrease in the rate of chloride extrusion after treatment with 100 mM $K^+$-ASW. Nevertheless, some extrusion persisted. Such results are consistent with a partial inhibitory effect by ouabain on an active chloride transport mecha-
The partial inhibition is difficult to assess because the depolarization due to ouabain also increases the electrochemical gradient for chloride. 2,4-dinitrophenol may have caused a partial inhibition, but the data do not permit a firm conclusion on this point. Acetazolamide and externally applied ammonium salts seemed to be without effect on the chloride transport process.

The accuracy of the chloride microelectrodes used in these experiments has been adduced from comparisons with other chemical analyses in a variety of tissues (Chow, Kunze, Brown, and Woodbury, 1970; Cornwall, Peterson, Kunze, Walker, and Brown, 1970). In addition, synaptic action on or application of acetylcholine to the L1–L6 neurons of this ganglion produces an inhibitory postsynaptic potential due solely to an increase in chloride conductance (Pinsker and Kandel, 1969; Kehoe and Ascher, 1970). Direct measurement of intracellular chloride activity using our chloride-sensitive microelectrodes shows that the chloride equilibrium potential equals the synaptic reversal potential in these neurons (Kunze and Brown, 1971).

The chloride permeabilities of $4.7 \times 10^{-8}$ to $36 \times 10^{-8}$ cm/sec calculated in the *Aplysia* giant neuron may be compared with those reported for squid axon which range from $1.1 \times 10^{-8}$ to $2.9 \times 10^{-8}$ cm/sec (Shanes and Berman, 1955) to $1.1 \times 10^{-8}$ to $2.9 \times 10^{-8}$ cm/sec (Hurlbut, 1970). In the subsequent paper, we calculated that the potassium permeability was $2.6 - 18.6 \times 10^{-8}$ cm/sec, which is therefore equivalent to the chloride permeability. This is of interest since it has been assumed that chloride permeability is either very much greater or very much smaller than other ionic permeabilities in molluscan neurons (Moreton, 1968; Gorman and Marmor, 1970).

The only other neuronal tissue in which comparable studies of chloride transport have been made was squid axon (Keynes, 1963), where it appeared that chloride was transported inwardly. Chloride transport by the *Aplysia* neuron R2 differs significantly in that (a) chloride was clearly extruded from the majority of neurons, although during September and October chloride may be transported into the neuron; (b) the effect of DNP was less; and (c) ouabain probably produces a partial inhibition of chloride extrusion.

The present demonstration of active chloride efflux in an *Aplysia* neuron, plus the indications of nonequilibrium chloride distributions in other neurons (see Introduction), and squid axon (Keynes, 1963) suggests that active transport of chloride may be a widespread phenomenon among neural tissues. As a result, the chloride equilibrium potential could differ significantly from the membrane potential. This is of considerable importance not only because many inhibitory synaptic potentials have been demonstrated to be chloride-dependent, but also because of the profound effects of carbon diox-
ide and hydrogen ions on excitable membranes. These effects are largely accounted for by changes in chloride or anion permeability (Reuben, Girardier, and Grundfest, 1962; DeMello and Hutter, 1966; Hutter and Warner, 1967; Hagiwara, Gruener, Hayashi, Sakata, and Grinnell, 1968; Strickholm, Wallin, and Schrager, 1969; Brown, Walker, and Sutton, 1970). Thus, the response of a given neuron to either of these substances will depend critically upon the relationship of the $E_{\text{Cl}}$ to the $E_m$. In turn, this relationship will be governed by the system responsible for the active transport of chloride.

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