Chloride and Sodium Influx: a Coupled Uptake Mechanism in the Squid Giant Axon

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ABSTRACT The squid giant axon was internally dialyzed while the unidirectional fluxes of either Cl or Na were measured. The effects of varying the internal or external concentration of either Na or Cl were studied. Chloride influx was directly proportional to the external Na concentration whereas Cl efflux was unaffected by changes of the external Na concentration between 0 and 425 mM. Neither Cl influx nor efflux were affected by changes of internal Na concentration over the range of 8-158 mM. After ouabain and TTX treatment a portion of the remaining Na influx was directly dependent on the extracellular Cl concentration. Furthermore, when the internal Cl concentration was increased from 0 to 150 mM, the influxes of Cl and Na were decreased by 14 and 11 pmol/cm²-s, respectively. The influx of both ions could be substantially reduced when the axon was depleted of ATP. The influxes of both ions were inhibited by furosemide but unaffected by ouabain. It is concluded that the squid axolemema has an ATP-dependent coupled Na-Cl co-transport uptake mechanism.

INTRODUCTION

A major portion of chloride influx across the axolemema of the squid giant axon depends upon cellular metabolism (Keynes, 1963) or, more directly, adenosine 5′-triphosphate (ATP; Russell, 1976). Inasmuch as the intracellular chloride concentration of the axon is much higher than expected from passive thermodynamic considerations (Bear and Schmitt, 1939); Webb and Young, 1940; Mauro, 1954; Koechlin, 1955; Deffner, 1961; Keynes, 1963; Brinley and Mullins, 1965 a and b; Russell, 1976), it seems likely that the ATP-dependent chloride influx represents an active transport process. Active chloride transport has been proposed for several excitable tissues (Keynes, 1963; Lux, 1971; Russell and Brown, 1972; Linas et al., 1974; Russell, 1976), but in contrast to the case for epithelial tissues (Zadunaisky, 1972; Burg et al., 1973; Nellans et al., 1973; Karnaky et al., 1977), little attention has been paid to the possible involvement of sodium in such transport processes. In this regard it is interesting to note that a portion of sodium influx into the squid giant axon requires ATP but does not seem to be the result of sodium-sodium exchange (Brinley and Mullins, 1968; Caldwell et al., 1960). Some of the ATP-stimulated sodium influx may be via either the Na-Ca exchanger (DiPolo, 1974; Blaustein and Santiago, 1977) or Na-Mg exchanger (Mullins et al., 1977), but these mechanisms cannot account for all the ATP-dependent sodium influx. It seemed possible that some of the ATP-
dependent sodium influx might be related to the ATP-dependent chloride influx.

Keynes (1963) examined the consequence of partially replacing external sodium on 36Cl uptake in two intact axons and found little or no effect. The technique of internal dialysis (Brinley and Mullins, 1967) provides significant advantages for the study of influxes, and in view of the evidence cited in the preceding paragraph, it seemed worthwhile to utilize this technique in order to reconsider the question of sodium-dependent chloride influx. In this study I have examined the effects of sodium on chloride influx as well as the effects of chloride on sodium influx. A significant portion of the influx of either ion requires the extracellular presence of the other ion. In addition, both of these co-ion-dependent influxes can be depressed by increasing the intracellular concentration of chloride. Finally, both fluxes are, to a large degree, dependent upon ATP. They are partially inhibited by the diuretic agent, furosemide, but are unaffected by ouabain. The similarities in the behaviors of the two fluxes strongly imply that a coupled Na-Cl uptake system, which requires ATP for its full expression, exists in the axolemma of the squid giant axon.

**METHODS**

**Axons**

These experiments were performed at the Marine Biological Laboratory, Woods Hole, Mass. during May and early June, 1976 through 1978. Live specimens of the squid, *Loligo pealei*, were decapitated, and the first stellar nerve was removed and placed in cold, Woods Hole seawater. After careful cleaning, the giant axons were mounted horizontally in a dialysis chamber.

The temperature of the bath was maintained at 15°C by means of a coolant fluid circulating from a Lauda K2/RD cooler (Brinkmann Instruments, Inc., Westbury, N.Y.) through the underside of the dialysis chamber; a thermistor (Fenwal Electronics, Framingham, Mass.) located just below the axon constantly monitored the bath temperature.

**Dialysis**

The technique of internal dialysis (Brinley and Mullins, 1967) was used in these experiments. The dialysis tube was a 12-cm length of hollow cellulose acetate tubing (140 μm o.d.; FRL, Inc., Dedham, Mass.) glued to a plastic T-tube. For influx experiments, the tube had a central region ~27 mm long which had been rendered porous by a 16-20-h soak in 0.1 N NaOH. The porous central region was 20 mm long for efflux experiments.

A 40-45 mm length of axon was cannulated at both ends, then the dialysis tube was guided through the axon until the porous region was positioned in the central portion of the axon. The axon was then roled onto grease dams at either end of the central slot in the bath. The grease was a mixture of Vaseline and mineral oil and it was also applied on top of the axon at the dam sites. Then greased plastic inserts were placed over the axon at these two points, isolating the central, dialyzed region of the axon from the cannulated ends.

**Influx Procedures**

The width of the central compartment between the grease dams was ~19 mm, whereas the axon was dialyzed over a length of ~27 mm. Thus, a region ~4 mm beyond the
central region was dialyzed on each end of the axon. This arrangement was designed to pick up isotope that diffuses laterally within the axon. If this is not done, the influx will be initially underestimated but will progressively increase as the undialyzed lateral regions begin to accumulate counts. The reservoir of counts in the undialyzed ends would serve to damp out the effects of a treatment which reduced the real influx.

The fluid bathing the axon could be withdrawn through ports located at the bottom of the central slot and connected to a peristaltic pump. In order to apply the appropriate radioactive external fluid, the following procedure was used. The axon was first washed with at least 5 ml (slot volume = 0.2 ml) of the appropriate nonradioactive solution. This was accomplished by applying the fluid at the top of the slot and withdrawing it through the bottom ports at a flow rate of ~1.7 ml/min. Then the fluid level in the slot was lowered until the meniscus was just over the axon and 0.2 ml of the radioactive solution was added. The level of fluid was again lowered and another 0.2 ml of radioactive fluid added. This process was repeated once more. The external fluids all contained phenol red so that any leaks through the grease seals into the end regions could be quickly detected and repaired. Specific activity samples were taken from the slot fluid bathing the axon.

Influx samples were taken by allowing the dialysis fluid (flowing at the rate of 1 ml/min) to fall directly into the scintillation vial after first passing through the axon. At the end of a timed interval (usually 6-8 min) the tip of the dialysis tube was washed with 1.0 ml of deionized water and the washings also collected in the vial. 10 ml of a 2:1 toluene: Triton X-100 counting cocktail (Nadarajah et al., 1969) containing 4 g/liter Omnifluor (New England Nuclear, Boston, Mass.) was added.

Efflux Procedures

For efflux experiments the isotope was presented via the dialysis fluid. External fluid entered the central compartment at a rate of 2.4 ml/min through the two bottom ports and was collected at the top after flowing around the axon. Two guard syringes, each withdrawing fluid at the rate of 50 μl/min, were connected to ports at either end of the central slot region. Thus, isotope efflux from the boundary area of the dialyzed region was collected and discarded. The remaining fluid (2.3 ml/min) was collected directly into scintillation vials mounted in a fraction collector which changed samples every 4 min. 10 ml of the toluene: Triton X-100 cocktail were added and a stiff gel quite suitable for counting resulted.

Both the 36Cl and 22Na samples were counted in a Packard Tricarb model 3255 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Each sample was counted until at least 1,000 counts were collected which allowed 95% confidence that the counts were accurate to within 6%. In most instances at least 2,000 counts were obtained.

Solutions

The compositions of the external solutions are listed in Table I. Intermediate chloride or sodium concentrations were obtained by mixing aliquots of the appropriate artificial seawater solutions (SSW). Chloride-36 as supplied by New England Nuclear or Amer-sham/Searle (Arlington Heights, Ill.) contains a considerable amount of carrier chloride, which was taken into account when making 36Cl-containing solutions. The influx solutions were made to contain ~30 μCi of 36Cl per millimole of total chloride. The 36Cl salt solutions provided by the suppliers were dried and then ashed at 450°C before being added to the experimental fluids. Sodium-22 was supplied as a carrier-free solution.

The compositions of the internal dialysis fluid stock solutions are given in Table II. Intermediate chloride concentrations were obtained by proportionately mixing the high and low chloride solutions. For experiments involving changes of internal sodium
concentration, sodium glutamate was added or subtracted at the osmotic expense of taurine; thus, internal potassium and chloride concentrations were maintained constant. For efflux experiments, NaCl was added as KCl taking into account its contribution to the total KCl content of the dialysis fluid. A final specific activity of ~50 μCi per millimole was used in the dialysis fluid.

TABLE I

| COMPOSITION OF THE ARTIFICIAL SEAWATERS |
|----------------------------------------|
| Ion | Na | Li | Choline | Mg | Mannitol | Gluconate | Methanesulfonate | SO₄ |
|-----|----|----|---------|----|----------|-----------|-----------------|-----|
| SSW | 425 | 118 | 425 | 425 | 840 |
| SSW | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Li | 425 | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Mg | 555 | 555 | 555 | 555 | 555 | 555 | 555 |
| Ca | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Cl | 435 | 435 | 435 | 435 | 435 | 435 | 435 |
| Gluconate | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| Methanesulfonate | 485 | 485 | 485 | 485 | 485 | 485 | 485 |
| Mannitol | 205 | 205 | 205 | 205 | 205 | 205 | 205 |

In addition to the major components listed above, all the artificial seawaters contained 0.5 mM phenol red, 0.1 mM EDTA, and 10 mM Hepes buffer titrated to pH 7.7 with NaOH. The osmolality of all the solutions was about 970 mosmol/kg.

TABLE II

| DIALYSIS FLUID STOCK SOLUTIONS* |
|----------------------------------|
| ATP | ATP-free |
|-----|----------|
| 0 Cl | 350 Cl | 0 Cl | 350 Cl |
| mM | mM | mM | mM |
| K | 350 | 350 | 350 | 350 |
| Na | 42 | 42 | 50 | 50 |
| Mg | 7 | 7 | 4 | 4 |
| Glutamate | 502 | 502 | 502 | 502 |
| Cl | 42 | 42 | 42 | 42 |
| SO₄ | 7 | 7 | 7 | 7 |
| Taurine | 200 | 200 | 205 | 205 |
| Hesper | 10 | 10 | 10 | 10 |
| EGTA | 0.5 | 0.5 | 0.5 | 0.5 |
| Phenol red | 0.5 | 0.5 | 0.5 | 0.5 |
| Cyanide | 2.0 | 2.0 | 2.0 | 2.0 |

* pH = 7.35; osmolality 935 mosmol/kg.
† 4 mM Na₂-ATP added immediately before use. pH was readjusted to 7.35 with KOH.

Furosemide was a gift of Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J. Adenosine 5'-triphosphate (ATP) was obtained from Sigma Chemical Co., St. Louis, Mo. In later experiments (1978) this was supplied essentially free of contamination by vanadium without any apparent difference in its effects on the present study.
**Membrane Potential**

The membrane potential was measured by momentarily placing a saturated KCl bridge connected to a calomel half-cell in contact with the dialysis fluid at the tip of the dialysis tube. The reference electrode was another calomel half-cell connected to the bath by a SSW salt-bridge. No attempt was made to correct for junction potentials, although it is believed these are small after the axoplasm has come into equilibrium with the dialysis fluid (Russell, 1976).

**RESULTS**

Chloride influx is inversely related to the intracellular chloride concentration (Russell, 1976). Therefore, in order to obtain a large influx, the present influx experiments were performed with a nominally zero internal chloride concentration unless otherwise specified.

**Effect of Varying [Na]o on Chloride Influx**

The first tests for sodium dependence were made using choline as the sodium substitute just as Keynes (1963) had done earlier. Replacement of 72% of the sodium in the SSW resulted in a reversible fall of chloride influx of ~30%. Lithium was also used as a sodium replacement in some early experiments. Complete replacement of external sodium by lithium resulted in about a 50% inhibition of chloride influx.

Because both choline and lithium are univalent cations, it seemed necessary to test for sodium dependency using a solution which contained only 10 mM potassium as the sole univalent cation. A solution designed by DeWeer (1970) for studying sodium fluxes was modified for use in the present experiments by keeping the external chloride concentration the same as the control Na-SSW. When all the external sodium was replaced by magnesium, the chloride influx in nine axons averaged $1.4 \pm 0.1$ pmol/cm$^2$·s (mean ± SEM), whereas when normal amounts of sodium were present (425 mM), it averaged $17.9 \pm 1.5$ pmol/cm$^2$·s. Qualitatively similar results were obtained from three axons dialyzed with 50 mM chloride. Complete replacement of external sodium using magnesium resulted in a fall of chloride influx from $11.0 \pm 0.7$ to $1.3 \pm 0.1$ pmol/cm$^2$·s. Fig. 1 A shows that a stepwise increase in external sodium concentration resulted in a proportional stepwise increase of chloride influx in fibers dialyzed with 0 mM chloride. In all, 12 axons were studied at several external sodium concentrations and their pooled results are seen in Fig. 1 B. A linear correlation between chloride influx and external sodium concentration in the range of 0–425 mM is evident. It may also be seen that substitution with lithium or choline resulted in substantially less reduction of chloride influx. This implies that lithium and choline can partially substitute for sodium in the activation of chloride influx. It may also help to explain why Keynes (1963) saw no effect when partially replacing sodium with choline.

**ATP Requirement for External Sodium-Dependent Chloride Influx**

An earlier study which demonstrated an ATP-dependent chloride influx was done in the presence of a normal external sodium concentration (Russell, 1976).
In that study, the average influx in the absence of ATP was reported to be 5.1 pmol/cm²·s. Recently, it has become obvious that a complete washdown of endogenous ATP using the cellulose acetate dialysis capillary tubes takes somewhat longer than was appreciated in the earlier study. It was found in the present experiments that ~70 min of dialysis with ATP-free, cyanide-containing fluids were required to reduce the chloride influx to a minimum steady value. In eight axons poisoned with cyanide and dialyzed with ATP-free fluid for no less than 80 min, chloride influx averaged 2.0 ± 0.4 pmol/cm²·s. Notice that this value is only slightly larger than the influx remaining after all the external sodium was replaced by magnesium. This similarity suggests that most of the sodium-dependent chloride influx also requires ATP.

![Figure 1](image)

**Figure 1.** The effect of replacing external Na on ATP-dependent Cl influx. (A) The effect on chloride influx of progressively increasing the external sodium concentration at the expense of the external magnesium concentration. The experiment was begun in Mg-mannitol SSW (Table I), then a mixture consisting of one-third NaCl-SSW, two-thirds MgCl₂-mannitol SSW was used, followed by two thirds NaCl-SSW, one-third MgCl₂-mannitol SSW. Axon diameter = 625 μM. (B) Effect on chloride influx of replacing extracellular sodium with three different cation substitutes in 15 axons. (●) Mg-mannitol; (△) choline; (□) lithium.

**Effect of Ouabain on Chloride Influx**

Ouabain was applied to three axons bathed in Na-SSW and dialyzed with ATP. The concentration used (10⁻⁵M) is 10–100 times that required to achieve 100% inhibition of active sodium efflux (Baker et al., 1969). Fig. 2 shows results typical of the three experiments. No inhibition of Cl influx could ever be detected after treatment with ouabain. This confirms a similar observation by Keynes (1963) who compared ³⁵Cl uptake in paired axons.

**Effect of Furosemide on Chloride Influx**

The diuretic agent furosemide inhibits chloride fluxes in a variety of epithelial tissues (Burg et al., 1973; Candia, 1973; Karnaky et al., 1977; Silva et al., 1977). In addition, this agent inhibits chloride fluxes in red blood cells (Brazy and Gunn, 1976), synaptosomes (Marchbanks and Campbell, 1976), and of more direct interest to the present study, in barnacle muscle (Boron et al., 1978).
Two external concentrations of furosemide were tested, 2 mM and 0.2 mM. In three axons, exposure to 2.0 mM furosemide resulted in a fall of Cl influx from $11.9 \pm 1.2$ to $3.3 \pm 1.0$ pmol/cm$^2$·s. However, at this concentration the membrane potential began to depolarize after 30-40 min of exposure and then the Cl influx would rapidly increase. In contrast, the effects of 0.2 mM furosemide were readily reversible as shown in Fig. 3. After the inhibition the axon was washed for 3 min with furosemide-free Na-SSW and then fresh $^{36}$Cl Na-SSW was reapplied. Such treatment almost completely reversed the inhibition within 20 min in this and one other axon similarly tested. In a total of six axons treated with 0.2 mM furosemide, chloride influx fell from $13.6 \pm 0.9$ to $2.9 \pm 0.4$ pmol/cm$^2$·s.

The average chloride influx in the presence of 0.2 mM furosemide was
slightly, though not significantly, higher than the average influx obtained from axons depleted of their ATP or bathed in Na free SSW (see above). This result suggests that furosemide blocks all the ATP-dependent, Na-dependent chloride influx, an hypothesis which was tested in the experiment illustrated in Fig. 4. First the axon was dialyzed with an ATP-containing dialysis fluid until a steady influx was obtained. The axon was then treated with 0.2 mM furosemide which caused the influx to fall from ~18 to ~3 pmol/cm²·s. The dialysis fluid was changed to one containing cyanide and no ATP. A 70-min dialysis with this fluid caused no further reduction of the influx. Thus, furosemide appears to block all the ATP-dependent chloride influx.

Effect of Na⁺ on Chloride Influx
The sodium concentration of the dialysis fluid was varied between 8 and 158 mM, a range which could be tested without changing the internal potassium concentration. Fig. 5 shows the results in one axon which are typical of those observed in two others. No discernible effect was noted when the internal sodium concentration was raised. It can be seen that although changing the internal sodium concentration was without effect, 0.2 mM furosemide caused a prompt fall of influx, thus showing that the axon was not simply a leaky one unable to respond to any treatment.

Effect of Cl⁻ on Chloride Influx
In view of the evidence that a large component of chloride influx is by a mediated transport mechanism, it was of interest to examine the relationship between chloride influx and the external chloride concentration. Three different substitute anions were used; gluconate, sulfate, and methanesulfonate. Fig. 6 is a composite of data obtained from nine axons, three axons with each anion substitute. The relationship between the chloride influx and external chloride concentration does not fit a simple, single-site carrier model, although it appears to saturate at external chloride concentrations greater than ~350-400 mM. In
view of the similarity of the data obtained using three rather different anions, it seems unlikely that the departure from ideal Michaelis-Menten kinetics can be attributed directly to the presence of the substitute anions. The possibility that these anions might bind sodium and reduce its chemical activity and thereby

\[ Na^{+} + e^{-} \rightarrow Na^{+}_{aq} \]  

indirectly affect chloride influx was tested by measuring the sodium ion activity of the chloride-free solutions using a sodium-sensitive glass electrode. No systematic difference among the solutions containing the anion substitutes and the Na-SSW was found. It is interesting to note that the apparent saturation of

\[ [Cl^{-}]_{o} \]

induced by raising the internal sodium concentration on chloride influx.

\[ \text{Inasmuch as this axon had a resting membrane potential of } -60 \text{ mV, the change of } [Na]_{i} \text{ from 8 to 158 mM reduced the net inward driving force of the sodium gradient from about-160 to about-85 mV, a 50% decline in the electrochemical gradient without any effect on chloride influx. However, treatment with 0.2 mM furosemide resulted in a prompt fall of chloride influx. Axon diameter = 625 \mu M.} \]

\[ \text{Figure 5. Effect of raising the internal sodium concentration on chloride influx.}\]

\[ \text{Figure 6. Effects on ATP-dependent chloride influx of reducing external chloride concentration with three different anion substitutes. These data represent nine axons, dialyzed with ATP-OCl-dialysis fluid.}\]
chloride influx occurs when the external chloride concentration is about equal to the external sodium concentration. This suggests that the external sodium concentration may be the limiting factor resulting in saturation of chloride influx.

**Lack of Effect of Sodium on Chloride Efflux**

The effects of changing either internal or external sodium concentrations upon Cl efflux were studied. In order to have an easily measurable efflux it was necessary to make the intracellular chloride concentration rather high, 150 mM.

| TABLE III |
| EFFECT OF SODIUM ON CHLORIDE EFFLUX IN ATP-FUELED AXONS (15°C) |

**A. External sodium**

| [Na]_o mM | 0 | 425 |
|-----------|---|-----|
| Axon      |   |     |
| 5038      | 4.6 | 4.5 |
| 5048A     | 5.7 | 5.8 |
| 5048B     | 3.9 | 3.7 |
| 5088A     | 5.6 | 5.9 |
| 5088B     | 5.9 | 5.1 |
| Mean      | 5.1 | 5.0 |
| ± SEM     | 0.4 | 0.5 |

**B. Internal sodium**

| [Na]_i mM | 8 | 58 | 158 |
|-----------|---|----|-----|
| Axon      |   |    |     |
| 5058A     | - | 4.8 | 5.0 |
| 5058B     | - | 5.7 | 5.8 |
| 5078A     | 5.5 | - | 6.1 |
| 5098A     | 5.2 | - | 4.4 |
| 5098B     | 5.8 | - | 6.1 |
| 5098C     | - | 5.6 | 5.1 |
| Mean      | 5.0 | 5.6 | 5.5 |
| ± SEM     | 0.5 | 0.5 | 0.4 |

Therefore, direct quantitative comparisons between the influx and efflux values in this report are not possible. Some preliminary experiments have suggested that chloride efflux is linearly related to the internal chloride concentration in the range of 14-150 mM. The results collected in Table III show that chloride efflux is unaffected by changes of either the intracellular sodium concentrations. The external sodium was replaced by magnesium just as in the chloride influx experiments. The internal sodium was changed by varying the sodium glutamate and taurine concentrations, thus maintaining the intracellular potassium and chloride concentrations, as well as the osmolality, constant.
The observations that chloride influx is dependent on external sodium and that it saturates when the external chloride concentration becomes essentially equal to the external sodium concentration leads one to suspect the presence of a coupled Na-Cl influx mechanism. If such coupling exists and if it is an obligatory coupling, then any treatment which affects the flux of one ion should similarly affect the other. It is known that chloride influx is reduced when the intracellular chloride concentration is increased (Russell, 1976). Sodium influx was measured from Na-SSW which also contained ouabain (10⁻²M) and tetrodotoxin (TTX; 10⁻⁶M). These agents were added to reduce sodium fluxes through the sodium pump and the conductance channels (Baker et al., 1969). Neither agent affects Cl⁻ influx (see Fig. 2). The dialysis fluid was nominally calcium-free and contained 0.5 mM EGTA to reduce possible Na-Ca counter-transport to a minimum. Fig. 7 shows the results of lowering the intracellular chloride concentration from 150 to 0 mM. Sodium influx increased when the internal chloride concentration was lowered to 0 mM, but declined toward control values when the intracellular chloride concentration was raised to 150 mM. Four other axons were similarly treated and it can be seen in Table IV that lowering the internal chloride concentration from 150 to 0 mM resulted in an average increase of sodium influx of ~11 pmol/cm²·s. Chloride influx, under similar conditions, increased by ~14 pmol/cm²·s (Russell, 1976).

**Effect of ATP on Sodium Influx**

Although it has previously been shown that there is an ATP-dependent sodium influx in the squid axon (Caldwell et al., 1960; Brinley and Mullins, 1968), it seemed important to demonstrate this dependence in the present experiments.
and to compare the magnitude of this flux to that of the ATP-dependent chloride influx. Fig. 8 shows the effects on sodium influx of dialyzing with an ATP-free dialysis fluid after first dialyzing with 4 mM ATP. This axon was poisoned with cyanide (2 mM) throughout the experiment. More than half the Na influx depended upon ATP. In four axons, the ATP-dependent sodium influx averaged 12 pmol/cm²·s (Table IV). This may be compared with the ATP-dependent chloride influx of 15–16 pmol/cm²·s.

### Table IV

| [Cl]₀, mM | 0 | 150 | 0 | 0 | 0 |
|----------|---|-----|---|---|---|
| ATP, mM  | 4 | 4   | 0 | 4 | 4 |
| [Cl]₀, mM | 555 | 555 | 555 | 0 | 555 |

| PMOL/cm²·s | + Furosemide |
|------------|--------------|
| Axon       |              |
| 5138A      | 26.2         | 8.9         | 12.3 |
| 5138B      | 17.4         | 9.6         | 8.8  |
| 5168A      | 14.1         | 6.7         | 7.4  |
| 5168B      | 16.2         | 12.8        | 8.2  |
| 5178B      | 18.1         | 5.3         | 12.8 |
| 5188A      | 16.7         | 7.5         | 12.8 |
| 5188B      | 17.4         | 12.8        | 8.2  |
| 5188C      | 15.7         | 12.8        | 8.2  |
| 5198A      | 15.7         | 12.8        | 8.2  |
| 5198B      | 18.2         | 12.8        | 8.2  |
| 5308A      | 22.2         | 12.8        | 8.2  |
| 5318A      | 20.9         | 12.8        | 8.2  |
| 5318B      | 14.1         | 12.8        | 8.2  |
| 6018A      | 20.0         | 12.8        | 8.2  |
| 6018B      | 22.5         | 12.8        | 8.2  |
| 6018C      | 16.8         | 12.8        | 8.2  |
| 6058B      | 19.7         | 12.8        | 8.2  |

Mean 18.2 7.0 7.5 9.1 7.3
SEM 0.8 1.0 1.9 1.2 0.8
n 17 5 4 6 5

* In all these experiments [Na]₀ = 425 mM and [Na]₀ = 50 mM.

### Effect of Varying Cl₀ on Sodium Influx

The results of the preceding two types of experiments strongly suggest a common link between portions of the chloride and sodium influxes. The effect on sodium influx of lowering the external chloride concentration was tested by substituting methanesulfonate for chloride. A total of seven axons were tested with various external chloride concentrations. Care was taken that every axon be tested with normal chloride concentration (555 mM) and at the end be tested with 0 mM external chloride concentration. The results of these experiments were normalized by subtracting the flux left in 0 mM chloride SSW from that obtained at each external chloride concentration. This difference was defined as the chloride-dependent sodium influx.
It should be pointed out that the protocol used was a conservative one that will tend to underestimate the size of the chloride-dependent flux because the 0 mM external chloride treatment was always the last treatment of each experiment. The possible underestimation arises because the chances of the axon becoming leaky increase with experimental time and any such leak flux would be counted as a non-chloride-dependent flux and assumed to be present throughout the experiment. The results of these experiments may be seen in Fig. 9 which shows that the external chloride-dependent sodium influx falls more or less linearly as the external chloride concentration is reduced. Table IV shows that ~50% or 9.1 pmol/cm²·s of the sodium influx from Na-SSW is dependent upon extracellular chloride.

*Effect of Furosemide on Sodium Influx*

It will be recalled that treatment with 0.2 mM furosemide reduced Cl influx by ~10 pmol/cm²·s. The effect of this agent on Na influx was studied in five axons. It was applied in ²²Na-SSW to axons being dialyzed with ATP-containing 0 Cl dialysis fluid so that the results are directly comparable to those obtained for chloride influx. A typical result is shown in Fig. 10. About 50% of the sodium influx was rapidly inhibited by the furosemide. It was somewhat more difficult to reverse the effect of the furosemide on the sodium influx than was previously noted for chloride influx. In the example shown in Fig. 10, two separate washout periods were required to return the influx to ~80% of its original value. The reason for difference in the ease of reversibility is unknown. On the average, furosemide inhibited the sodium influx by ~8 pmol/cm²·s (Table IV).

**Discussion**

*Na-Cl Co-Transport?*

These results clearly show that extracellular sodium is a requirement for a large fraction of chloride influx into the squid giant axon. Similarly, some sodium
influx has an obligatory requirement for extracellular chloride. In that a large portion of the influx of each of these ions depends upon the presence of the other ion, it is tempting to suggest that they share a common transport mechanism. In the present set of experiments, a number of qualitative similarities were found between chloride and sodium influxes. A portion of both

**Figure 9.** Accumulated data from 10 axons showing the nearly linear relationship between the external chloride-dependent sodium influx and extracellular chloride concentration in ATP-fueled axons. In all instances the chloride was replaced by methanesulfonate.

**Figure 10.** This shows the inhibition of sodium influx caused by 0.2 mM furosemide and the difficulty in reversing the effect. The axon was washed twice in furosemide-free Na-SSW. Axon diameter = 530 μM.

chloride and sodium influx requires ATP. Both fluxes are inversely related to the intracellular chloride concentration. Thus, as the electrochemical gradient against which chloride must be moved is increased, the rate of the inward movement of both chloride and sodium is reduced. A fraction of both influxes can be blocked by furosemide, an agent generally considered to be an inhibitor of chloride fluxes in epithelia. Neither influx is affected by ouabain. Finally,
Table V shows that the quantitative comparisons between the influxes for these common properties is also quite close. Only the Cl\textsubscript{0}-dependent sodium influx seems significantly less than its counterpart, the Na\textsubscript{0}-dependent chloride influx. This would occur if methanesulfonate possessed a certain ability to substitute for chloride on the transport mechanism. In general, the sodium fluxes were always slightly less than the corresponding chloride fluxes. This is probably due to the fact that the cell interior is electrically negative making cation influxes particularly sensitive to increases in nonspecific membrane “leakiness.” The general experimental protocol was to reserve for the end of any given experiment that treatment which would define the residual or baseline flux. Therefore, it is likely that by the time this treatment was performed, the leakiness was greater than it had been earlier in the experiment. In this way the relevant sodium influx might be systematically underestimated.

The foregoing results clearly demonstrate that a portion of the influx of both Cl and Na have many properties in common. The simplest model to explain this correspondence is a Na-CI co-transport mechanism. Such a mechanism would require the simultaneous presence of both Na and Cl and would result in a one-to-one coupling ratio for their influxes (see Table V).

As pointed out in the Introduction, axoplasmic chloride levels are too high to be explained by a thermodynamically passive mechanism. The observations that metabolic poisons (Keynes, 1963) or intracellular ATP-depletion (Russell, 1976) inhibit \textsuperscript{36}Cl uptake have suggested that the proximate energy source for the net Cl uptake mechanism is ATP. However, we must, for the present, admit our ignorance as to whether the energy resident within the ATP molecule is actually used to power this thermodynamically “uphill” movement of chloride into the axon. It may be that ATP serves a catalytic purpose such as changing the external site affinities for chloride and (or) sodium in a manner analogous to that suggested for the sodium-dependent glutamate uptake system (Baker and Potashner, 1973) and the Na-Ca counter-transport system (DiPolo, 1976; Blaustein and Santiago, 1977).

Is There a Role for the Sodium Gradient?

In view of the external Na requirement and the many Na gradient-dependent transport processes that have been described (e.g., Crane, 1977), we must consider whether the Na gradient could be responsible for the uptake of Cl against an electrochemical gradient. If one assumes a one-for-one coupling of Na and Cl influx, then, in principle, the Na gradient could drive intracellular Cl to as high as 4.0 M (i.e., \( E_{\text{Cl}} = E_{\text{Na}} \); \( \text{Na}^+ = 58 \text{ mM} \); \( \text{Na}_o = 425 \text{ mM} \); \( \text{Cl}_o = 555 \text{ mM} \)). Because the actual reported values for intracellular Cl are around 100 mM, it is clear that the Na gradient possesses sufficient energy to account for the transmembrane distribution of Cl.

Although the Na gradient hypothesis has not been rigorously tested in the present experiments, two kinds of experiments were performed which do address this issue. These experiments tested the effects of changing either intracellular Na concentrations on Cl efflux as compared to similar experi-
ments testing Cl influx. In general, the Na gradient hypothesis requires that the
direction of the net flow of the co-transported species be the same as that of Na
moving down its electrochemical gradient.

Thus, changing the direction the Na electrochemical gradient ought to
change the direction of the net flux. Obviously, a change in a net flux can result
from changes in the unidirectional influx or efflux or both. In the present
experiments data were obtained on both Cl influx and efflux under conditions
where the Na electrochemical gradient favored net Na (and if coupled,
presumably Cl, as well) loss from the cell interior. When external Na was zero
and internal Na = 58 mM, a condition which favors net efflux, we saw that Cl
efflux was unaffected (Table III A) while influx was inhibited (Fig. 1), a set of
conditions that should promote net Cl efflux. Thus, these data support the idea
of a role for the Na gradient in net Cl uptake. However, neither Cl efflux nor
influx were affected by varying Na\textsubscript{i} between 8 and 158 mM when Na\textsubscript{o} was
normal (425 mM). Since raising Na\textsubscript{i} reduces the inward Na gradient, one might
expect a combination of effects on unidirectional fluxes to favor net Cl efflux.
The fact that such did not occur may be explained if the proposed coupled Na-

\begin{table}
\centering
\caption{Comparison of Cl and Na Influxes in the Squid Axon}
\begin{tabular}{lllll}

|                | ATP dependent | [Cl\textsubscript{i}] dependent | [Na\textsubscript{i}] dependent | [Cl\textsubscript{o}] dependent | Furosemide inhibited |
|----------------|---------------|---------------------------------|---------------------------------|-------------------------|---------------------|
| Cl influx, pmol/cm\textsuperscript{2}.s\textsuperscript{-1} | 16            | 14*                             | 16.5                            | 11                      |                     |
| Na influx, pmol/cm\textsuperscript{2}.s\textsuperscript{-1} | 12            | 11                              | 9                               | 8                       |                     |

* Estimated from Russell (1976) using a Q_{10} of 1.65 (Russell, unpublished observations).
\end{tabular}
\end{table}

Cl unidirectional fluxes are a function of the product of the two ion concentra-
tions. If this is the case, then Cl efflux might not be expected to be much
affected by changing Na\textsubscript{i} from 8-158 mM, inasmuch as the Na\textsubscript{i}.Cl\textsubscript{i} product is
comparable to the Na\textsubscript{o}.Cl\textsubscript{o} product when Na\textsubscript{o} = 2-43 mM, a range of Na\textsubscript{o} where
Cl influx was little affected (Fig. 1 B).

**Possible Role for a Coupled Sodium Anion Uptake**

As has been discussed previously (Keynes, 1963; Russell, 1976), the function of
an active chloride uptake mechanism is unknown. However, it has been shown
in the squid axon that the extrusion of an intracellular acid load depends
critically upon the presence of intracellular chloride, extracellular HCO\textsubscript{3}, and
ATP (Russell and Boron, 1976). In other preparations, it has become clear that the
acid-extruding mechanism also depends upon extracellular sodium
(Thomas, 1977; Boron and Roos, 1978); in fact, a net uptake of sodium has been
demonstrated in the snail neuron (Thomas, 1977) after an intracellular acid
load. In view of the present finding of a sodium-dependent chloride influx, one
may speculate as to its role in the acid-extruding mechanism. Perhaps the system
studied in this work could, under appropriate conditions, engage in sodium-
Coupled Na-Cl Transport in Squid Axon

Dependent bicarbonate influx. Thus, in the face of an intracellular acid load, sodium and bicarbonate move inward via the system described in the present report and chloride leaves, perhaps with a proton. Such a combination of fluxes is well-suited to raise intracellular pH and has been suggested by Thomas (1977).

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