Cation-coupled Chloride Influx in Squid Axon

Role of Potassium and Stoichiometry of the Transport Process

JOHN M. RUSSELL

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT Evidence is presented showing that the Cl^- uptake process in the squid giant axon is tightly coupled not only to Na^+ uptake but also to K^+ uptake. Thus, removal of external K^+ causes both Cl^- and Na^+ influxes to be reduced, particularly when $[Cl^-]_i$ is low, that is, under conditions previously shown to be optimal for Cl^-/Na^+-coupled influx. In addition, there exists a ouabain-insensitive K^+ influx, which depends on the presence of external Cl^- and Na^+, is inversely proportional to $[Cl^-]_i$, and is blocked by furosemide/bumetanide. Finally, this ouabain-insensitive K^+ influx appears to require the presence of cellular ATP. The stoichiometry of the coupled transport process was measured using a double-labeling technique combining in the same axon either ^{36}Cl and ^{42}K or ^{22}Na and ^{42}K. The stoichiometry of the flux changes occurring in response either to varying $[Cl^-]_i$ between 150 and 0 mM or to treatment with 0.3 mM furosemide is, in both cases, ~3:2:1 ($Cl^-/Na^+/K^+$). Although these fluxes require ATP, they are not inhibited by 3 mM vanadate. In addition, treatment with DIDS has no effect on the fluxes.

INTRODUCTION

Evidence for cation-coupled chloride transport processes is found in a variety of cells (e.g., Ellory et al., 1982; Warnock and Eveloff, 1982). The squid giant axon is no exception. Thus, it has been shown that unidirectional Cl^- influx requires not only intracellular ATP (Russell, 1976) but also extracellular Na^+ (Russell, 1979). Furthermore, it has been shown that a component of ouabain-insensitive Na^+ influx requires ATP and extracellular Cl^-. Finally, both Cl^- and Na^+ influxes were found to be inversely related to $[Cl^-]_i$ and blocked by furosemide or bumetanide. In view of the similar properties of the Cl^- and Na^+ influxes, it seemed reasonable to consider that they had a common transport process that rather tightly coupled the movements of Cl^- and Na^+.

While performing the experiments just summarized, it was noted that the magnitude of the Cl^- influx mediated by the co-transport mechanism was

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/83/06/0909/17 $1.00 909
Volume 81 June 1983 909-925

always greater than its Na^+ counterpart (Russell, 1979, 1980). This suggested the possibility that another ion might be involved in the overall process. An involvement of potassium in cation-coupled chloride transport processes has been reported for red blood cells and a variety of epithelial cells (e.g., Ellory et al., 1982). Thus, a role for potassium in the cation-coupled Cl^- influx process in squid axon seemed possible.

To demonstrate convincingly a direct role for K^+ in the Cl^- uptake transport process, it was first necessary to determine that the influxes of both Cl^- and Na^+, in the presence of ouabain, required external K^+. Furthermore, it was necessary to show that a portion of K^+ influx has properties in common with the Cl^-/Na^+ uptake process identified earlier, i.e., (*a*) it should require the simultaneous presence of external Cl^- and Na^+, (*b*) it should be inversely dependent upon $[Cl^-]_i$, (*c*) it should be inhibited by furosemide, and (*d*) it should be dependent on ATP. Inasmuch as all these conditions were met, it was of interest to measure the apparent stoichiometry of this cation-coupled anion transport process. The response of the fluxes of the three ions to two different treatments was measured using a double-labeling technique. It was found that both increasing $[Cl^-]_i$ from 0 to 150 mM and treatment with 0.3 mM furosemide resulted in a decrease of all three fluxes in the following ratio: $3Cl^-/2Na^+/1K^+$.

Some of these results were presented at the 25th annual meeting of the Biophysical Society (Russell, 1981).

METHODS

Axons

These experiments were performed at the Marine Biological Laboratory, Woods Hole, MA, during May and early June, 1979–1982. Live specimens of the squid, *Loligo pealei*, were decapitated, and the first stellar nerves were 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 17°C by means of a coolant fluid circulating from a Lauda K2/RD cooler (Brinkman Instruments, Inc., Westbury, NY) through the underside of the dialysis chamber; a thermistor (Fenwal Electronics, Framingham, MA) 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 OD; FRL, Inc., Dedham, MA) glued to a plastic T tube. For influx experiments, the tube had a central region ~27 mm long that had been rendered porous by a 16–20-h soak in 0.1 N NaOH.

A 40–45 mm length of axon was cannulated at both ends, and 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 lowered onto grease dams at either end of the central slot in the bath. The grease was a mixture of vaseline and mineral oil and its 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 length of the central slot 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 apparently increase progressively as the undialyzed lateral regions begin to accumulate radioactive isotope. The reservoir of radioisotope in the undialyzed ends would serve to damp out the effects of a treatment that reduced the actual 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. 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 and the slot fluid was gently stirred by repeatedly withdrawing and ejecting ~100 μl of fluid with a mechanical pipette. The level of fluid was again lowered and another 0.2 ml of radioactive fluid was added and stirred. This process was repeated once more. The radioactive 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 μl/min) to fall directly into the scintillation vial after first passing through the axon. At the end of a time interval (usually 6–8 min), the tip of the dialysis tube was washed with 1.0 ml of deionized water and the washings were 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, MA) was added.

Solutions

The compositions of the external solutions or squid seawaters (SSW) are given in Table I. ^{36}Cl as supplied by New England Nuclear or Amersham/Searle (Arlington Heights, IL) contains a considerable amount of carrier chloride, which was taken into account when making ^{36}Cl-containing solutions. The influx solutions were made to contain ~30 μCi ^{36}Cl/mM total Cl. The ^{36}Cl salt solution provided by the suppliers was dried and then ashed at 450°C before being added to the experimental fluids. ^{22}Na was supplied as a carrier-free solution. It was added directly to the appropriate SSW to attain a final specific activity of ~35 μCi/mM of total Na^+. ^{42}K was obtained from New England Nuclear as the "very high specific activity nuclide." An aliquot was evaporated to dryness and the appropriate SSW was added to attain a final specific activity (at zero decay time) of 12.5 mCi/mM of total K^+. The much higher ^{42}K specific activity was necessary because of the short half-life of this nuclide (12.4 h) and it made easier the calculations for double-labeling experiments, inasmuch as the first time the samples were counted, essentially all the counts came from ^{42}K.

The internal dialysis fluids had the following compositions in millimoles per liter: 413 K^+; 0 Na^+; 7 (ATP-containing) or 4 (ATP-free) Mg^{++}; 0 or 150 Cl^-; 414 or 264

glutamate; 10 HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; pK = 7.55); 2 EGTA; 190 glycine; 0.5 phenol red; 0 or 4 ATP. The pH was ~7.35 and the osmolality was ~960 mosmol/kg. Adenosine 5'-triphosphate (ATP) was obtained vanadium-free from Sigma Chemical Co. (St. Louis, MO). SITS (4-acetamido-4'-isothiocyano-2,2'stilbene-disulfonic acid) and DIDS (4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid) were obtained from Pierce Chemical Co. (Rockford, IL). Furosemide was a gift of Hoechst-Roussel Pharmaceuticals, Inc. (Sommerville, NJ). Bumetanide was a gift of Dr. H. C. Palfrey, University of Chicago, Chicago, IL. Vanadate, free of V_2O_5, was a gift of Dr. L. J. Mullins, University of Maryland, Baltimore, MD.

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.

TABLE I

COMPOSITION OF EXTERNAL FLUIDS*

Ion	SSW	0-K^+ SSW	0-Cl^- SSW	0-Na^+ SSW
		mM		
Na	425	435	425	—
K	10	0	10	10
Mg	62.5	62.5	62.5	275
Ca	3	3	3	—
Cl	561	561	—	560
Methanesulfonate	—	—	561	—
Mannitol	—	—	—	195

* In addition to the major components listed above, all the external squid seawater (SSW) contained 0.5 mM phenol red, 0.1 mM EDTA, and 10 mM EPPS (*N*-2-hydroxyethylpiperazine propane sulfonic acid; pK = 8.0); pH = 8.0; osmolality ≅ 970 mosmol/kg.

RESULTS

External K^+ Is Required for Chloride Influx

Chloride influx via the cation-coupled uptake system is highest when $[Cl^-]_i \cong 0$ and is near zero when $[Cl^-]_i$ is raised to 150 mM (Russell, 1976). This inverse, *trans*-side dependence of the co-transport fluxes was useful for identifying an external K^+ dependence for these co-transport fluxes. Fig. 1 shows the effect on Cl^- influx of replacing K^+ in the external bathing fluid of two axons. The data in panel A show that an axon dialyzed with a Cl^--free fluid has a very large external K^+-dependent Cl^- influx. However, panel B shows that if an axon is dialyzed with a fluid that is otherwise identical but contains 150 mM Cl^-, external K^+ has very little upon Cl^- influx. Under the latter conditions, Cl^- influx is very small regardless of whether extracellular K^+ is present or not. Thus, the external K^+-dependent Cl^- influx is inversely related to $[Cl^-]_i$ just as is the external Na^+-dependent Cl^- influx (Russell, 1979). The

results from nine axons treated as in Fig. 1A are shown in Table IIA. External K^+-dependent Cl^- influx averaged almost 17 pmol/cm^2·s.

External K^+ Is Required for Ouabain-insensitive Na^+ Influx

The Na^+ influx of interest to this study is that portion which, in the continued presence of ouabain (3×10^{-5} M) and TTX (10^{-7} M), requires extracellular Cl^- (Russell, 1979). This external Cl^--dependent Na^+ influx is inversely related to $[Cl^-]_i$ just as is the external Na^+-dependent Cl^- influx. Fig. 2

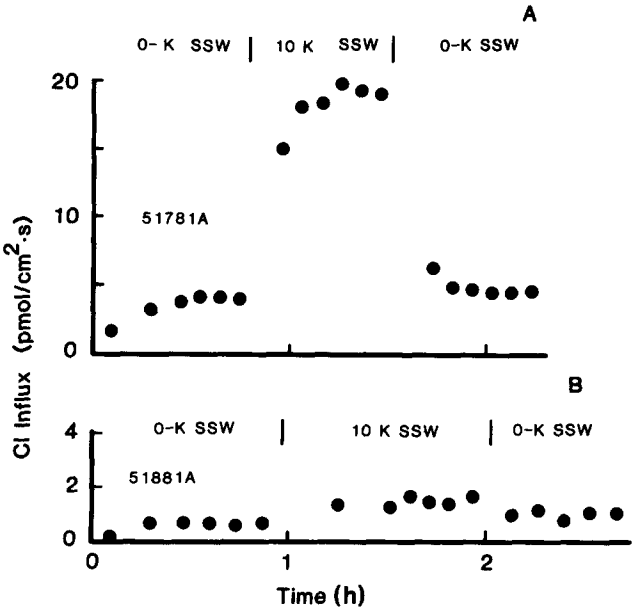

FIGURE 1. (A) $[K^+]_o$-dependent Cl^- influx. This axon was dialyzed with a Cl^-- and Na^+-free solution containing 4 mM ATP. Replacing 10 mM Na^+ with K^+ in the external fluid caused Cl^- influx to increase from ~4 pmol/cm^2·s to ~19.5 pmol/cm^2·s. Upon return to K^+-free external fluid, Cl^- influx declined to ~4.5 pmol/cm^2·s. Axon diameter, 575 μM. (B) Lack of effect of changing $[K^+]_o$ on Cl^- influx when $[Cl^-]_i$ = 150 mM. When the axon was exposed to 10 mM K^+, Cl^- influx only increased from 0.7 pmol/cm^2·s to 1.6 pmol/cm^2·s; subsequent removal of external K^+ resulted in a Cl^- influx of 1.0 pmol/cm^2·s. Axon diameter, 510 μM.

illustrates that this anion-coupled Na^+ influx is also dependent on extracellular K^+. Although the axon was dialyzed with 150 mM Cl^-, the addition of 10 mM K^+ to the external fluid had only a slight effect upon Na^+ influx. However, after 73 min of dialysis with a Cl^--free solution, treatment with an external fluid containing 10 mM K^+ resulted in a large stimulation of Na^+ influx. A total of 12 axons were treated according to the protocol of the latter half of Fig. 2. The collated results presented in Table IIB show that the external K^+-dependent Na^+ influx averages ~11 pmol/cm^2·s. This value is significantly less than the comparable value for Cl^- influx (17 pmol/cm^2·s).

TABLE II

EXTERNAL K^+-DEPENDENT INFLUXES*

	0 mM K_o^+	10 mM K_o^+	0 mM K_o^+	K_o^+-dependent influx
(A) Cl^- influx ($n = 9$)	8.5±1.6	26.4±1.6	10.6±1.7	16.9±1.1
(B) Na^+ influx ($n = 12$)	7.0±0.6	18.3±0.9	8.6±0.8	10.9±1.1

* $[Cl^-]_i$ = 0 mM; 3×10^{-5} M ouabain and 10^{-7} M TTX were present throughout; fluxes are reported as pmol/cm^2·s ± SE.

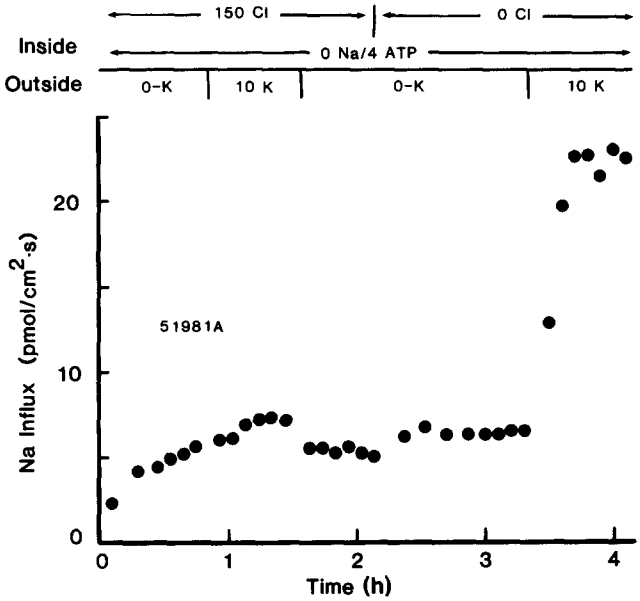

FIGURE 2. $[K^+]_o$-dependent Na^+ influx. While the axon was dialyzed with 150 mM Cl^-, removal of external K^+ resulted in only a small reversible increase of Na^+ influx, from 5.2 to 7.2 back to 5.3 pmol/cm^2·s. However, after 73 min of dialysis with a Cl^--free fluid, addition of 10 mM K^+ caused Na^+ influx to increase from 6.5 to 22.5 pmol/cm^2·s. Axon diameter, 510 μM.

Such a difference between Cl^- and Na^+ fluxes via the purported coupled anion-cation uptake system is in keeping with the earlier observation that Na_o^+-dependent Cl^- influx was greater than the Cl_o^--dependent Na^+ influx (Russell, 1980).

Properties of K^+ Influx

The dependence of Cl^- and Na^+ influxes upon external K^+ plus the apparent lack of equality of the coupled anion and cation fluxes suggested that a portion of K^+ influx might also be mediated by the coupled anion-cation

transport process. This hypothesis was tested in the following series of experiments designed to characterize K^+ influx with respect to known properties of coupled Cl^-/Na^+ uptake. Ouabain (3×10^{-5} M) was always present to rule out effects on K^+ fluxes through the Na^+/K^+ pump. TTX (10^{-7} M) was also always present. Membrane potential changes caused by the various experimental protocols were always small (never greater than 3–4 mV), thereby reducing any possible contributions of K^+ conductance pathways to the observed K^+ fluxes.

DEPENDENCE ON EXTRACELLULAR CL$^-$ Fig. 3 shows an experiment in which

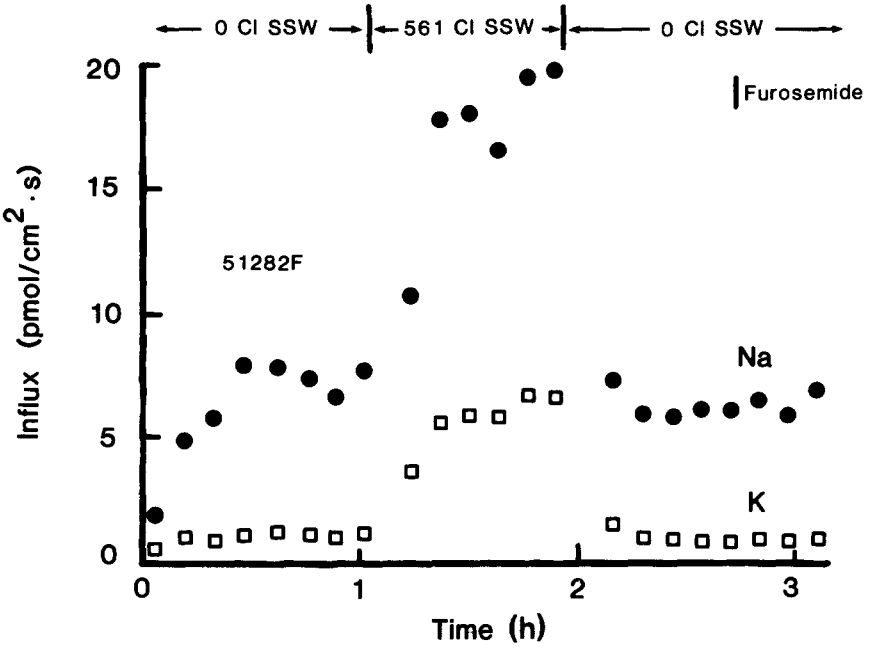

FIGURE 3. $[Cl^-]_o$-dependent K^+ and Na^+ influxes. This axon was dialyzed with a Cl^-- and Na^+-free dialysis fluid containing 4 mM ATP. External Cl^- was replaced by methanesulfonate ($CH_3SO_3^-$). In this axon the external Cl^- dependent fluxes were K^+, 5.7 pmol/cm^2·s; Na^+, 13.2 pmol/cm^2·s. Notice that in the absence of external Cl^-, treatment with 0.3 mM furosemide had no effect on either K^+ or Na^+ influx (cf. Figs. 4, 6, and 7). Axon diameter, 600 μM.

the external Cl^- requirement of both K^+ and Na^+ influxes was measured in the same axon. In the absence of external Cl^-, both K^+ and Na^+ influxes were relatively low. However, replacement of the external methanesulfonate with Cl^- resulted in a stimulation of both K^+ and Na^+ influxes. The stimulation of both influxes was reversible upon removal of external Cl^-. In the absence of external Cl^-, 0.3 mM furosemide had no effect on the residual K^+ or Na^+ fluxes, which indicates that furosemide inhibits the Cl_o^--dependent component of these fluxes. Finally, notice that the ratio of the change in Na^+ influx to that of K^+ influx was 2.3 (see below) in this experiment. In previous studies on the Na^+ influx, the use of gluconate and sulfate as Cl^- substitutes gave the

same results as methanesulfonate (Russell, 1979). Thus, unless all three of these anions directly interact with the transport process in exactly the same way, it seems the effect is due to external Cl^- removal. In a total of five axons, the external Cl^--dependent K^+ influx averaged 9.5 pmol/cm^2·s (Table IIIA).

DEPENDENCE UPON EXTERNAL NA^+ The cation-coupled Cl^- uptake has already been shown to depend on the presence of external Na^+. The experiment illustrated in Fig. 4 shows a similar external Na^+ dependency of K^+ influx. In a total of three axons, the external Na^+-dependent K^+ influx averaged 9.3 pmol/cm^2·s (Table IIIB). Fig. 4 also shows that K^+ influx, in the presence of full external Na^+, is inhibited by 0.3 mM furosemide to the same extent as that observed upon removal of external Na^+.

DEPENDENCE ON INTRACELLULAR CL^- Both Cl^- and Na^+ influx are inversely related to $[Cl^-]_i$. The preceding experiments were all performed upon axons dialyzed with a 0-Cl^- dialysis fluid, a treatment that should result in a maximal K^+ influx if a portion of K^+ influx is via the coupled transport system. This prediction was directly tested in the experiment illustrated in

TABLE III

EXTERNAL ION DEPENDENCIES OF K^+ INFLUX*

(A) Dependence upon Cl_o^- (n = 5) K^+ influx in	0-Cl^- SSW[1]	SSW	0-Cl^- SSW	Cl_o^--dependent K^+ influx
	3.4±1.7	12.9±3.7	3.3±1.7	9.5±2.1
(B) Dependence upon Na_o^+ (n = 3) K^+ influx in	**Na^+ SSW[2]**	**SSW**	**0-Na^+ SSW**	**Na_o^+-dependent K^+ influx**
	2.6±0.9	13.4±1.6	4.8±0.5	9.3±1.5

* $[Cl^-]_i$ = 0 mM; 3 × 10^{-5} M ouabain and 10^{-7} M TTX present throughout. [1]Cl_o^- replaced by methanesulfate; [2]Na_o^+ replaced by Mg-mannitol. Fluxes are reported as pmol/cm^2·s ± SE.

Fig. 5. In this experiment K^+ and Cl^- influxes were simultaneously measured while $[Cl^-]_i$ was varied between 150 and 0 mM. Clearly, reduction of $[Cl^-]_i$ to nominally 0 mM caused both influxes to increase, with Cl^- influx being increased the most. In this example, the stimulated Cl^- influx was almost three times that of the stimulated K^+ influx. It should be pointed out that during the course of this experiment the membrane resting potential changed very little, hyperpolarizing from −60 at the beginning to −61.5 at the end. Inhibition of K^+ influx by intracellular Cl^- seems a particularly compelling bit of evidence linking a portion of K^+ influx to Cl^- (and Na^+) influx.

DEPENDENCE UPON ATP A requirement for ATP in the intracellular fluid has been clearly shown for the Cl^- + Na^+ uptake process (Russell, 1979). Certainly, the results of the preceding sections are consistent with the idea that K^+ also is inwardly transported by this same process. Therefore, it would be expected that K^+ influx would likewise exhibit a dependence upon ATP.

In the experiment illustrated in Fig. 6, the axon had been dialyzed with an ATP-free dialysis fluid for 42 min before the zero time in the figure. It can be

seen that both K^+ and Cl^- influxes decline during the initial treatment phase of the figure. Presumably, this reflects the gradual reduction of cellular ATP levels. When 4 mM ATP was introduced via the dialysis fluid, both K^+ and Cl^- fluxes were stimulated. Once again, the pattern is that Cl^- flux is stimulated to a greater degree than K^+ flux; in this case, Cl^- influx was increased by an amount 4.6 times that by which K^+ influx was increased. That the increase of fluxes was not simply due to a nonspecific permeability increase is seen from the fact that furosemide inhibited both fluxes to levels actually lower than obtained during the ATP-washout phase of the experi-

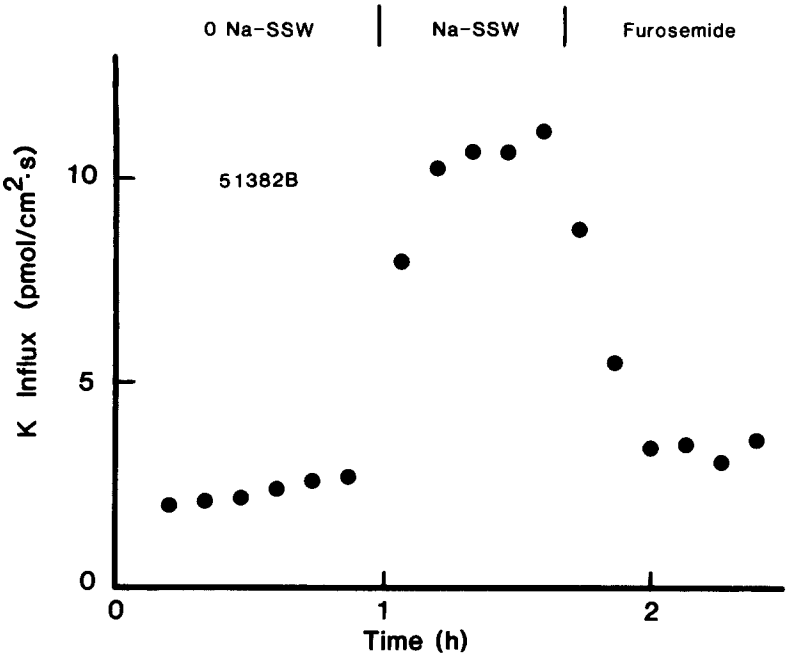

FIGURE 4. $[Na^+]_o$-dependent K^+ influx. This axon was dialyzed with Cl^-- and Na^+-free dialysis fluid containing 4 mM ATP. In Na^+-free seawater (Mg^{++} replacing Na^+), K^+ influx was ~2.6 pmol/cm^2·s. When Na^+ returned to the seawater, K^+ influx increased to ~10.7 pmol/cm^2·s. Treatment with 0.3 mM furosemide reduced K^+ influx to 3.4 pmol/cm^2·s. Axon diameter, 525 μM.

ment. The ratio of furosemide-sensitive Cl^-/K^+ fluxes in this case was 2.45. These results are typical of a total of three axons. Qualitatively, these results show that ATP removal does reduce K^+ influx but the fact that the ratio of ATP-sensitive Cl^-/K^+ fluxes was greater than the furosemide-sensitive Cl^-/K^+ fluxes may suggest a less than straightforward relationship among the fluxes of the three ions and their ATP requirements. Nonetheless, an ATP requirement for a ouabain-insensitive K^+ influx was identified, and in the three axons studied using the same protocol as that for the axon of Fig. 6, the average size of this ATP-dependent flux was 4.5 ± 0.2 pmol/cm^2·s. As we shall soon see, this is rather less than the furosemide- or $[Cl^-]_i$-dependent K^+ influxes. The significance of this difference is unknown.

EFFECT OF INHIBITORS Several examples of the inhibitory effect of 0.3 mM furosemide on the anion-cation-coupled transport system have already been shown (Figs. 3, 4, and 6). In a total of 15 axons treated with furosemide, the K^+ influx was reduced by 8.4 ± 0.5 pmol/cm^2·s. Three axons were treated with 10 μM bumetanide, which reduced K^+ influx by 8.8 ± 0.3 pmol/cm^2·s. Furosemide or bumetanide treatment resulted in a slight hyperpolarization of the membrane resting potential, averaging <1 mV.

Fig. 7A shows that treatment with 50 μM DIDS had no effect upon either K^+ or Cl^- influx, whereas subsequent treatment with 0.3 mM furosemide resulted in a prompt inhibition of both fluxes, with the furosemide-sensitive Cl^- influx being 3.2 times greater than the inhibitor-sensitive K^+ influx. Thus,

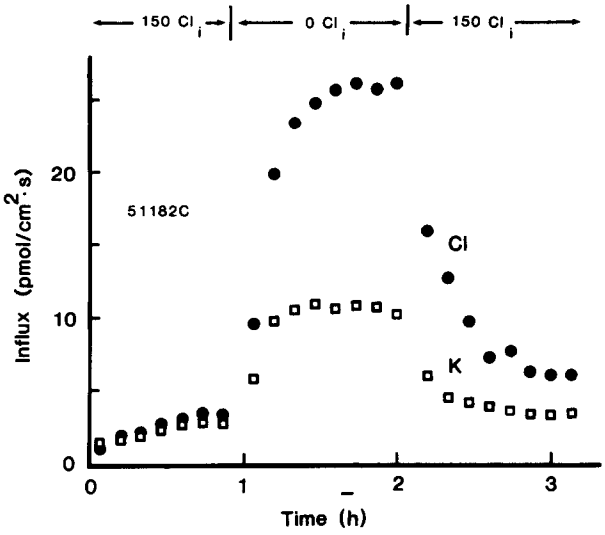

FIGURE 5. Effect of changing $[Cl^-]_i$ on K^+ and Cl^- influxes. This axon was dialyzed with a Na^+-free dialysis fluid containing 4 mM ATP. When the dialysis fluid contained 150 mM Cl^-, Cl^- influx = 3.3 pmol/cm^2·s, K^+ influx = 2.8 pmol/cm^2·s. Dialysis with a Cl^--free dialysis fluid increased both influxes, Cl^- influx = 26 pmol/cm^2·s, K^+ influx = 10.7 pmol/cm^2·s. Axon diameter, 475 μM.

DIDS, which can inhibit intracellular pH regulation and its associated fluxes of Cl^- and Na^+ (Boron and Russell, 1983), is without effect on the coupled anion-cation uptake process in the axon.

Fig. 7B illustrates the lack of effect of 3 mM vanadate (VO_4^{3-}) applied intracellularly compared with the usual effect of 0.3 mM furosemide on K^+ and Cl^- influxes. This result is typical of a total of three axons. Two axons were treated with 0.3 mM VO_4^{3-} with a similar lack of effect on the influxes.

Stoichiometry of $Cl^-/Na^+/K^+$ Uptake

The results of the foregoing experiments support the hypothesis that Cl^-, Na^+, and K^+ share a coupled uptake mechanism. I measured the stoichiometry of the change in the fluxes resulting from two different treatments. One treatment

was to reduce $[Cl^-]_i$ from 150 mM to nominally 0. The other treatment was exposure to 0.3 mM furosemide. All the experiments were conducted as double-label experiments measuring K^+ and either Cl^- or Na^+ influxes simultaneously. The flux changes could then all be normalized to the change of K^+ influx and the relative flux changes were expressed as a ratio.

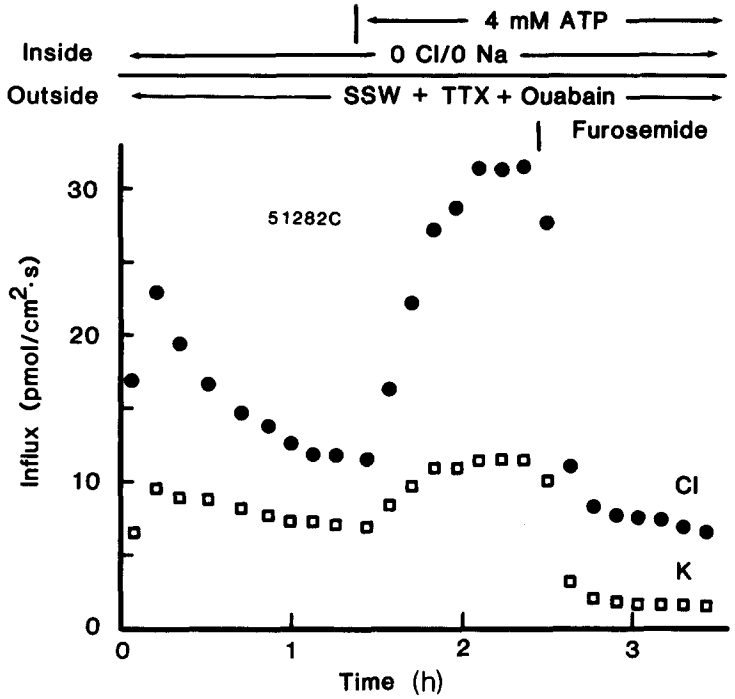

FIGURE 6. ATP-dependent K^+ and Cl^- influx. The axon was dialyzed with an ATP-free dialysis fluid for 42 min before the isotopes were applied at the beginning of the figure. Both fluxes, after an initial rise, relax towards lower values; for K^+ influx, ~7 pmol/cm^2·s and for Cl^- influx, ~12 pmol/cm^2·s. When 4 mM ATP was added to the dialysis fluid (Cl^- and Na^+ free throughout the experiment), both influxes increased, K^+ influx to ~11.5 pmol/cm^2·s and Cl^- influx to ~31 pmol/cm^2·s. Treatment with 0.3 mM furosemide reduced both influx to substantially lower values than seen under nominally ATP-free conditions. This suggests that $[ATP]_i$ in this fiber after ~130 min dialysis with ATP-free fluid was still high enough to support a significant fraction of Cl^-/Na^+/K^+ influx. Nevertheless, this result qualitatively demonstrates that both K^+ and Cl^- influxes are stimulated by ATP in the presence of ouabain. Axon diameter, 475 μM.

Table IVA shows the results from 14 axons in which $[Cl^-]_i$ was varied from 150 to 0 to 150 mM. The results show that lowering $[Cl^-]_i$ to nominally 0 mM stimulated the influxes of Cl^-, Na^+, and K^+, respectively, in the ratio of 3.33:1.83:1. Table IVB shows the results of furosemide inhibition on the same three influxes when $[Cl^-]_i$ = 0 mM. The ratio of Cl^-, Na^+, and K^+ fluxes sensitive to furosemide proved to be 3.08:2.2:1. The results of these two series

of experiments suggest that the coupled anion-cation uptake process has a stoichiometry of $3Cl^-/2Na^+/1K^+$.

DISCUSSION

The foregoing results show that not only is external K^+ required for coupled Cl^-/Na^+ uptake, but, in fact, the coupled uptake process also transports K^+. Table V summarizes the results of the present study and collates them with the results of an earlier study on coupled Cl^- and Na^+ influxes (Russell, 1979). These data clearly illustrate the relationships among the three ions and

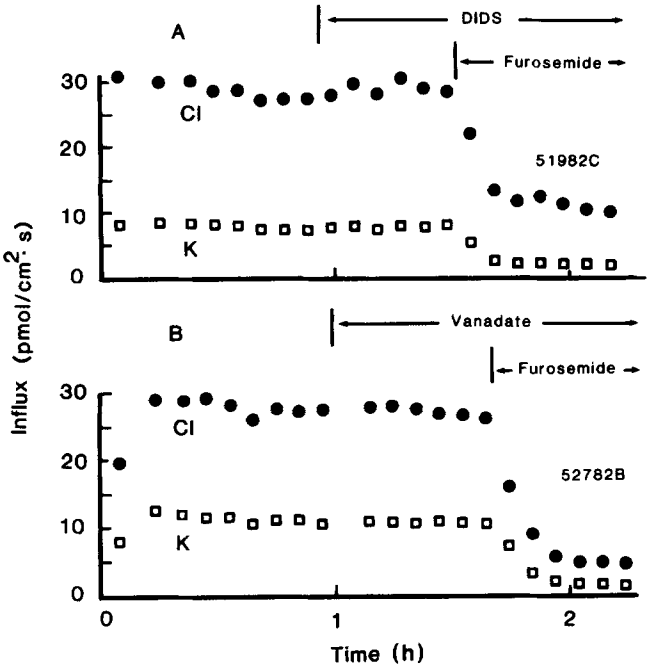

FIGURE 7. (A) Lack of effect of 50 μM DIDS applied externally on K^+ and Cl^- influxes. Subsequent treatment with 0.3 mM furosemide inhibited both influxes as usual. Axon diameter, 500 μM. (B) Lack of effect of 3.0 mM vanadate on K^+ and Cl^- influxes. Once again, 0.3 mM furosemide inhibited both influxes. Axon diameter, 575 μM.

provide strong evidence for a rather tight coupling of the three ion fluxes. Thus, a portion of the fluxes of all three ions is absolutely dependent upon the simultaneous presence of all three ions. Similarly, the fluxes of all three ions are inversely dependent on the intracellular chloride concentration. That Cl^-, Na^+, and K^+ influxes should all be inversely related to $[Cl^-]_i$ would be unexpected in the absence of a coupled transport process.

Stoichiometry

Table V also illustrates that a quantitative pattern exists among the three fluxes. Thus, the coupled influx of Cl^- is always greater than that of Na^+, which in turn exceeds that of K^+. However, when comparing individual ion

TABLE IV

DOUBLE-LABEL STOICHIOMETRY EXPERIMENTS*

	150 mM $[Cl^-]_i$		0 mM $[Cl^-]_i$		150 mM $[Cl^-]_i$		Stoichiometry
(A) Internal Cl^--dependent influxes							
(1) Cl^-/K^+ influxes	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-	Cl^-/K^+
(n = 7)	2.3±0.1	3.7±0.6	11.1±1.3	31.7±3.3	4.8±1.3	12.5±1.4	3.33±0.27
(2) Na^+/K^+ influxes	K^+	Na^+	K^+	Na^+	K^+	Na^+	Na^+/K^+
(n = 7)	2.2±0.3	2.9±0.6	10.2±1.2	19.4±2.4	3.2±0.5	7.4±1.0	1.83±0.1
(B) Furosemide-sensitive fluxes[1]							
(1) Cl^-/K^+ influxes	Control				0.3 mM furosemide		Stoichiometry
	K^+	Cl^-			K^+	Cl^-	Cl^-/K^+
(n = 5)	10.6±1.2	34.8±2.1			2.0±0.3	10.1±1.5	3.08±0.24
(2) Na^+/K^+ influxes	K^+	Na^+			K^+	Na^+	Na^+/K^+
(n = 4)	10.3±1.6	25.4±1.0			1.9±0.5	7.1±1.8	2.2±0.30

* Fluxes are reported as pmol/cm^2·s ± SE.
[1] $[Cl^-]_i$ = 0 mM.

TABLE V

SUMMARY OF COUPLED Cl^-:Na^+:K^+ FLUXES*

	K_o-dependent	Na_o-dependent	Cl_o-dependent	Cl_i-dependent	Furosemide inhibited
	pmol/cm^2·s				
Cl influx	17.7±1.1 (11)	16.5‡±1.0 (9)	—	23.6±1.9 (7)	17.1§±1.1 (11)
Na influx	12.0±0.9 (12)	—	9.1‡±1.3 (6)	13.0§±0.8 (12)	12.0§±1.0 (9)
K influx	—	9.3±1.5 (3)	9.5±2.1 (5)	7.3±0.7 (21)	8.4±0.5 (15)

* $[Cl^-]_o$ = 0 at all times except during Cl_i-dependent experiments; [ATP] = 4 mM.
‡ From Russell, 1979.
§ Combined data from this paper and Russell, 1979. Numbers in parentheses refer to number of axons from which data were taken.

fluxes obtained from different axons, it is risky to assign a stoichiometry. Biological variation from axon to axon is one reason. Another is that the absolute magnitude of the fluxes varies with the time of the season in which the animals are captured. Thus, all three fluxes are larger in late May through early June than they are in late April through early May. Consistent with this is the report by Brinley and Mullins (1965) in which they noted a higher

$[Cl^-]_i$ in axoplasm from animals captured during the warmer months compared with the values from the animals captured earlier in the year. Therefore, for example, comparing Cl^- fluxes obtained in early May to K^+ fluxes obtained in early June could result in a systematic underestimate of the Cl^-/K^+ coupling ratio. Thus, to obtain reliable estimates of stoichiometry, it was necessary to measure the influx of two of the three ions simultaneously and always to measure K^+ influx. In this way, the ratio of the fluxes could be normalized to that of K^+ influx. In the present study, stoichiometric measurements were made of the coupled fluxes in response to two different perturbations, namely changing $[Cl^-]_i$ and treatment with furosemide. The results of these two kinds of experiments designed to directly measure the coupling ratio of Cl^-/K^+ and Na^+/K^+ were similar. In both cases, the Cl^-/K^+ ratio was slightly greater than 3.0, whereas the Na^+/K^+ ratio was near 2.0. These results suggest that the anion-cation uptake process promotes the coupled transport of $Cl^-/Na^+/K^+$ in the ratio of 3:2:1. Such a stoichiometry would be electroneutral, if somewhat cumbersome.

The issue of whether this mechanism is rigorously electroneutral was not specifically addressed in this study. Changes in membrane potential directly attributable to changes in co-transport rate were not observed. Neither is there any sensitivity of the bumetanide-inhibited Cl^- influx to a 25-mV membrane depolarization (Russell, 1983). Nevertheless, it should be kept in mind that given the normally low membrane resistance of the squid axolemma, it would be difficult to detect changes in membrane resting potential that would arise from a less than perfectly electroneutral stoichiometry.

Energetics of $Cl^-/Na^+/K^+$ Uptake

Given that the transport process being studied involves the coupled movement of three ions, it is logical to consider whether the overall process is energized by the relevant ion transmembrane gradients. Although we actually measured unidirectional fluxes in this study, the fact that $[Na^+]_i \cong 0$ would make it impossible for this process to run backwards (even if it were capable of doing so under favorable conditions; see below), and thus it is reasonable to consider that these unidirectional fluxes represent net fluxes mediated by the coupled $Cl^-/Na^+/K^+$ transport process. Given a stoichiometry of $3Cl^-/2Na^+/1K^+$ and assuming electroneutrality, the net driving force available from the relevant chemical gradients can be represented as follows:

$$\Delta G_{net} = RT \ln \frac{[Cl]_i^3 \cdot [Na]_i^2 \cdot [K]_i}{[Cl]_o^3 \cdot [Na]_o^2 \cdot [K]_o}.$$

Under physiological conditions, i.e., $[Cl]_i = 120$ mM, $[Cl]_o = 560$ mM, $[Na]_i = 50$ mM, $[Na]_o = 425$ mM, $[K]_i = 350$ mM, $[K]_o = 10$ mM, the calculation reveals a large excess of energy available to the system, $-5.35RT$. Whether or not the ion gradients serve as the sole source of driving energy is presently unknown. In fact, there is some evidence against a totally gradient-driven model. First, we have the observation that ATP is required. However, it can be argued that the ATP requirement could be indirect. An example of this view would have ATP be required as a source of cyclic AMP and phosphate

for the subsequent protein kinase-mediated phosphorylation of the "carrier" protein. However, experiments that directly tested that hypothesis failed to yield supportive data (Russell, 1980). Second, a gradient-energized process would be expected to exhibit unidirectional fluxes in both directions, with the net direction being determined by the particular set of gradients. However, in an earlier study, no effect on Cl^- efflux was noted while changing $[Na^+]_i$ from 8 to 158 mM (Russell, 1979). Although it is not conclusive, this result does not support the idea of a reversible, gradient-driven process. It is obvious that the final resolution of the energy source for this transport process will require further experiments. In particular, it will be important to test for "backflux" or efflux of Cl^-, Na^+, and K^+ via a coupled system with the same set of requirements as are known for the influx mode.

Comparison with Other Coupled $Cl^-/Na^+/K^+$ Transport Processes

The coupled transport of Cl^-, Na^+, and K^+ has been suggested for a variety of different cell types, including erythrocytes (e.g., Dunham et al., 1980; Haas et al., 1982), kidney cells (Greger and Schlatter, 1981), flounder intestine (Musch et al., 1982) ascites tumor cells (Geck et al., 1980), and a variety of other cultured cells (Aiton et al., 1981). Thus far, all the coupled transport systems identified share several general properties. First, they are all insensitive to ouabain, an inhibitor of the Na^+/K^+ exchange pump. Second, they are all inhibited by furosemide. Third, all but one (flounder intestine; Musch et al., 1982) are reported to be electroneutral. In at least three reports, the anion transport inhibitors SITS or DIDS do not inhibit the coupled $Cl^-/Na^+/K^+$ transport process (Aiton et al., 1981; Haas et al., 1982; this report). Thus, the present findings in squid axons are in good agreement with these general properties. However, with respect to stoichiometry there appears to be disagreement. In Ehrlich cells (Geck et al., 1980) and duck red cells (Haas et al., 1982), the reported stoichiometry has been $2Cl^-/1Na^+/1K^+$. In both of these studies the reported stoichiometry is of net fluxes rather than unidirectional fluxes and thus the data may not be strictly comparable to the data in the present report.

With the addition of nerve to the list of cells exhibiting coupled $Cl^-/Na^+/K^+$ transport, it begins to seem likely that such a transport process may be a general cellular feature. If true, this implies that the process must subserve some fundamental cellular process. In red cells and Ehrlich cells the process appears to be involved in cell volume regulation by promoting cell re-swelling under hyperosmotic conditions that would tend passively to shrink cells (Schmidt and McManus, 1977; Geck et al., 1980). In the case of squid giant axons we do not yet know whether the cell is capable of such cell-volume regulation. In epithelial cells the system may function to promote net transcellular movements of Na^+ and Cl^- (Greger and Schlatter, 1981).

Comparison with Other Results on Squid Axon

Keynes (1963), in his landmark paper on Cl^- in the squid giant axon, measured the effects on Cl^- uptake of either removing external K^+ or raising its concentration. His results showed that both treatments stimulated Cl^- influx. The finding that K^+-free SSW enhanced Cl^- influx is unexpected in

view of the present results. The fact that Keynes (1963) studied Cl^- uptake into intact or undialyzed axons may offer a partial explanation. That such a method lacks the sensitivity of the internal dialysis technique was evidenced by his failure to detect any effect of removal of external Na^+ on Cl^- influx. Furthermore, exposure of an undialyzed axon to K^+-free media would be expected to result in some changes of the intracellular ionic milieu (i.e., K^+, Na^+, and Ca^{++}). On the other hand, bathing axons in 52 mM external K^+ is likely to enhance Cl^- influx via at least two modes. First, since Cl^- influx is known to depend on external K^+, it is not unreasonable to suppose that raising $[K^+]_o$ would stimulate the cation-coupled Cl^- uptake. Second, raising $[K^+]_o$ will cause the resting membrane potential to depolarize, which would enhance Cl^- entry via furosemide/bumetanide-insensitive conductance pathways (Russell, 1983). Thus, the effect of raising $[K^+]_o$ on Cl^- influx reported by Keynes (1963) is not inconsistent with what is now known about Cl^- movements across the axolemma.

Mullins and Brinley (1969) studied K^+ fluxes in dialyzed squid axons. Although their reasons for such a study were related to questions about the Na^+/K^+ exchange pump, some of their findings are pertinent to the present study. Even though they did not specifically test for an ATP-dependent, ouabain-insensitive component of K^+ influx, they presented data showing an ATP-dependent K^+ influx when $[Na^+]_i \cong 0$. In five such axons dialyzed with 8 mM Cl^-, they found an ATP-stimulated K^+ influx of ~3 pmol/cm^2·s (see Table III, Mullins and Brinley, 1969). Such a flux is somewhat smaller than expected from the present results; nevertheless, it is consistent with the presence of an ATP-dependent K^+ uptake via the $Cl^-/Na^+/K^+$ uptake mechanism. Furthermore, they present data that indicate that K^+ influx in the presence of ATP is greater in axons dialyzed with 8 mM Cl^- than in axons dialyzed with 88 mM Cl^- (see Table V, Mullins and Brinley, 1969), a finding that is also consistent with the present results. On the other hand, Mullins and Brinley (1969) were unable to detect any effect on K^+ influx of replacing external Na^+ with Li^+ or choline. This result is unexpected not only in view of the present findings on Na^+-dependent K^+ influx, but also because in red blood cells external Na^+ has been shown to inhibit ouabain-sensitive K^+ influx (Garrahan and Glynn, 1967). It should be noted that the experiments of Mullins and Brinley (1969) were conducted in the absence of an inhibitor of the Na^+/K^+ exchange pump. Thus, it is possible that the apparent lack of effect of external Na^+ removal could be the result of two offsetting effects. Removal of external Na^+ would be expected to reduce K^+ influx via the $Cl^-/Na^+/K^+$ uptake mechanism, but would enhance K^+ uptake via the Na^+/K^+ exchange pump. Such a possibility indicates the importance of being aware of this anion-cation-coupled transport process and properly controlling for it when the objective is to study Na^+ or K^+ fluxes mediated by other mechanisms.

I wish to thank the Director and Staff of the Marine Biological Laboratory for the facilities placed at my disposal. I also wish to thank Dr. H. C. Palfrey for first suggesting to me the possibility of K^+ involvement in the Cl^- transport mechanism of the squid.
This work was supported by grant NS-11946 from the National Institutes of Health.

Received for publication 3 January 1983 and in revised form 15 March 1983.

REFERENCES

Aiton, J.F., A. R. Chipperfield, J. F. Lamb, P. Ogden, and N. L. Simmons. 1981. Occurrence of passive furosemide-sensitive transmembrane potassium transport in culture cells. *Biochim. Biophys. Acta.* 646:389–398.

Boron, W. F., and J. M. Russell. 1983. Stoichiometry and ion dependence of the intracellular pH-regulating mechanism in squid giant axons. *J. Gen. Physiol.* 81:373–399.

Brinley, F. J., Jr., and L. J. Mullins. 1965. Variations in the chloride content of isolated squid axons. *Physiologist.* 8:121.

Brinley, F. J., Jr, and L. J. Mullins. 1967. Sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* 50:2303–2332.

Dunham, P. B., G. W. Stewart, and J. C. Ellory. 1980. Chloride-activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA.* 77:1711–1715.

Ellory, J. C., P. B. Dunham, P. J. Logue, and G. O. Stewart. 1982. Anion-dependent cation transport in erythrocytes. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* 299:483–495.

Garrahan, P. J., and I. M. Glynn. 1967. The sensitivity of the sodium pump to external sodium. *J. Physiol. (Lond.).* 192:175–188.

Geck, P., C. Pietrzyk, B.-C. Burckhardt, B. Pfeiffer, and E. Heinz. 1980. Electrically silent cotransport of Na^+, K^+ and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta.* 600:432–477.

Greger, R., and E. Schlatter. 1981. Presence of luminal K^+, a prerequisite for active NaCl transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch. Eur. J. Physiol.* 392:92–94.

Haas, M., W. F. Schmidt III, and T. J. McManus. 1982. Catecholamine-stimulated ion transport in duck red cells: gradient effects in electrically neutral [Na + K + 2Cl] co-transport. *J. Gen. Physiol.* 80:125–147.

Keynes, R. D. 1963. Chloride in the squid giant axon. *J. Physiol. (Lond.).* 169:690–705.

Mullins, L. J., and F. J. Brinley, Jr. 1969. Potassium fluxes in dialyzed squid axons. *J. Gen. Physiol.* 53:704–740.

Musch, M. W., S. A. Orellana, L. S. Kimberg, M. Field, D. R. Halm, E. J. Krasny, Jr., and R. A. Frizzell. 1982. Na^+-K^+-Cl^- co-transport in the intestine of a marine teleost. *Nature (Lond.).* 300:351–353.

Nadarajah, A., B. Leese, and G. F. Joplin. 1969. Triton X-100 scintillant for counting calcium-45 in biological fluids. *Int. J. Appl. Radiat. Isot.* 20:733–735.

Russell, J. M. 1976. ATP-dependent chloride influx into internally dialyzed squid giant axons. *J. Membr. Biol.* 28:335–349.

Russell, J. M. 1979. Chloride and sodium influx: a coupled intake mechanism in the squid giant axon. *J. Gen. Physiol.* 73:801–818.

Russell, J. M. 1980. Anion transport processes in neurons. *Ann. NY Acad. Sci.* 341:510–523.

Russell, J. M. 1981. K^+-dependent Na + Cl uptake by squid giant axon. *Biophys. J.* 33:40*a*. (Abstr.)

Russell, J. M. 1983. Chloride in the squid giant axon. *Curr. Top. Membr. Transp.* In press.

Schmidt, W. F., and T. J. McManus. 1977. Ouabain-insensitive salt and water movements in duck red cells. III. The role of chloride in the volume response. *J. Gen. Physiol.* 70:99–121.

Warnock, D. G., and J. Eveloff. 1982. NaCl entry mechanisms in the luminal membrane of the renal tubule. *Am. J. Physiol.* 242:F561–F574.