Stoichiometry and Ion Dependencies of the Intracellular-pH-regulating Mechanism in Squid Giant Axons

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ABSTRACT The ion transport system responsible for intracellular pH (pH_i) regulation in squid giant axons was examined in experiments with pH-sensitive microelectrodes and isotopic fluxes of Na^+ and Cl^-. In one study, axons were acid-loaded and the rate of the subsequent pH_i recovery was used to calculate the acid extrusion rate. There was an absolute dependence of acid extrusion on external Na^+, external HCO_3^- (at constant pH), and internal Cl^-. Furthermore, the dependence of the acid extrusion rate on each of these three parameters was described by Michaelis-Menten kinetics. Acid extrusion was stimulated by an acid pH_i, required internal ATP, and was blocked by external 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS). Under a standard set of conditions (i.e., $[HCO_3^-]_o$ = 12 mM, pH_o = 8.00, $[Na^+]_o$ = 425 mM, $[Cl^-]_i$ = 150 mM, $[ATP]_i$ = 4 mM, pH_i = 6.5, and 16°C), the mean acid extrusion rate was 7.5 $pmol \cdot cm^{-2} \cdot s^{-1}$. In a second study under the above standard conditions, the unidirectional Na^+ efflux (measured with ^{22}Na) mediated by the pH_i-regulating system was found to be ~0, whereas the mean influx was about 3.4 $pmol \cdot cm^{-2} \cdot s^{-1}$. This net influx required external HCO_3^-, internal Cl^-, an acid pH_i, internal ATP, and was blocked by SITS. In the final series of experiments under the above standard conditions, the unidirectional Cl^- influx (measured with ^{36}Cl) mediated by the pH_i-regulating system was found to be ~0, whereas the mean efflux was ~3.9 $pmol \cdot cm^{-2} \cdot s^{-1}$. This net efflux required external HCO_3^-, external Na^+, an acid pH_i, internal ATP, and was blocked by SITS. We conclude that the pH_i-regulating system mediates the obligate net influx of HCO_3^- (or equivalent species) and Na^+ and the net efflux of Cl^- in the stoichiometry of 2:1:1. The transport system is stimulated by intracellular acid loads, requires ATP, and is blocked by SITS.

INTRODUCTION

The importance of intracellular pH (pH_i) regulation is self-evident in view of the pH sensitivity of virtually all cellular processes studied (see Roos and

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Boron, 1981). Although the normal pH_i of most cells is in the range 7.0–7.3 (Roos and Boron, 1981), the pH_i that would prevail if H^+ and HCO_3^- were passively distributed across the cell membrane is generally 6.0–6.8. Thus, there is a tendency for cells to be acidified by the passive fluxes of H^+ and HCO_3^-, as well as by the production of acid by cellular metabolism. This tendency must be counteracted by primary or secondary active transport processes (i.e., "acid extrusion"). Such transport processes, which presumably serve to regulate pH_i, have been identified in a number of invertebrate cells: the squid giant axon (Russell and Boron, 1976), the snail neuron (Thomas, 1977), the barnacle muscle fiber (Boron et al., 1979, 1981), and the crayfish neuron (Moody, 1981). Common characteristics of the pH_i-regulating transport systems of these cells[1] are (*a*) an absolute dependence on external HCO_3^- and Na^+ and on internal Cl^-, (*b*) stimulation by relatively acidic pH_i, and (*c*) sensitivity to inhibitors of anion transport such as 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). In addition, membrane potential data suggest that acid extrusion is electroneutral in snail neurons (Thomas, 1976) and barnacle muscle (Boron, 1977). These general properties are incorporated into the four models of Fig. 1. Although these models differ from one another in some details, they all lead to several common predictions. First, the fluxes of Na^+, Cl^-, and HCO_3^- (or an equivalent species) ought to be mutually dependent upon one another's presence on the appropriate side of the cell membrane. Second, the net influxes of Na^+ and HCO_3^- and the net efflux of Cl^- ought to be inversely related to pH_i and blocked by SITS. Furthermore, for the squid axon, in which acid extrusion apparently requires ATP, all of the aforementioned fluxes ought to depend on ATP. Finally, the stoichiometry ought to be one equivalent of Na^+ entering the cell for each equivalent of Cl^- leaving and for every two equivalents of intracellular acid neutralized.

In the present study, we have tested all of the above predictions for the pH_i-regulating system of the squid giant axon. Although some of these predictions had previously been examined in this or in other preparations, never had all of them been tested in the same cell type. Furthermore, whereas some of the pioneering studies on the ionic mechanism of pH_i regulation had used ion-sensitive microelectrodes to assess net fluxes of Na^+ and Cl^-, we have determined these net fluxes from the difference between the unidirectional influxes and effluxes of the radioisotopes ^{22}Na and ^{36}Cl. Thus, the objection that possible cell-volume changes may alter intracellular Na^+ and Cl^- activities independently of net ion fluxes is circumvented by the use of radioisotopes. Our results confirm all the aforementioned predictions (Fig. 1).

Some of these data have been presented to the Society of General Phsyiologists (Russell and Boron, 1979) and at a Kroc Foundation Symposium (Russell and Boron, 1982).

[1] Not all properties have yet been identified in all preparations. The crayfish neuron apparently has an Na-H exchanger which is totally independent of the HCO_3^--dependent system discussed in this paper.

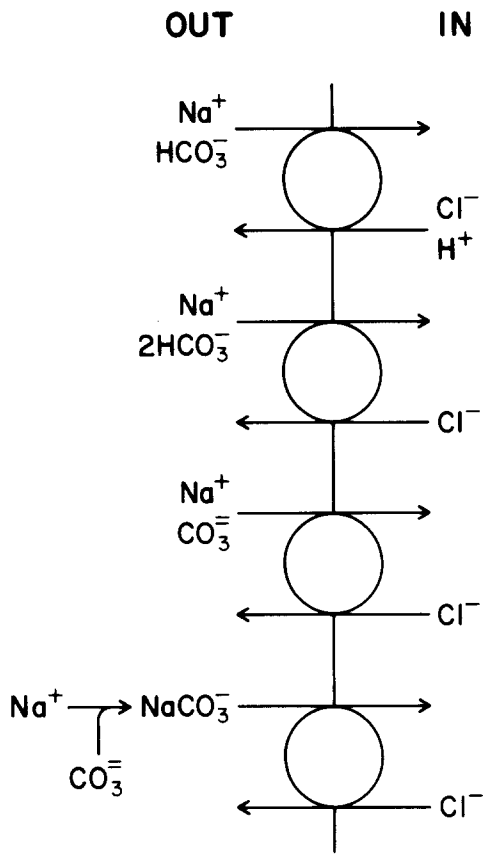

FIGURE 1. Four models for the mechanism of acid extrusion. The first, proposed by Thomas (1977), has external Na^+ and HCO_3^- exchanging for internal Cl^- and H^+. The second is a variant in which the exit of H^+ is replaced by the entry of a second HCO_3^-. In the third, the entry of a single $CO_3^=$ replaces that of two HCO_3^-. Finally, the fourth model (Becker and Duhm, 1978) has the entry of the $NaCO_3^-$ ion pair replacing the separate entry of Na^+ plus $CO_3^=$. All models are equivalent thermodynamically and predict an electroneutral exchange of ions.

METHODS

General

The experiments were conducted at the Marine Biological Laboratory, Woods Hole, MA, from late April through early June, 1978–82, inclusive. Live specimens of the squid *Loligo pealei* were decapitated, and the first stellar nerve from each side was removed and placed in cold, Woods Hole seawater. A 4–5-cm length of giant axon (generally 400–650 μm diam) was isolated from the nerve by microdissection, cannulated at both ends, and mounted horizontally in a chamber (Fig. 2) designed for internal dialysis (Brinley and Mullins, 1965). The temperature, controlled by a

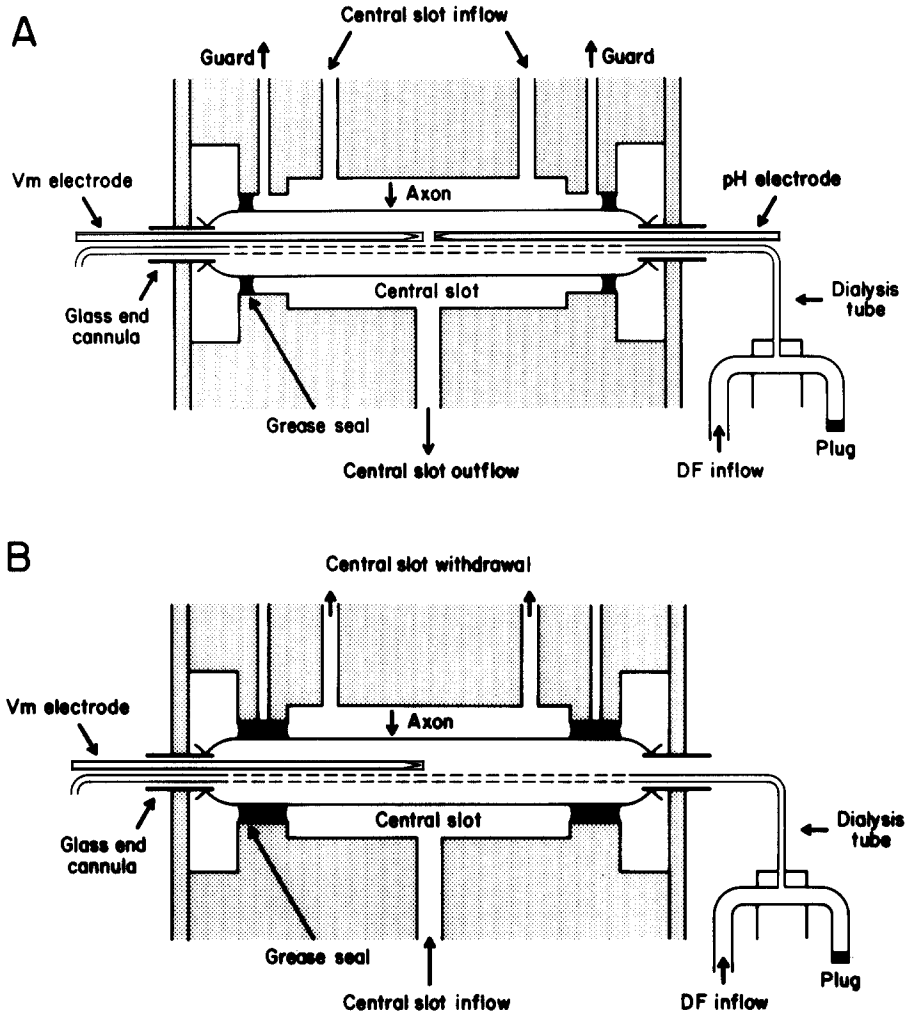

FIGURE 2. Schematic diagrams of axon and chamber. (A) pH_i and isotope efflux experiments. The axon was mounted horizontally in the chamber and cannulated at both ends. The dialysis tube, threaded down the length of the axon, was permeable in the region indicated by the broken lines. A voltage-measuring (i.e., V_m) electrode was introduced through one cannula and lay next to the dialysis tube. In pH_i experiments only, a pH-sensitive electrode was also introduced through the opposite cannula. Artificial SSW continuously flowed through the central slot. In the isotope experiments only, ~2.5% of the SSW entering the central slot was withdrawn by laterally placed guards. Grease seals isolated the central slot from the unperfused end wells. (B) Isotope influx experiments. SSW solutions were changed as described in the text; during the actual measurements, the SSW in the slot was stationary. Grease seals completely obliterated the guard region. Note that the hydrolyzed region of the dialysis tube extended well into the guard region.

circulating water bath connected to the water jacket on the underside of the dialysis chamber, was 22°C for the kinetic studies and 16°C for the stoichiometry and isotopic flux experiments.

Solutions

The standard external fluid (i.e., squid seawater, SSW) had the following composition (in mM): 425 Na^+, 12 K^+, 10 Ca^{++}, 50 Mg^{++}, 542 Cl^-, 15 of the anionic form of 4-[2-hydroxyethyl]-1-piperazine-propane sulfonic acid (EPPS), and 15 of the neutral form of EPPS ($pK \cong 8.0$). The SSW had an osmolality of ~970 mosmol/kg and was buffered to pH 8.0. When HCO_3^- was used, it replaced Cl^- mole for mole except in one series of experiments (the internal Cl^- kinetics study, Figs. 7 and 8), in which 12 mM $NaHCO_3$ was added in addition to the usual components. For all HCO_3^- seawaters, the solution was first brought up to volume with all components except the HCO_3^- salt. After the solution was titrated to pH 8.0, the HCO_3^- salt was added as a powder and the pH was readjusted if necessary by briefly gassing the solution with 100% O_2 or 5% CO_2/95% O_2. The solution was drawn up into a gas-tight syringe and delivered to the chamber via CO_2-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI). When the Na^+ concentration was reduced, Na^+ was replaced mole for mole by choline, *N*-methyl-D-glucammonium, or Li^+. The choline solutions were made immediately before use from choline chloride crystals previously washed in an activated-charcoal suspension and then re-crystallized from isopropanol (Boron et al., 1981).

The standard internal dialysis fluid (DF) had the following composition (in mM): 350 K^+, 50 Na^+, 7 Mg^{++}, 150 Cl^-, 264 glutamate, 210 taurine, 10 *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), 1.0 ethyleneglycol-*bis*(beta-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), 0.5 phenol red, and 4.0 ATP. The solutions had an osmolarity of 950–960 mosmol/kg and were titrated to the appropriate pH with either KOH or glutamic acid. ATP was added to the DF just before use from a 400-mM (pH 7.0) stock solution kept at −5°C. In experiments involving changes in $[Cl^-]_i$, Cl^- and glutamate were exchanged mole for mole. In those involving changes in $[Na^+]_i$, Na^+ and K^+ were exchanged mole for mole.

SITS and 4,4'-diisothiocyanostilbene-2,2'-disolfonic acid (DIDS) were both obtained from Pierce Chemical Co. (Rockford, IL). Vanadium-free ATP was obtained from either Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Indianapolis, IN). Furosemide was a gift from the Hoechst-Roussel Pharmaceutical Corp. (Bridgewater, NJ).

Internal Dialysis

The internal dialysis technique permits control of the intracellular ionic environment as well as measurement of radioisotopic influx or efflux (see Fig. 2). Our dialysis capillaries (140 μm outer diam) were made of cellulose acetate tubing (Fisher Research Laboratories, FRL, Inc., Los Banos, CA). The central region was renderd porous to low-molecular-weight solutes by soaking it in 0.1 N NaOH for 18–24 h. For influx experiments, the porous central region was ~24 mm long, whereas for efflux and pH_i experiments, the porous region was ~18 mm long. Insertion of the dialysis tubing into the axon was facilitated by stiffening the tubing with a length of tungsten wire placed in the lumen. The stiffened tubing was then guided through the axon until the capillary's porous region was located in the central portion of the axon, after which the tungsten wire was removed. Electrodes for measuring membrane potential (V_m)

and, if desired, pH_i, were then introduced through opposite cannulas. The central portion of the axon was physically isolated from the cannulated ends by grease seals (a mixture of Vaseline and mineral oil). These were formed by first lowering the axon onto grease dams located at either end of the central slot in the chamber and then applying grease on the top of the axon at the dam sites. Finally, plastic inserts were placed over the grease seals. Dialysis fluid perfused the dialysis capillary at the rate of 1 μl/min.

Measurement of Intracellular pH

In experiments in which pH_i was measured, a pH-sensitive microelectrode was introduced into the axon through one cannula and an open-tipped reference electrode was introduced through the other. The electrode tips were located in the central portion of the axon and were within 500 μm of one another. In the stoichiometry study, as well as in experiments in which the pH_i recovery was studied as a function of $[Cl^-]_i$, the axons were also dialyzed (see Fig. 2A). However, in experiments in which the recovery of pH_i from acid loads was studied as a function of $[HCO_3^-]_o$, $[Na^+]_o$, or pH_i, the axons were not dialyzed; the arrangement of the apparatus in these experiments was thus as shown in Fig. 2A, except for the omission of dialysis tubing. The pH-sensitive electrodes were of the design of Hinke (1967). They were filled with 0.1 M HCl and fitted with a Ag/AgCl half-cell. The tapered portion of the electrodes had outer diameters of ~125 μm or less for at least the terminal 3 cm. The pH-sensitive tips had outer diameters of ~50 μm at the glass-glass seal, and generally had lengths of 200–300 μm. The internal reference electrodes had dimensions similar to those of the pH electrodes, but had open tips (~10 μm outer diam). They were filled with 0.5 M KCl except for the internal Cl^- kinetics study, in which case they were filled with 1.0 M K^+-glutamate. The junction potential of a glutamate-filled electrode is about −15 mV in SSW or DF and is stable and easily compensated for. This electrode was fitted with a calomel half-cell. A second calomel half-cell, the tip of which was placed at the chamber's outlet port, served as the external reference electrode. The system was grounded through a platinum wire in the bath. The signals from the pH, internal reference, and external reference electrodes were amplified by high-impedance (10^{14}–10^{15} Ω) electrometers. The electronically obtained difference between the signals from the pH and internal reference electrodes is the voltage due solely to pH_i and was plotted on one channel of a strip-chart recorder. The difference between the signals from the internal and external reference electrodes is membrane potential (V_m) and was also plotted on a strip-chart recorder. The pH electrodes were calibrated in high ionic strength buffers, as previously described (Boron and De Weer, 1976*a*).

Calculation of Acid Extrusion Rate

It was not possible, under the conditions of our experiments, to measure isotopic fluxes of HCO_3^- (or equivalent species). Furthermore, the pH_i-regulating system in question may transport H^+ in addition to an HCO_3^--like species. We therefore used the rate of pH_i recovery from an acid load to calculate the equivalent net influx of HCO_3^- plus the net efflux, if any, of H^+. We term this the "acid extrusion rate" and calculate it as the product of the rate of pH_i recovery from an imposed acid load (dpH_i/dt), the volume-to-surface ratio, and the intracellular buffering power (β). dpH_i/dt, the slope of the pH_i change, was determined directly from the strip-chart recording. The volume-to-surface ratio is one-fourth the axon's diameter, assuming the axon to be a cylinder. β is dB/dpH, where dB is the amount of strong base that must be added to

the axoplasm to raise pH_i by dpH. This was determined as follows (see Boron, 1977). The axon was first acid-loaded by dialysis (mean pH_i = 6.65 ± 0.08, n = 8) and exposed to the same solutions as it would have been in an ordinary experiment, except that SITS was present in the SSW to block the pH_i-regulating mechanism. The axon was then exposed to SSW containing 0.2 mM of NH_4^+ at pH_o 7.75. Such a solution contains a small amount of NH_3 which enters the axon and then combines with H^+ to form NH_4^+, thereby raising pH_i. For each NH_4^+ so formed, one H^+ has been removed from cellular buffers. Thus, the amount of strong base added to the axoplasm (i.e., dB in the above definition of β) is simply $d[NH_4^+]_i$. We allowed the exposure of the axon to NH_4^+ SSW to continue until pH_i reached a new steady level, ~0.10–0.15 higher than the initial one. At this point, the amount of strong base added to the cells was $\Delta[NH_4^+]_i$, which is the same as $[NH_4^+]_i$ since the initial $[NH_4^+]_i$ was zero. $[NH_4^+]_i$ was calculated from pH_i and pK, assuming that $[NH_3]_i = [NH_3]_o$. Although β could be calculated from $[NH_4^+]_i$ and the rise of pH_i produced by application of NH_4^+ SSW, such a value for ΔpH_i is somewhat artificially reduced, owing to the passive entry of NH_4^+ during the exposure to NH_4^+ SSW (see Boron, 1977). Therefore, we took as ΔpH_i the change in pH_i upon removal of external NH_4^+. The average β (i.e., $\Delta[NH_4^+]_i/\Delta pH_i$) in eight experiments was 11.2 ± 1.1 mM.

Radioisotope Influx Experiments

For influx experiments, the radioisotope was presented to the exterior of the axon in the central slot between the grease dams, thereby exposing a 17–19-mm length (measured with calipers) of axon. The axon was dialyzed not only in the central region, where the isotope was applied, but also for a length of 4–5 mm on either side (Fig. 1B). This permits collection of isotope which enters the central region of the axon and then diffuses laterally (see Russell, 1976). It is crucial that extracellular isotope not leak from the central slot, past the grease seals, and into the end chambers containing the cannulated regions of the axon; this would increase the surface area for isotope influx in an uncontrolled manner. The detection of such grease-seal leaks was facilitated by the addition of 0.5 mM phenol red to all isotope-containing SSWs. When a leak was noted, the lateral grease seal was quickly repaired and the end-region was washed with isotope-free SSW. This procedure often allowed the experiment to be salvaged.

Because it was not economically feasible to superfuse axons with radioisotope-containing SSW continuously, the following procedure was adopted to change external solutions. Ports located at the bottom of the central chamber slot in which the axon was suspended were used to withdraw the fluid bathing the axon. At the same time, the new SSW was carefully added from the top. Care was taken never to allow the meniscus of the external solution to drop below the axon. The axon was first rinsed with 5 ml of an isotope-free version of the new SSW (slot volume = 0.2 ml). The isotope-containing version of the new SSW was then applied in three 0.2-ml aliquots. After each application the solution in the central slot was mixed several times by gently withdrawing it into a mechanical pipette and then ejecting it. The first two aliquots were withdrawn through the bottom ports and discarded. The third 0.2-ml aliquot of radioisotope-containing SSW was allowed to remain in contact with the axon. This entire solution-changing procedure took ~2 min.

For each new application of isotope-containing SSW, two samples were taken directly from the fluid bathing the axon for determination of specific activity, one soon after the fluid was applied and the other 30–40 min later (i.e., about halfway through the experiment). If these two samplings yielded specific activities differing by

>5%, the data were discarded. All isotope-containing SSWs were made up to have the same specific activity. The specific activity of the SSW actually in contact with the axon was generally ~15% less than that of the original solution, because of dilution of the isotope by isotope-free SSW previously in the central slot.

For experiments in which HCO_3^- was included in the external fluid, the appropriate concentration of water-saturated CO_2 was gently and continuously blown across the surface of the SSW bathing the axon.

Influx samples were taken by allowing the dialysis fluid, after passing through the axon, to fall directly into a scintillation vial. At the end of a suitable time interval (usually 5 min), the tip of the dialysis tube was rinsed with 1.0 ml of deionized water directly into the vial. To this was added 10 ml of a 2:1 mixture of toluene to Triton X-100 counting cocktail (Nadarajah et al., 1969) containing 4 g/liter of Omnifluor (New England Nuclear, Boston, MA). Samples were counted in a Beckman model LS 7500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA) until sufficient counts were accumulated for a 3% counting error.

Radioisotope Efflux Experiments

For efflux experiments, isotope-containing DF was presented to the interior of the axon via the dialysis capillary. External fluid (i.e., SSW) continuously entered the chamber's central slot at a rate of 2.4 ml/min through the two bottom ports and was collected at the top of the slot after having flowed around the axon. Fluid was separately withdrawn from each of two "guard" regions (i.e., just medial to the grease seals) at the rate of 50 μl/min (see Fig. 2A) and discarded. This fluid represents isotope efflux from regions of the axon where solute control by dialysis was poor. The fluid withdrawn at the central slot outflow (i.e., 2.3 ml/min) was collected in scintillation vials mounted in a fraction collector. 10 ml of the cocktail described above was added, and the resulting stiff gel was counted until a 3% error was achieved.

Radioisotopes

Chloride-36 was obtained as aqueous solutions of $Na^{36}Cl$ from Amersham/Searle (Arlington Heights, IL) or of $K^{36}Cl$ from New England Nuclear. Each solution was evaporated to dryness at low heat (~90°C), then ashed at 450°C to remove organic contaminants. The resulting powder contained a significant amount of nonradioactive Cl^-, which was taken into account in determining the final $[Cl^-]$ of ^{36}Cl-containing solutions. Seawaters for studying Cl influx contained ^{36}Cl at a final specific activity of ~100 μCi/mmol of total chloride, whereas ^{36}Cl-containing dialysis fluids had a specific activity of ~50 μCi/mmol total chloride.

Sodium-22 (New England Nuclear) was supplied as a carrier-free aqueous solution of $^{22}NaCl$, which was added directly to either external or internal solutions. ^{22}Na-containing seawaters had a final specific activity of ~90 μCi/mmol total sodium, whereas the dialysis fluids used to study Na efflux had a specific activity of 50 μCi/mmol.

RESULTS

Acid Extrusion Rate

Intracellular pH regulation is best studied by acid-loading a cell and then monitoring the subsequent recovery of pH_i, which is due to the extrusion of acid across the cell membrane. The term "acid extrusion" includes the influx of HCO_3^- (or related species) or the influx of OH^- or the efflux of H^+, or a

combination of these. The acid extrusion rate is calculated from the rate of pH_i recovery (see Methods).

DEPENDENCE ON EXTERNAL HCO_3^- The recovery of pH_i from an imposed acid load is known to depend on extracellular HCO_3^- (Boron and De Weer,

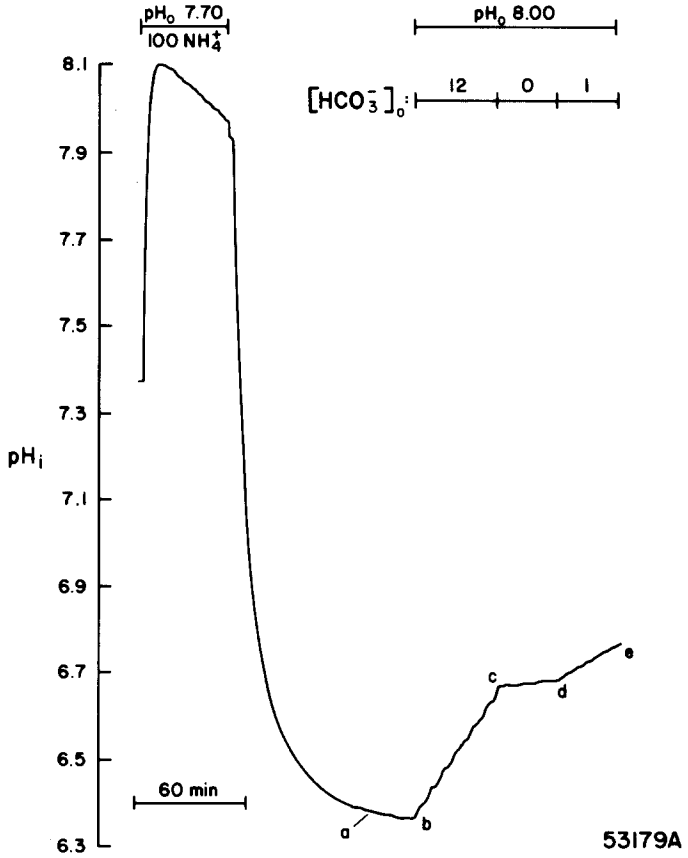

FIGURE 3. Effect on pH_i recovery of altering $[HCO_3^-]_o$ at constant pH_o. A nondialyzed axon was acid-loaded by pretreating it for ~50 min with SSW containing 100 mM NH_4^+ (pH_o = 7.70). After removal of external NH_4^+, pH_i fell far below its initial level, but failed to recover in the nominal absence of HCO_3^- (segment *ab*). With the introduction of 12 mM HCO_3^- into the SSW (pH_o = 8.00), pH_i recovered rapidly (*bc*). Removal of the HCO_3^- at constant pH_o greatly slowed the pH_i recovery (*cd*). Finally, addition of 1 mM HCO_3^- at constant pH_o stimulated the pH_i recovery (*de*), but not to the extent produced by the 12 mM HCO_3^-.

1976*b*). We have extended this observation by quantitating the dependence of the acid extrusion rate on the external HCO_3^- concentration ($[HCO_3^-]_o$) in experiments in which external pH (pH_o) was held constant by proportionally varying the P_{CO_2} and $[HCO_3^-]_o$. Fig. 3 illustrates a typical experiment, in

which a nondialyzed axon was acid-loaded by pretreating it with SSW containing 100 mM NH_4^+ (NH_4^+ replacing Na^+; pH = 7.7; NH_4^+/SSW). This procedure and its theoretical basis[2] have been described earlier (Boron and De Weer, 1976*a*, *b*). Note that after the axon was acid-loaded, pH_i failed to recover when bathed in nominally HCO_3^--free SSW (Fig. 3, segment *ab*). When the axon was exposed to SSW containing 12 mM HCO_3 equilibrated with 0.5% CO_2 (pH_o = 8.0; 12 HCO_3/SSW), however, pH_i recovered at a relatively high rate (segment *bc*). From the rate of pH_i recovery, as well as axoplasmic buffering power and axon diameter, we calculate that the acid extrusion rate during segment *bc* was 10.7 pmol·cm^{-2}·s^{-1}. At point *c*, the nominal $[HCO_3^-]_o$ was reduced to 0 mM (pH_o = 8.0), causing the acid extrusion rate to fall to 0.6 pmol·cm^{-2}·s^{-1}. The remaining small flux was probably due to a small amount of HCO_3^- in the solution immediately surrounding the axon's membrane. When $[HCO_3^-]_o$ was raised to 1 mM, still holding pH_o at 8.0, the calculated acid extrusion rate increased to 3.8 pmol·cm^{-2}·s^{-1}. It had previously been shown in the squid giant axon that pH_i recovery from an acid load is accelerated by simultaneously increasing $[HCO_3^-]_o$ and pH_o at a constant P_{CO_2}, (Boron and De Weer, 1976*b*). The present result demonstrates that increasing $[HCO_3^-]_o$ alone is sufficient to enhance acid extrusion.

In a total of 30 similar experiments performed on 13 nondialyzed axons, we determined the dependence of the acid extrusion rate on $[HCO_3^-]_o$ at a constant pH of 8.0, a pH_i of ~6.7, and a $[Na^+]_o$ of 425 mM. The $[Cl^-]_i$ of nondialyzed axons is ~100 mM (Keynes, 1963; Russell, 1976); as will be shown below, net Cl^- fluxes associated with acid extrusion are too small to change $[Cl^-]_i$ significantly during an experiment. Our results, collated in Fig. 4 and summarized in Table I, show that the dependence of the acid extrustion rate on $[HCO_3^-]_o$ can be described by simple Michaelis-Menten kinetics, with an apparent K_m for external HCO_3^- of 2.3 mM and an apparent V_{max} of 10.6 pmol·cm^{-2}·s^{-1}.

DEPENDENCE ON EXTERNAL NA^+ The $[Na^+]_o$ dependence of acid extrusion, predicted by all models of Fig. 1, is examined in the experiment of Fig. 5. A nondialyzed axon was acid-loaded by the NH_4^+ pretreatment technique discussed above. When the axon was bathed in nominally HCO_3^--free media containing 425 mM Na^+ (segment *ab*), there was no recovery of pH_i from the acid load. The addition of 12 mM HCO_3^- (0.5% CO_2, pH_o = 8.0) produced a relatively rapid recovery of pH_i (*bc*), as already noted in Fig. 3. In this case the calculated acid extrusion rate was 9.7 pmol·cm^{-2}·s^{-1}. Reducing $[Na^+]_o$ to 15 mM decreased the acid extrusion rate to 1.1 pmol·cm^{-2}·s^{-1} (segment *cd*),

[2] When an axon is initially exposed to the NH_4^+/SSW, NH_3 rapidly enters and causes an increase in pH_i. Later, the passive entry of NH_4^+ predominates, causing a slow fall of pH_i, as NH_4^+ partially dissociates to NH_3 and H^+. When the external NH_4^+ is removed, intracellular NH_4^+ dissociates to form NH_3 (which readily leaves the cell) and H^+ (which is trapped within). Thus, the axoplasm is greatly acidified. The degree of acid loading is determined by the previous net influx of NH_4^+.

whereas raising $[Na^+]_o$ to 100 mM increased the rate to 6.4 pmol·cm^{-2}·s^{-1} (segment *de*). The results of 21 similar experiments on 15 axons are collated in Fig. 6 and summarized in Table I. They show that the dependence of acid extrusion rate upon $[Na^+]_o$ is described by simple Michaelis-Menten kinetics,

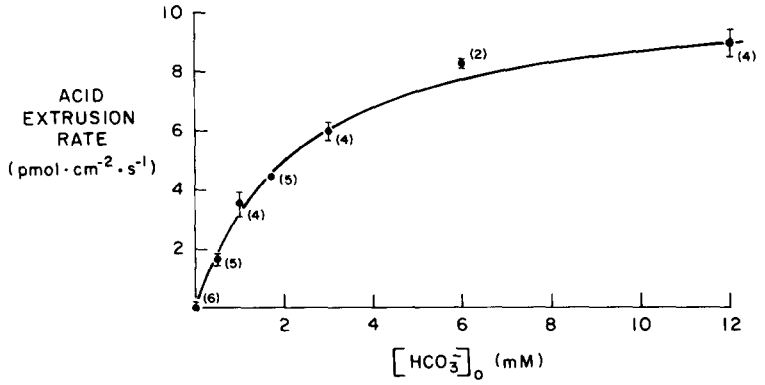

FIGURE 4. Dependence of acid extrusion rate on $[HCO_3^-]_o$. The acid extrusion rate was calculated (see Methods) from the rate of pH_i recovery (in experiments on 13 axons, similar to that of Fig. 3), intracellular buffering power, and axon diameter. $[Cl^-]_i$ was ~100 mM in these undialyzed axons. pH_o was 8.0, $[Na^+]_o$ was 425 mM, and the pH_i recovery rates were determined at a pH_i of ~6.7. Data were normalized to the acid extrusion rate under "standard" conditions (i.e., $[HCO_3^-]_o$ = 12 mM) for each axon and then scaled to the mean acid extrusion rate for all axons under standard conditions (9.52 ± 1.77 pmol·cm^{-2}·s^{-1}; n = 28 axons). The number of determinations is given in parentheses; vertical bars indicate standard errors. The curve through the points is a nonlinear least-squares fit to the Michaelis-Menten equation; K_m = 2.3 ± 0.2 mM, V_{max} = 10.6 ± 0.4 pmol·cm^{-2}·s^{-1}.

TABLE I

KINETICS OF ACID EXTRUSION*

Parameter varied	$[HCO_3^-]_o$	$[Na^+]_o$	$[Cl^-]_i$	Apparent K_m	Apparent V_{max}
	mM	*mM*	*mM*	*mM*	*pmol·cm^{-2}·s^{-1}*
$[HCO_3^-]_o$	—	425	~100	2.3±0.2	10.6±0.4
$[Na^+]_o$	12	—	~100	77±12	10.3±0.6
$[Cl^-]_i$	12	425	—	84±15	19.6±1.2

* pH_o = 8.0, pH_i = 6.7.

with an apparent K_m for external Na^+ of 77 mM and an apparent V_{max} of 10.3 pmol·cm^{-2}·s^{-1}.

DEPENDENCE ON INTERNAL CL$^-$ The models of Fig. 1 predict that there ought to be an absolute dependence of acid extrusion on intracellular Cl^- as verified in an earlier study on squid axons (Russell and Boron, 1976). We

have now extended this finding by studying the recovery of pH_i from acid loads at several different values of $[Cl^-]_i$. In these experiments, both the acid-loading and the establishment of various levels of $[Cl^-]_i$ were achieved by dialyzing the axon with a low-pH DF of the appropriate $[Cl^-]_i$ until a pH_i of ~6.7 was reached. Control experiments with Cl^--sensitive microelectrodes showed that the time required for pH_i to reach ~6.7 is also sufficient for $[Cl^-]_i$ to reach $[Cl^-]_{DF}$.

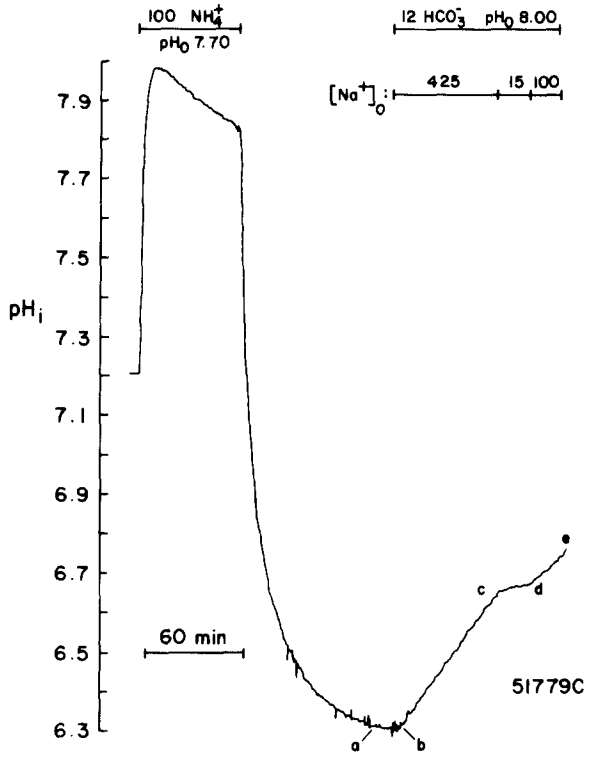

FIGURE 5. Effect on pH_i recovery of altering $[Na^+]_o$. A nondialyzed axon was acid-loaded by pretreating it for ~60 min with SSW containing 100 mM NH_4^+ (pH_o = 7.70). After removal of the external NH_4^+, pH_i fell far below its initial value, but failed to recover in the nominal absence of HCO_3^- (segment *ab*). In the simultaneous presence of 12 mM HCO_3^- and 425 mM Na^+ in the SSW (pH_o = 8.00), pH_i recovered rapidly (*bc*). Reducing $[Na^+]_o$ to 15 mM greatly reduced the rate of pH_i recovery (*cd*), whereas raising $[Na^+]_o$ to 100 mM increased the pH_i recovery rate (*de*), though not to the initial level.

In the experiment illustrated in Fig. 7A, the pH_i prior to dialysis was 7.35. At point *a*, dialysis was begun with a pH 6.6 fluid containing 4 mM ATP and 200 mM Cl^-; 30 min of dialysis reduced pH_i to ~6.67. At this point (*b*), flow of the dialysis fluid was halted, returning control of pH_i to the axon. As can be seen in segment *bc*, there was no recovery of pH_i while the axon was bathed in HCO_3^--free SSW. However, when 12 HCO_3/SSW was presented, pH_i rose

rather rapidly. The calculated acid extrusion rate during the initial portion of segment *cd* was 15.0 pmol·cm^{-2}·s^{-1}.

In a total of 38 similar experiments on 38 axons, we measured the acid extrusion rate at several different values of $[Cl^-]_i$. The inset of Fig. 7 illustrates examples of the pH_i recovery from acid loads after dialysis with fluids containing 0, 100, and 350 mM Cl^-, and shows that pH_i recovery rates increase as $[Cl^-]_i$ increases. As indicated by Fig. 8, a summary of all the data, the dependence of acid extrusion rate on $[Cl^-]_i$ follows simple Michaelis-Menten kinetics, with an apparent K_m for internal Cl^- of 84 mM, and an apparent V_{max} of 19.6 pmol·cm^{-2}·s^{-1} (see Table I). The reader will note that the apparent V_{max} for acid extrusion in these experiments is nearly twice that for those experiments in which $[HCO_3^-]_o$ and $[Na^+]_o$ were varied (see Figs. 4

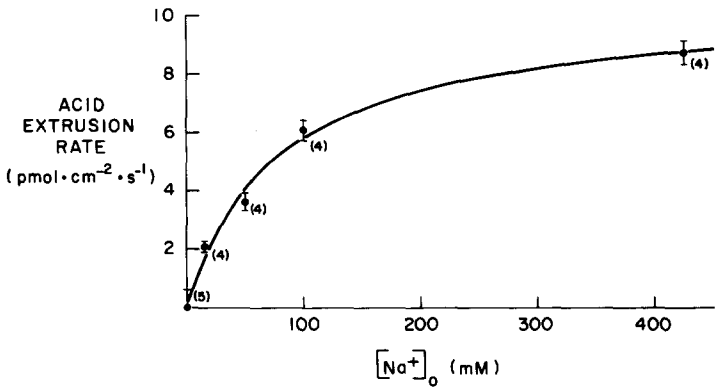

FIGURE 6. Dependence of acid extrusion rate on $[Na^+]_o$. This represents a collation of data from experiments similar to that of Fig. 5 on 15 axons. The calculation of the acid extrusion rates and normalization of the data was as described for Fig. 4. $[Cl^+]_i$ was ~100 mM in these nondialyzed axons, pH_o was 8.00, $[HCO_3^-]_o$ was 12 mM, and the pH_i recovery rates were determined at a pH_i of ~6.7. The number of determiantions is given in parentheses; vertical bars indicate standard errors. The curve through the points is a nonlinear least-squares fit to the Michaelis-Menten equation; K_m = 77 ± 13 mM, V_{max} = 10.3 ± 0.6 pmol·cm^{-2}·s^{-1}.

and 6, and Table I). This discrepancy is the result of the rather low $[Cl^-]_i$ (i.e., ~100 mM) prevailing in the latter experiments, which were performed on nondialyzed axons. For example, we see from Fig. 4 that the fitted acid extrusion rate of $[HCO_3^-]_o$ of 12 mM and a $[Na^+]_o$ of 425 mM is 8.9 pmol·cm^{-2}·s^{-1}. An examination of Fig. 8, which summarizes the $[Cl^-]_i$ data obtained at the aforementioned $[HCO_3^-]_o$ and $[Na^+]_o$ values, reveals that an acid extrusion rate of 8.9 pmol·cm^{-2}·s^{-1} corresponds to a $[Cl^-]_i$ of 70 mM. This is within the range of reported $[Cl^-]_i$ values for nondialyzed axons, especially for those obtained in the month of May (Brinley and Mullins, 1965), as were those in the present study. Thus, the $[Cl^-]_i$ study performed on dialyzed axons is consistent with the $[HCO_3^-]_o$ and $[Na^+]_o$ studies performed on nondialyzed axons.

DEPENDENCE ON pH_i The pH_i-regulating mechanisms of barnacle muscle (Boron et al., 1979) and snail neurons (Thomas, 1977) exhibit a steep dependence on pH_i, their apparent rates of acid extrusion being approximately zero at normal pH_i and rising steadily at lower pH_i values. The pH_i dependence of acid extrusion in squid giant axons was examined in five experiments in the present study. In each case, a nondialyzed axon was first exposed to pH

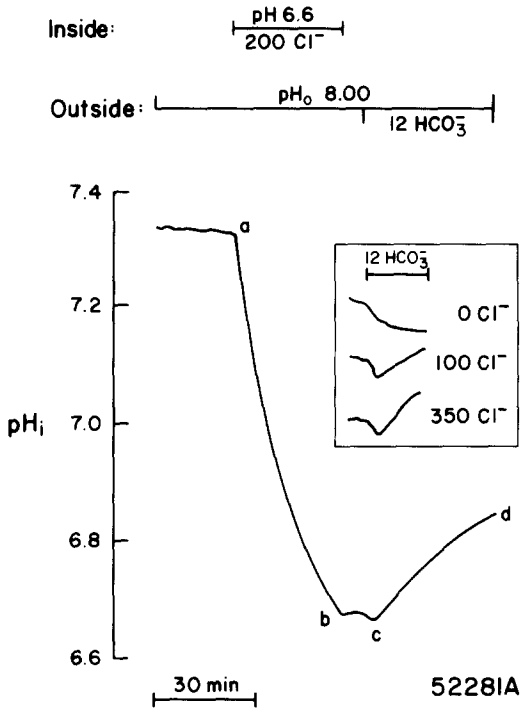

FIGURE 7. pH_i recovery at different values of $[Cl^-]_i$. At point *a*, dialysis ws begun with a fluid containing 200 mM Cl^- at pH 6.6. Halting dialysis (point *b*) returned control of pH_i to the axon, but produced only a very slow pH_i recovery (*bc*). The addition of 12 mM HCO_3^- to the SSW at a constant pH_o of 8.00 caused pH_i to recover (*cd*). The inset shows the results of similar experiments (comparable to segments *bc* and *cd*) on axons of approximately the same diameter. Although 12 mM HCO_3^- failed to stimulate pH_i recovery in the axon previously dialyzed with 0 mM Cl^- (top), the recovery rate was greater in axons dialyzed with 100 (middle) and 350 mM Cl^- (bottom).

8.00 SSW containing 10 mM HCO_3^-. This caused an initial fall in pH_i (due to the influx of CO_2), followed by a slower recovery (due to acid extrusion). From the rate of pH_i recovery, we calculated the acid extrusion rate (see Methods), assuming an intrinsic intracellular buffering power of 9 mM·pH^{-1} (Boron and De Weer, 1976*a*). At a mean pH_i of 7.36 ± 0.04, the mean acid extrusion rate was 3.4 ± 0.6 pmol·cm^{-2}·s^{-1}. We then acid-loaded the axon with a pulse of NH_4^+ (see above) and exposed it to the pH 8.00/10 mM

HCO_3^- SSW for a second time. At a mean pH_i of 6.75 ± 0.14, the mean acid extrusion rate was 7.0 ± 0.6 pmol·cm^{-2}·s^{-1}. Thus, the acid extrusion rate of squid axons is inversely related to pH_i.

DEPENDENCE ON INTERNAL ATP We have previously demonstrated that acid extrusion by the squid axon requires intracellular ATP (Russell and Boron, 1976). This observation has been confirmed in the present study.

EFFECT OF PHARMACOLOGIC AGENTS We have previously reported that recovery of pH_i from an acid load is blocked by 0.5 mM SITS (Russell and Boron, 1976). This has been repeatedly confirmed in the present study. In two additional experiments we found that acid extrusion is reversibly inhibited ~85% either by 1 mM DNDS or by 0.6 mM of the diuretic agent furosemide.

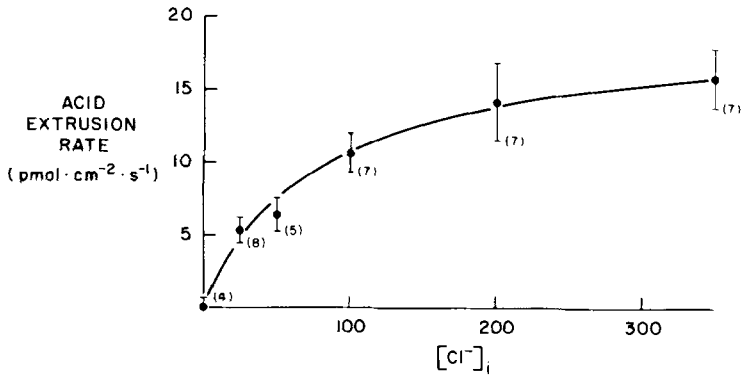

FIGURE 8. Dependence of acid extrusion rate on $[Cl^-]_i$. This represents a collation of data from 38 experiments similar to the one of Fig. 7. Only one data point was obtained per axon. Acid extrusion rates were calculated as described for Fig. 4. The plotted points represent mean values of non-normalized acid extrusion rates. The number of determinations is given in parentheses; vertical bars represent standard error. The curve through the points is a nonlinear, least-squares fit to the Michaelis-Menten equation; K_m = 84 ± 15 mM, V_{max} = 19.6 ± 1.2 pmol·cm^{-2}·s^{-1}. $[Na^+]_o$ was 437 mM, pH_o was 8.00, $[HCO_3^-]_o$ was 12 mM, and pH_i recovery rates were obtained at a pH_i of ~6.7.

EFFECT OF CHANGES IN MEMBRANE POTENTIAL The models of Fig. 1 are of electroneutral transport systems, which ought not to be influenced by changes in membrane potential (V_m). In the experiment of Fig. 9, an axon was dialyzed (segment *ab*) with a pH 6.5 solution containing 400 mM Cl^- and 0 mM Na^+. When pH_i had fallen to ~6.55, dialysis was halted (point *b*), returning control of pH_i to the axon. No recovery of pH_i occurred (*bc*), however, until 10 mM HCO_3^- was added to the pH 8.0 SSW. This elicited a rapid rise in pH_i (*cd*) corresponding to an acid extrusion rate of 21.1 pmol·cm^{-2}·s^{-1}. This rather high rate is a consequence of the previous period of dialysis with 400 mM Cl^-. Subsequently raising $[K^+]_o$ from 10 to 200 mM (K^+ replacing Na^+) caused V_m to rise from approximately −51 mV to approximately −17 mV, but had

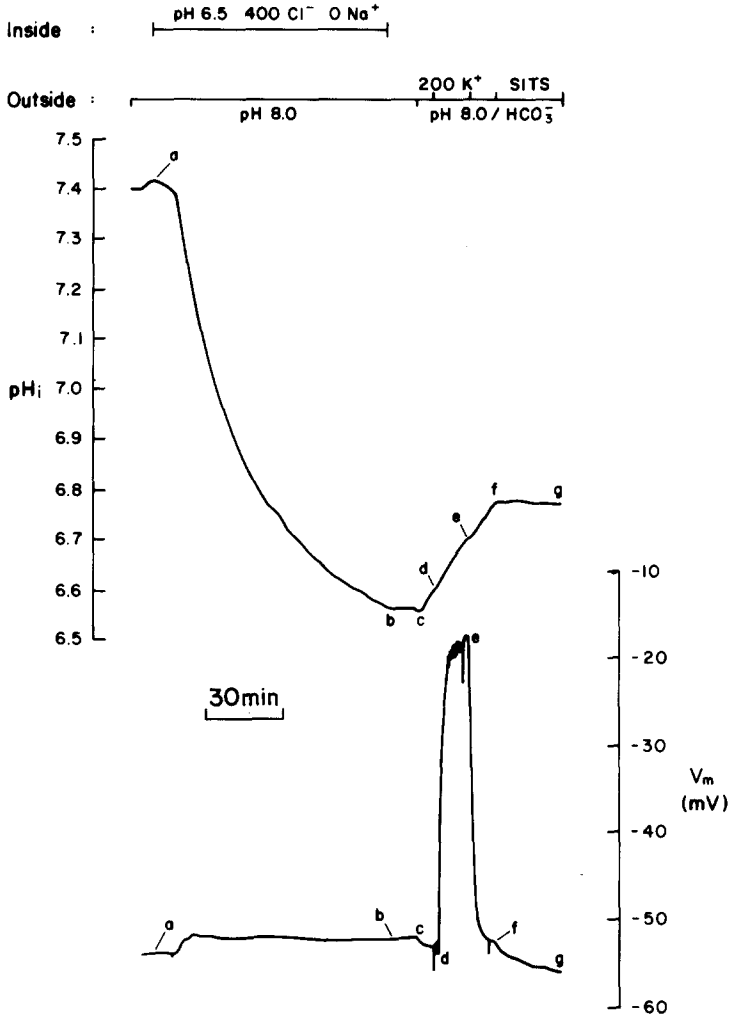

FIGURE 9. Effect of depolarization on pH_i recovery. The axon was acid-loaded by dialyzing with a pH 6.5 solution containing 400 mM Cl^- and 0 mM Na^+ (segment *ab*). After dialysis was halted (point *b*), returning control of pH_i to the axon, there was no pH_i recovery (*bc*) until 12 mM HCO_3^- was added to the SSW (*cd*). When $[K^+]_o$ was increased from 10 to 200 mM (holding $[HCO_3^-]_o$ and pH_o constant) at point *d*, there was only a slight decrease in the pH_i recovery rate (*de*), even though V_m changed from approximately −51 to −17 mV. Returning $[K^+]_o$ to 10 mM (*ef*) restored V_m to its initial value, but had only a slight effect on the pH_i recovery rate. Finally, application of SITS completely blocked the pH_i recovery (*fg*).

only a slight effect on the acid extrusion rate (*de*), which fell to 19.2 pmol·cm^{-2}·s^{-1}. This 11% inhibition of acid extrusion is reasonably close to the value of 9% predicted from the accompanying decrease in $[Na^+]_o$ (from 425 to 235 mM) and the apparent K_m for external Na^+ (i.e., 77 mM). Returning $[K^+]_o$

to 10 mM caused a recovery of V_m, but had only a slight effect on the pH_i recovery (acid extrusion rate: 20.4 pmol·cm^{-2}·s^{-1}). Finally, the addition of 0.5 mM SITS to the SSW blocked further recovery of pH_i.

STOICHIOMETRY The models of Fig. 1 predict that two equivalents of intracellular acid be neutralized for each equivalent of Na^+ taken up and each equivalent of Cl^- extruded. The experiments of the previous subsection are consistent with such an electroneutral transport process. To determine the stoichiometry of the transport system directly, we measured the acid extrusion rate and the net fluxes of Na^+ and Cl^- (using radioisotopes), all under identical conditions of incubation. These experiments were conducted on dialyzed axons at 16°C, the lower temperature being required to maintain stable isotopic fluxes from continuously dialyzed axons. Extraneous Na^+ fluxes were minimized by the following precautions. (*a*) Diffusion through the voltage-dependent Na^+ channel was blocked by application of 10^{-7} tetrodotoxin (TTX). (*b*) Fluxes mediated by the Na-K pump were inhibited by application of 10^{-5} ouabain. (*c*) Na^+ influx via the coupled Na-Cl uptake process (Russell,

TABLE II
STOICHIOMETRY OF ACID EXTRUSION*

Parameter	Net flux
	pmol·cm^{-2}·s^{-1}
Acid extrusion	7.5±0.6 (n = 15)
Net Na^+ influx	3.4±0.4 (n = 13)
Net Cl^- efflux	3.9±0.2 (n = 17)

* Conditions: $[Na^+]_o$ = 425 mM, $[HCO_3^-]_o$ = 12 mM, pH_o = 8.00, pH_i = 6.7, $[Cl^-]_i$ = 150 mM, $[Na^+]_i$ = 50 mM, $[ATP]_i$ = 4 mM, T = 16°C.

1979) was largely inhibited by elevating $[Cl^-]_i$ to 150 mM. The other conditions are listed in the footnote to Table II.

An accurate determination of the acid extrusion rate requires not only a measurement of the pH_i recovery rate, but also knowledge of the total axoplasmic buffering power (β_T) under identical conditions. In a CO_2-containing solution, β_T is the sum of the CO_2 buffering power (β_{CO_2}), which can be calculated, and the intrinsic intracellular buffering power (β_I), which must be determined empirically (see Methods). In separate experiments, eight axons were dialyzed with a fluid having a pH of 6.5; the other conditions were identical to those given in Table II, except that 0.5 mM SITS was present and CO_2 and HCO_3^- were absent. Dialysis was halted when pH_i reached ~6.6, and β_I was determined as outlined in Methods, yielding a value of 11.2 ± 1.1 mM.

In a second series of experiments we measured the acid extrusion rate under conditions identical to those under which the buffering power was determined. The time course of the pH_i decline due to dialysis was similar to that shown in Fig. 7. After dialysis was halted, pH_i failed to recover as long as the axons were bathed in HCO_3^--free SSW, but increased relatively rapidly when 12

mM HCO_3^- was added to the SSW. For 15 axons, the average acid extrusion rate was 7.5 ± 0.6 pmol·cm^{-2}·s^{-1} (see Table II). In an earlier study involving only three axons, the calculated acid extrusion rate under similar conditions was only 4.8 ± 0.9 pmol·cm^{-2}·s^{-1} (Russell and Boron, 1976), a value arrived at by *assuming* a buffer power of 9 mM (i.e., the value for undialyzed axons). Had the correct buffering power (i.e., determined in these experiments) been used, the calculated acid extrusion rate would have been 6.0 pmol·cm^{-2}·s^{-1}. This is reasonably close to the present estimate, given the small sample size in the earlier study.

Na^+ Fluxes

The models of Fig. 1 predict that acid extrusion should be accompanied by a net influx of Na^+. We measured this net flux by determining the difference between unidirectional Na^+ influx and efflux, using ^{22}Na as an isotopic marker. Extraneous or background Na^+ fluxes were minimized by the previously mentioned precautions.

NA$^+$ EFFLUX Fig. 10 illustrates an experiment in which Na^+ efflux was first allowed to reach a steady value in an axon dialyzed with a pH 6.7 fluid containing 4 mM ATP and 150 mM Cl^-. The SSW was HCO_3^- free and contained TTX but no ouabain. The application of 10^{-5} M ouabain reduced Na^+ efflux from ~20 to ~2 pmol·cm^{-2}·s^{-1}, which reflects inhibition of the Na-K pump. When 12 mM HCO_3^- was added to the SSW, however, there was no effect upon unidirectional Na^+ efflux, even though acid extrusion should have been greatly stimulated. Similar results were obtained in five other axons. Thus, the axon's pH_i-regulating system does not mediate a unidirectional Na^+ efflux under the conditions of these experiments. The *net* Na^+ flux produced by this transporter can therefore be taken as the unidirectional Na^+ influx.

NA$^+$ INFLUX: DEPENDENCE ON EXTERNAL HCO_3^- Fig. 11 illustrates an experiment in which an axon was dialyzed with a fluid of the same composition as in the experiment of Fig. 10. The Na^+ influx was measured as the SSW was changed from 0 mM HCO_3^- to 12 mM HCO_3^- and then back to 0 mM HCO_3^-. As shown above, axons treated in such a way extrude acid only during the exposure to 12 mM HCO_3^-. Fig. 11 shows that the application of HCO_3^- triggers an increase in the Na^+ influx, which is reversed upon removal of HCO_3^-. This HCO_3^--stimulated Na^+ influx is presumably the postulated Na^+ flux through the pH_i-regulating system (Fig. 1). In a total of 13 similar experiments, reversible increases of unidirectional Na^+ influx always accompanied the application of 12 mM HCO_3^-. The average increase was 3.4 ± 0.4 pmol·cm^{-2}·s^{-1} (see Table II).

NA$^+$ INFLUX: DEPENDENCE ON INTERNAL CL^- The models of Fig. 1 predict that the Na^+ influx linked to acid extrusion ought to require intracellular Cl^-. To test this hypothesis, we dialyzed axons with a pH 6.7 DF containing 4 mM ATP and 0 mM Cl^- (glutamate replacing Cl^-). Dialysis with this Cl^--free solution was performed for ~1 h before isotopic flux studies were begun to

ensure that $[Cl^-]_i$ was as low as possible.[3] In five axons treated in this manner, exposure to 12 HCO_3^-/SSW resulted in an average increase in Na^+ influx of only 0.3 ± 0.2 pmol·cm^{-2}·s^{-1}. Thus, the HCO_3^--dependent Na^+ influx has an absolute requirement for internal Cl^-.

NA$^+$ INFLUX: DEPENDENCE ON PH$_i$ Because the rate of acid extrusion is inversely related to pH_i, we would expect the HCO_3^--dependent Na^+ influx to be inhibited at relatively high (i.e., normal) pH_i values. This hypothesis was

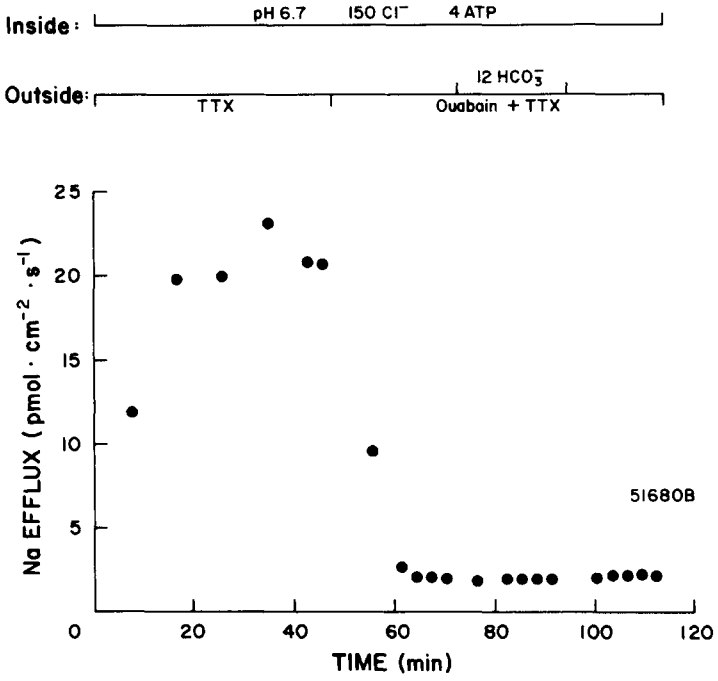

FIGURE 10. Na^+ efflux. The axon was dialyzed with a solution at pH 6.7, containing 150 mM Cl^-, 50 mM Na^+, and 4 mM ATP. In the continuous presence of 10^{-7} M TTX, Na^+ efflux rose and leveled off as the isotope came into equilibrium in the axoplasm. The subsequent addition of 10^{-5} M ouabain to the SSW caused a large fall in Na^+ efflux. There was no change when acid extrusion was stimulated by the application of 12 mM HCO_3^-.

tested in five axons dialyzed with a fluid of pH 7.3, containing 4 mM ATP and 150 mM Cl^-. When 12 mM HCO_3^- was applied, influx changed by an average of −0.1 ± 0.1 pmol·cm^{-2}·s^{-1}. Thus, the appearance of the HCO_3^--dependent Na^+ influx requires that pH_i be lower than the physiological value.

[3] Experiments with Cl^--selective liquid ion-exchanger microelectrodes confirmed that such a pretreatment reduced $[Cl^-]_i$ to <5 mM. In view of the high K_m of the acid extrusion process for intracellular Cl^- (i.e., ~84 mM), such a pretreatment seems adequate for testing the dependence of Na^+ influx on $[CL^-]_i$.

NA^+ INFLUX: DEPENDENCE ON ATP Previous experiments had demonstrated the ATP requirement of acid extrusion in squid axons (Russell and Boron, 1976). To test the ATP dependence of the HCO_3^--stimulated Na^+ influx, we depleted axons of their ATP by (*a*) continuously exposing their entire surface (i.e., the cannulated end-regions as well as the central dialyzed portion) to SSW containing 2 mM cyanide, beginning at the time of cannulation, and (*b*) dialyzing with an ATP-free fluid which also contained 2 mM cyanide. Previous studies had shown that ~70 min of such dialysis is sufficient to block the axon's ATP-dependent Na-Cl uptake system (Russell, 1979). In the present experiments, the axons were dialyzed with the aforementioned DF, which also contained 150 mM Cl^- and was titrated to pH 6.7 for 75 min

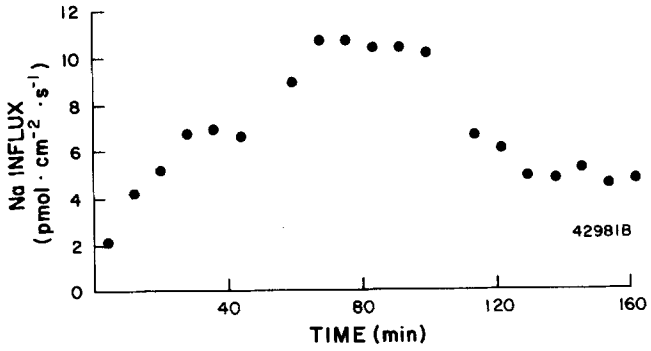

FIGURE 11. The effect of HCO_3^- on Na^+ influx. The axon was dialyzed with a fluid containing 150 mM Cl^- and 4 mM ATP at pH 6.7. In addition, it was exposed to 10^{-7} M TTX and 10^{-5} M ouabain in the SSW. Addition of 12 mM HCO_3^- to the SSW (holding pH_o constant at 8.0) caused the Na^+ influx to rise by ~4 pmol·cm^{-2}·s^{-1}, whereas removal of the HCO_3^- had the opposite effect.

before the influx measurements were begun. In a total of four axons depleted of ATP, the average increase of Na^+ influx upon exposure to 12 HCO_3^-/SSW was 0.1 ± 0.4 pmol·cm^{-2}·s^{-1}. Thus, ATP is required for the HCO_3^--dependent Na^+ influx.

NA^+ INFLUX: EFFECT OF PHARMACOLOGIC AGENTS Seven axons were pretreated with 0.5 mM SITS for 45–60 min before being dialyzed with a pH 6.7 DF containing 4 mM ATP and 150 mM Cl^-. When 12 mM HCO_3^- was applied, the Na^+ influx increased by an average of only 0.1 ± 0.2 pmol·cm^{-2}·s^{-1}. Thus, SITS blocks the HCO_3^--dependent Na^+ influx.

STOICHIOMETRY As noted above, Na^+ influx increased by an average of 3.4 ± 0.4 pmol·cm^{-2}·s^{-1} in 13 axons stimulated by the external application

of 12 mM HCO_3^- (see Table II). Inasmuch as no stimulation of Na^+ *efflux* occurred under identical conditions, this increased, unidirectional influx represents a net influx. Furthermore, this extra Na^+ influx shares all the properties of net acid extrusion: dependence on HCO_3^-, ATP, internal Cl^-, a low pH_i, as well as inhibition by SITS. We therefore conclude that this component of Na^+ influx is directly coupled to the 7.5 ± 0.6 pmol·cm^{-2}·s^{-1} of net acid extrusion measured under identical conditions. The stoichiometry is thus 2.2 equivalents of acid extruded for each equivalent of Na^+ taken up, very near the 2:1 stoichiometry predicted from the models of Fig. 1.

Cl^- Fluxes

The models of Fig. 1 predict that a net efflux of Cl^- ought to accompany acid extrusion. In the following experiments, unidirectional Cl^- influxes and efflux were measured using ^{36}Cl under conditions identical to those used in the Na^+ flux and net acid extrusion stoichiometric studies.

CL$^-$ INFLUX In five axons dialyzed with a pH 6.7 fluid containing 4 mM ATP and 150 mM Cl^-, application of 12 mM HCO_3^- caused the ^{36}Cl influx to rise by 0.1 ± 0.2 pmol·cm^{-2}·s^{-1}. Thus, stimulation of acid extrusion produces no significant change in the unidirectional Cl^- influx, under the conditions of these experiments. The *net* Cl^- flux produced by the transporter can therefore be taken as the unidirectional Cl^- efflux.

CL$^-$ EFFLUX: DEPENDENCE ON EXTERNAL HCO_3^- We have previously shown that application of external HCO_3^- stimulates Cl^- efflux in squid axons, provided the pH_i is relatively low (Russell and Boron, 1976). We have confirmed this finding in the present study. In 17 axons incubated under conditions identical to those used in the above Cl^- influx study, treatment with 12 mM HCO_3^- caused the Cl^- efflux to rise by an average of 3.9 ± 0.3 pmol·cm^{-2}·s^{-1}.

CL$^-$ EFFLUX: DEPENDENCE UPON EXTERNAL NA^+ The models of Fig. 1 predict that the Cl^- efflux linked to acid extrusion ought to require extracellular Na^+. This was tested in five axons which were continuously bathed in Na^+-free SSW (choline replacing Na^+) while being dialyzed with a pH 6.7 fluid containing 4 mM ATP and 150 mM Cl^-. When 12 mM HCO_3^- was added to the external fluid, the Cl^- efflux increased by an average of only 0.4 ± 0.2 pmol·cm^{-2}·s^{-1}. Thus, the HCO_3^--dependent Cl^- efflux requires extracellular Na^+.

CL$^-$ EFFLUX: DEPENDENCE ON PH_i. Because both acid extrusion and the HCO_3^--stimulated Na^+ influx are inversely related to pH_i, we examined the pH_i dependence of the HCO_3^--stimulated Cl^- efflux. Fig. 12 illustrates an experiment in which an axon was initially dialyzed with a fluid containing 4 mM ATP and 150 mM Cl^-, and titrated to pH 7.3. When 12 mM HCO_3^- was applied, Cl^- efflux failed to increase. However, after lowering the pH of the DF to 6.7, the addition of 12 mM HCO_3^- to the SSW increased Cl^- efflux by ~4 pmol·cm^{-2}·s^{-1}. Thus, HCO_3^--dependent Cl^- efflux is inversely related to pH_i.

CL$^-$ EFFLUX: DEPENDENCE ON ATP We have previously demonstrated that

in the absence of ATP, exposure to HCO_3^--containing external fluid has no effect on the Cl^- efflux from axons dialyzed with an acid DF containing 150 mM Cl^- (Russell and Boron, 1976).

CL⁻ EFFLUX: EFFECT OF PHARMACOLOGIC AGENTS In an earlier study (Russell and Boron, 1976), we reported that pretreatment with 0.5 mM SITS inhibits the HCO_3^--dependent Cl^- efflux. We have now confirmed this observation in six axons pretreated with 0.5 mM SITS, for which the average increase of Cl^- efflux caused by 12 mM HCO_3^- SSW was only 0.1 ± 0.1 pmol·cm^{-2}·s^{-1}. Fig. 13 illustrates an experiment demonstrating that 50 μM DIDS is also an effective inhibitor of the HCO_3^--dependent Cl^- efflux.

STOICHIOMETRY As noted above, when stimulated by the application of 12 mM external HCO_3^-, the Cl^- efflux rose by 3.9 pmol·cm^{-2}·s^{-1}. This increased, unidirectional Cl^- efflux represents a *net* efflux, because the HCO_3^--

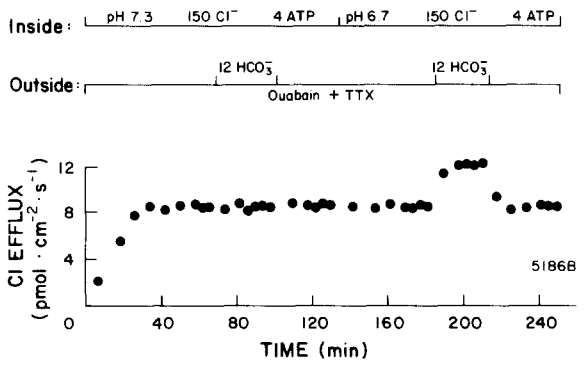

FIGURE 12. Low-pH_i dependence of the HCO_3^--stimulated Cl^- efflux. In the first part of the experiment, the axon was dialyzed with a pH 7.3 solution containg 150 mM Cl^- and 4 mM ATP. The SSW contained ouabain (10^{-5} M) and TTX (10^{-7} M) throughout. At a pH_i of 7.3, application of 12 mM HCO_3^- had no effect on Cl^- efflux. After dialysis with a solution identical to the first, but titrated to pH 6.7, HCO_3^- application stimulated Cl^- efflux by ~4 pmol·cm^{-2}·s^{-1}.

stimulated Cl^- influx was zero. Inasmuch as this efflux shares the same properties as acid extrusion and the net Na^+ influx (i.e., dependence on HCO_3^-, external Na^+, ATP, and a low pH_i, as well as inhibition by SITS and DIDS), we conclude that it is directly coupled to acid extrusion. The ratio of acid extruded (i.e., 7.5 pmol·cm^{-2}·s^{-1}) to Cl^- extruded (i.e., 3.9 pmol·cm^{-2}·s^{-1}) is 1.9, reasonably close to that predicted by the models of Fig. 1, 2:1.

DISCUSSION

pH_i-regulating System of the Squid Axon

The results of the present study, as well as earlier work, demonstrate that acid extrusion by the squid axon (*a*) has an absolute requirement for external Na^+, external HCO_3^-, and for internal Cl^-; (*b*) is stimulated at low values of pH_i;

(*c*) requires internal ATP; and (*d*) is inhibited by the stilbene derivatives. In addition, we have shown that all of the above properties are shared by a net Na^+ influx and a net Cl^- efflux. These data indicate that the process of acid extrusion involves the obligatory net transport of Na^+, Cl^-, and HCO_3^- (or an equivalent species). Furthermore, the relationship among the acid extrusion rate, the net Na^+ influx, and the net Cl^- efflux indicate that the stoichiometry of the transport system is one equivalent of Na^+ entering the cell for each equivalent of Cl^- leaving the cell and for each two equivalents of acid neutralized intracellularly.

These results support all models of Fig. 1. To distinguish among the four, one must examine kinetic data. The results of Figs. 4 and 6, which show the

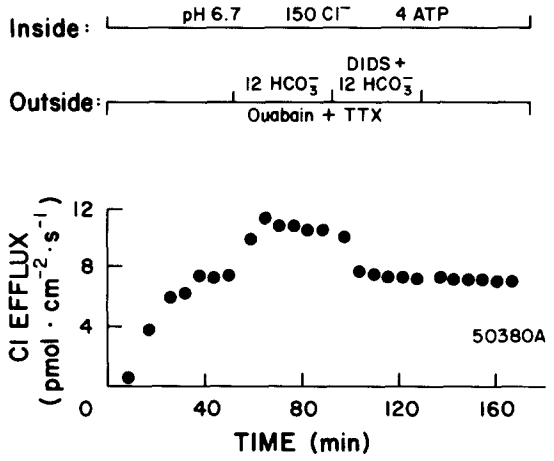

FIGURE 13. DIDS sensitivity of the HCO_3^--stimulated Cl^- efflux. The axon was dialyzed with a pH 6.7 solution containing 150 mM Cl^- and 4 mM ATP. The SSW contained ouabain (10^{-5} M) and TTX (10^{-7} M) throughout. The addition of 12 mM HCO_3^- to the SSW produced an increase in Cl^- efflux which amounted to ~4 pmol·cm^{-2}·s^{-1}. However, when DIDS (50 μM) was added to the SSW, in the continued presence of HCO_3^-, the Cl^- efflux returned to the value prevailing before the addition of HCO_3^-.

dependence of acid extrusion rate on $[HCO_3^-]_o$ and $[Na^+]_o$, respectively, are sufficient to test one of the predictions of model 4. When these data are replotted (Fig. 14) as a function of $[NaCO_3^-]_o$ (calculated from the stability-constant data of Garrels et al., 1961) both sets fall on a single Michaelis-Menten curve, with an apparent K_m for $NaCO_3^-$ of 74 ± 3 μM, and an apparent V_{max} of 10.6 ± 0.2 pmol·cm^{-2}·s^{-1}. Although model 4 predicts that the two sets of data indeed should fall on the same curve, our confirmation of this prediction by no means proves the model. A stronger case could be made only if the model were supported by additional kinetic data, such as an examination of the $[Na^+]_o$ dependence at various values of $[HCO_3^-]_o$ and pH_o, or an examination of the $[HCO_3^-]_o$ dependence at various values of $[Na^+]_o$ and pH_o. In this regard, it is of interest to note that when barnacle-muscle

data analogous to our squid data of Figs. 4 and 6 are replotted as a function of $[NaCO_3^-]_o$, they, too, fall on a single Michaelis-Menten curve (Boron et al., 1981). However, when the $[Na^+]_o$ dependence at pH_o 8.0 was examined at two values of $[HCO_3^-]_o$, the data, when replotted as a function of $[NaCO_3^-]_o$, fell on two quite different Michaelis-Menten curves. Thus, model 4 has been ruled out for barnacle muscle. Further kinetic studies clearly are required to test the squid-axon models adequately.

Comparison with Other Systems Transporting H^+ and/or HCO_3^-

In this paper, we describe a pH_i-regulating transport system that tightly couples the movement of Na^+, Cl^-, HCO_3^- (or an equivalent species), and possibly H^+. A similarly tight coupling of these ions appears to exist for the pH_i-regulating systems of both barnacle muscle (Roos and Boron, 1982) and the snail neuron (Thomas, 1982). However, the data on the interdependencies

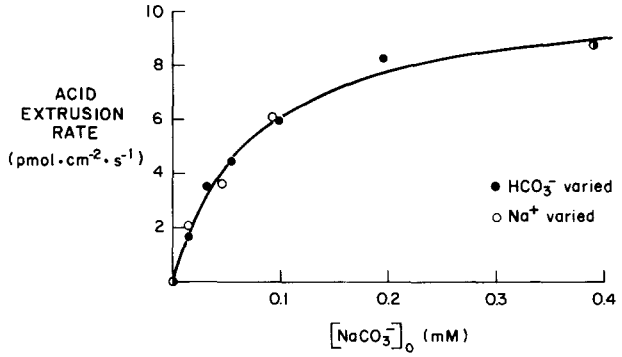

FIGURE 14. Replot of acid-extrusion-rate data as a function of $[NaCO_3^-]_o$. The data of Fig. 4 ($[HCO_3^-]_o$ varied; closed circles) and Fig. 6 ($[Na^+]_o$ varied; open circles) were replotted as a function of the $[NaCO_3^-]_o$ calculated from the data of Garrels et al. (1961). Note that both sets of data are fitted by the same Michaelis-Menten curve; $K_m = 74 \pm 3$ μM, $V_{max} = 10.6 \pm 0.2$ pmol·cm^{-2}·s^{-1}.

of the net Na^+ and Cl^- fluxes in the last two preparations are not as complete as for the squid axon. In experiments with ion-sensitive electrodes on snail neurons, Thomas (1977) found that pH_i recovery from an acid load is accompanied by an increase of the intracellular Na^+ activity and a decrease of the intracellular Cl^- activity. It could be objected that the activity changes, measured with microelectrodes, were in fact not representative of net Na^+ and Cl^- fluxes tightly coupled to acid extrusion, but rather, of cell volume changes. However, it is not clear how a simple volume change could have produced both an increase in Na^+ activity and a decrease in Cl^- activity. Furthermore, the present results indicate that even if volume changes did take place, they did not obscure the fundamental observation that net Na^+ and Cl^- fluxes do occur during acid extrusion.

From their ionic requirements and the apparent interdependencies of the ion fluxes, it appears that the pH_i-regulating systems of squid axons, snail

neurons, and barnacle muscle are very similar. However, we can identify two subtle differences. In the first place, whereas ATP is required for the squid system, the snail system is unaffected by the metabolic inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone, applied alone or in combination with intracellular injections of orthovanadate (Thomas, 1982). In barnacle muscle, the ATP dependence of acid extrusion has yet to be examined. Second, we have been unable to identify in the squid axon either a unidirectional Na^+ efflux or a unidirectional Cl^- influx associated with acid extrusion. In contrast, acid extrusion in barnacle muscle is accompanied by a significant Na^+ efflux[4] and Cl^- influx (Boron et al., 1978), which is consistent with the hypothesis that the barnacle's pH_i-regulating system also mediates an apparent Na-Na and Cl-Cl exchange.

Inasmuch as the pH_i-regulating mechanism of squid, snail, and barnacle superficially resembles other transport systems currently being studied, it is useful to distinguish among them.

PURPORTED PH_i-REGULATING SYSTEMS A pH_i-regulating system which has a requirement for Na^+ is the amiloride-sensitive Na-H exchanger, which has been identified in a number of preparations (see Roos and Boron, 1981). Unlike the pH_i-regulating system of the squid axon, however, Na-H exchange is unaffected by application of SITS or by removal of Cl^- (Boron and Boulpaep, 1982).

A Cl-HCO_3 exchange has been identified in sheep cardiac Purkinje fibers (Vaughan-Jones, 1979) and has been postulated for mouse soleus muscle (Aickin and Thomas, 1977). The Purkinje fiber's transporter requires both HCO_3^- and Cl^- and is blocked by SITS. Unlike the squid axon's pH_i-regulating system, however, the Cl-HCO_3 exchanger is independent of Na^+ (Vaughan-Jones, 1982), is apparently not inactivated at high pH_i (Vaughan-Jones, 1982), and probably mediates net HCO_3^- *efflux* under normal conditions.

In mouse soleus muscle (Aickin and Thomas, 1977), recovery of pH_i from an acid load is apparently mediated by both Na-H exchange and Cl-HCO_3 exchange, the former accounting for about two-thirds of the cell's acid-extruding capacity. Thus, acid extrusion is inhibited by amiloride, which acts on the Na-H exchanger, and is only partially blocked by application of SITS or by removal of HCO_3^-, which affect the presumed Cl-HCO_3 exchanger. If such a parallel arrangement of Na-H and Cl-HCO_3 exchangers existed for squid axons, then we would not have observed the absolute requirement of acid extrusion for HCO_3^-, Na^+, and Cl^-, nor the total blockade by application of SITS.

Recently (Boron and Boulpaep, 1982), a transport system has been identified in the basolateral membrane of salamander proximal tubule cells, in which the movements of Na^+ and HCO_3^- (or an equivalent species) are tightly coupled. Although this transporter is blocked by SITS, it is apparently independent of Cl^-. Furthermore, it moves net negative charge in the same

[4] Russell, J. M., N. F. Boron, and M. S. Brodwick. Intracellular pH and Na fluxes: evidence for reversibility of the pH_i-regulating mechanism. Manuscript submitted for publication.

direction as Na^+ and HCO_3^-, and normally mediates the net *efflux* of HCO_3^- (or an equivalent species).

PURPORTED VOLUME-REGULATORY SYSTEMS When *Amphiuma* erythrocytes are shrunken in a hypertonic solution, their volume spontaneously recovers in a HCO_3^--dependent process involving the net uptake of Na^+ and Cl^- (Cala, 1980). It has been suggested that this regulatory volume increase is mediated by an amiloride-sensitive Na-H exchanger (Siebens and Kregenow, 1978, 1980; Cala, 1980) in parallel with a SITS-sensitive Cl-HCO_3 exchanger (Cala, 1980). The latter may be identical to the band III anion exchanger of erythrocytes. If the Na-H and Cl-HCO_3 exchange rates are fortuitously identical, then the net effect would be the isohydric uptake of NaCl. In view of (*a*) the lack of obligatory coupling observed between Na^+ and Cl^-, and (*b*) the observation that there is a net Cl^- influx rather than a net efflux, this system also appears to be distinct from that of the squid axon.

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