J. Physiol. (1986), **381**, *pp.* 241–261
With 12 *text-figures*
Printed in Great Britain

REGULATION OF INTRACELLULAR pH IN RETICULOSPINAL NEURONES OF THE LAMPREY, *PETROMYZON MARINUS*

BY MITCHELL CHESLER

From the Department of Physiology and Biophysics, New York University Medical Center, 550 First Avenue, New York, NY 10016, U.S.A.

(*Received 7 November 1985*)

SUMMARY

1. The regulation of intracellular pH (pH_i) in lamprey reticulospinal neurones was investigated with pH-sensitive micro-electrodes based on a neutral carrier liquid membrane. Experiments were performed using an *in vitro* brain-stem preparation.

2. In HEPES-buffered solutions, extracellular pH (pH_o) was consistently more acidic than the pH of the bathing solution (pH_b). In HCO_3^--buffered solutions, the brain was also relatively acidic, but the brain pH gradient was smaller.

3. In HEPES- and HCO_3^--buffered solutions, mean pH_i was 7·40–7·50. This range was too high to be explained by a passive distribution of H^+, OH^- or HCO_3^-.

4. In nominally HCO_3^--free, HEPES-buffered solution, cells were acid loaded by addition and subsequent withdrawal of NH_4^+ from the superfusate. pH_i recovered from acid loading by an energy-dependent process in 10–20 min. Recovery from acid loading in HEPES-buffered solutions was blocked by exposure to amiloride.

5. Removal of extracellular Na^+ caused a slow, accelerating fall of pH_i. Return of Na^+ to the bath caused an immediate reversal of this acidification, followed by a slow recovery of pH_i. Measurement with Na^+-sensitive micro-electrodes during acid loading showed a rapid rise in the intracellular Na^+ activity ($[Na^+]_i$).

6. Following acid loading, transition from HEPES- to HCO_3^--buffered solutions caused an increase in the acid extrusion rate of at least 48%. The effect of these solution changes was dependent on pH_o. After blocking pH_i recovery with amiloride, transition from HEPES- to HCO_3^--buffered Ringer plus amiloride produced a slow recovery of pH_i.

7. Recovery from acid loading in HCO_3^--buffered solutions was inhibited 65% by the anion transport blocker DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid). Recovery from acid loading after incubation in Cl^--free solution was slower than recovery after replenishment of Cl^-.

8. It is concluded that in HCO_3^--free solutions, pH_i regulation is accomplished by a Na–H exchange mechanism. In the presence of extracellular HCO_3^- an additional mechanism can operate to extrude intracellular acid.

INTRODUCTION

The regulation of intracellular pH (pH_i) has been studied extensively in recent years. Among nerve cells, investigations have been limited to invertebrate species. In snail neurones (Thomas, 1977), squid axon (Boron & Russel, 1983) and crayfish

stretch receptor (Moser, 1985), the net extrusion of acid is accomplished by a single Na^+-, HCO_3^-- and Cl^--dependent transport system. In central neurones of the crayfish (Moody, 1981) and leech (Schlue & Thomas, 1985), two mechanisms of acid transport are found; here cells utilize Na–H exchange in addition to a HCO_3^--dependent mechanism.

A dual mechanism of pH_i regulation using Na–H exchange and HCO_3^--dependent transport was first described in mammalian striated muscle (Aickin & Thomas, 1977*a*). Subsequent studies demonstrated that two mechanisms were not a universal feature of vertebrate cells. In mammalian cardiac muscle (Vaughan-Jones, 1982; Vanheel, De Hemptinne & Leusen, 1984) and salamander kidney cells (Boron & Boulpaep, 1983*a*), the net extrusion of acid is mediated strictly by Na–H exchange.

The study of pH_i regulation has yet to be extended to vertebrate central neurones. A report of amiloride-sensitive Na^+ uptake in cultured neuroblastoma cells (Moolenaar, Boonstra, van der Saag & de Laat, 1981) suggested that a Na–H exchange mechanism may be utilized. However, because of the small size of cells, direct, continuous monitoring of pH_i has not been performed. Among C.N.S. neurones, the reticulospinal cells of the lamprey are a notable exception. The large cell bodies of these nerve cells have been the subject of numerous electrophysiological studies utilizing the entire *in vitro* brain stem (Rovainen, 1979). I have used this preparation to study pH_i regulation for the first time in vertebrate central neurones. My results indicate that in HCO_3^-- free solutions, pH_i is regulated by a Na–H exchanger. Evidence is presented for an additional mechanism of acid extrusion which operates in HCO_3^--containing media. Portions of this work have appeared in preliminary form (Chesler & Nicholson, 1984; Chesler & Nicholson, 1985).

METHODS

General. Larvae of *Petromyzon marinus* (9–15 cm length) were captured from the Delaware and Connecticut River systems and maintained at 5 °C in aerated aquaria. Animals were anaesthetized by immersion in 0·2 % tricaine methanesulphonate (Sigma). The brain and 1 cm of spinal cord were dissected under ice-cold HCO_3^--buffered Ringer, pinned dorsal side up in a chamber, then superfused with room temperature (23 °C) HCO_3^--Ringer. The choroidal tissue overlying the ventricles was removed, the isthmic tectum split, and the brain stem pinned flat. The reticulospinal neurones were trans-illuminated and impaled under visual control at 40 × magnification. The large (50–100 μm) Muller and Mauthner neurones of the bulbar reticulospinal group (Rovainen, 1979) were used exclusively.

Superfusion solutions. Normal HCO_3^- Ringer was equilibrated with 5 % CO_2 in O_2 and had the following composition (mM): NaCl, 90; KCl, 2 or 5; $CaCl_2$, 2·6; $MgCl_2$, 1·8; glucose, 4; $NaHCO_3$, 23 (pH = 7·35). HEPES-buffered solutions (HCO_3^--free) were bubbled with 100 % O_2 for 15 min, then sealed in glass bottles. These solutions were made more alkaline than HCO_3^- Ringer in order to control extracellular pH (see Results). In 5 mM-HEPES Ringer, NaCl replaced $NaHCO_3$ and the solution was titrated with NaOH (pH = 7·40–7·50 or 7·80). In 23 mM-HEPES Ringer (pH = 7·40 or 7·60–7·70), NaCl was reduced to compensate for the addition of NaOH. In NH_4^+-containing solutions, NH_4Cl replaced NaCl. Na^+-free Ringer contained choline or bis(2-hydroxyethyl)dimethyl ammonium (BDA^+, Kodak) as Na^+ substitutes. HEPES-buffered, Na^+-free solutions contained 5 mM-HEPES and were titrated with KOH, requiring omission of 2 mM-KCl to maintain K^+ approximately constant. Cl^--free Ringer contained isethionate or methanesulphonate as the main Cl^- substitutes, as well as (mM): K_2SO_4, 1; Ca^{2+} gluconate, 2·6; and $MgSO_4$, 1·8. As these solutions were not compensated for Ca^{2+} binding, the Ca^{2+} activity of isethionate Ringer was estimated to be one-third less than normal Ringer (Christofferson & Skibsted, 1975) and that of methanesulphonate Ringer decreased by 14 % (Kenyon & Gibbons, 1977). Solutions containing amiloride

(Merck Pharmaceuticals, West Point, PA, U.S.A) were prepared the day of use. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid, Pierce Chemical Co., Rockford, IL, U.S.A.) was added directly within 3 h of use. Solutions were gravity fed to the experimental chamber via CO_2-impermeable Saran tubing (Clarkson Equipment and controls, Detroit, MI, U.S.A.). Fluid volume was maintained at 0·3 ml and exchanged at a rate of 3 chamber volumes per minute.

Ion-sensitive micro-electrodes. H^+ and Na^+-sensitive micro-electrodes were fabricated from 1 mm diameter borosilicate glass (A–M Systems, Everett, WA, U.S.A.). Two pipettes were heated on a vertical micro-electrode puller, twisted 360 deg, then pulled to form double-barrelled micropipettes. The future H^+- or Na^+-sensitive barrels were backfilled with phosphate buffer (pH 7·4) or 150 mM-NaCl respectively. In early work, reference barrels for intracellular electrodes were filled with 1 M-KCl, 3 M-K acetate or 3 M-Li acetate. In later experiments 0·10 M-K acetate was used (see below). Reference barrels of extracellular pH electrodes contained 0·15 M-NaCl. Electrodes were broken to a tip diameter of 0·5–2·0 μm. The remainder of the fabrication procedure has been described (Kraig, Ferreira-Filho & Nicholson, 1983). The neutral carrier H^+ exchanger (Amman, Lanter, Steiner, Schulthess, Shijo & Simon, 1981) was a gift of Professor W. Simon, Swiss Federal Institute of Technology. Later experiments utilized the same solution purchased from Fluka Chemical Corp. (Buchs, Switzerland). The Na^+-exchanger, based on the ligand ETH 227 (Steiner, Oehme, Amman & Simon, 1979) was purchased ready-made from Fluka.

pH micro-electrodes lasted up to 4 days but were typically used on the day they were made. Before each experiment, the response in HEPES- and CO_2-containing Ringer was compared against a glass pH macro-electrode. Neither the electrode slope nor the base-line potential (at constant pH) were sensitive to 5 % CO_2. At the termination of an impalement, a d.c. drift on the pH electrode was usually noted. Experiments were not analysed if the drift exceeded 5 mV. pH calibrations were performed before and after each experiment using 50 mM-K phosphate buffers of pH 6·0, 7·0 and 8·0. Na^+-sensitive electrodes were calibrated in solutions of 5, 10, 20 and 40 mM-NaCl with a constant 100 mM-KCl background. Electrode responses were fitted to the Nikolsky equation (Eisenman, 1961) to determine theoretical slope and interference parameters. Slopes of pH and Na^+ electrodes were not affected by the type of buffer (phosphate, HEPES or HCO_3^-). The mean slope of pH micro-electrodes was 49·1 ± 0·8 mV/pH unit ($n = 65$, ± S.E. of mean), with negligible cationic interference. The slope of Na^+ micro-electrodes ranged between 56 and 58 mV per decade with a selectivity for Na^+ over K^+ of 50–100:1. Intracellular Na^+ activity ($[Na^+]_i$) was calculated with respect to the free concentration of Na^+ in HEPES Ringer.

Reference filling solution. With prolonged impalements, leakage of filling solution into cells was inevitable. Use of 1 M-KCl caused a significant accumulation of Cl^-, judged by a large depolarizing response to superfusion of GABA (see also Gold & Martin, 1982). When 3 M-acetate solutions were used, cells gradually alkalinized. This was presumably due to efflux of acetic acid from the cell. To avoid artifacts which might be caused by leakage of concentrated electrolytes, reference barrels were filled with 0·10 M-K acetate (adjusted with HCl to a pH of 7·1). To determine the magnitude of the junction potential shift with this solution (Caldwell, 1968), reticulospinal neurones were impaled with double-barrelled electrodes (1–2 μm tip size) containing 0·10 M-K acetate in one side and 3 M-Li acetate in the other. Li acetate was used because the limiting conductivity of Li^+ and acetate are well matched (Dean, 1979) and because 3 M-KCl electrodes were not as stable in the tissue. In fifty-three determinations, using four electrodes and two animals, the membrane potential recorded on the K acetate barrel was more positive by 9 ± 2 mV (mean ± S.D.). A 0·10 M-K acetate filling solution was therefore used with a 9 mV junction potential correction. A correction error of two standard deviations (4 mV) would correspond to a base-line pH_i and $[Na^+]_i$ error of roughly 0·07 and 2 mM respectively. The greatest deviation from the mean was 6 mV, which would correspond to a pH error of about 0·10. For the purposes of this investigation, a maximum recording error of this magnitude was acceptable (see Results).

Electronics and data storage. Input stages for monitoring potentials on the ion and reference barrels utilized conventional operational amplifiers wired for unity gain (Analog Devices, 515L and 515J respectively). A 1 M-KCl, 3 % agar bridge fitted with an Ag–AgCl junction served as the bath indifferent electrode. Reference signals were subtracted with a differential instrumentation amplifier (Analog Devices 520), filtered, and recorded simultaneously on a Gould 220 chart recorder and a Nicolet digital oscilloscope. Data was analysed directly from the chart record. The digitized signal was sampled at 5 or 10 s intervals (which accounted for most of the low-pass filtering) and stored on floppy disk. Figures were prepared on a conventional X–Y plotter after analog conversion of stored data. In some cases, the trace was photographed directly from the chart record.

Acid loading. In HEPES-buffered Ringer, cells were acid loaded using the NH_4^+ pre-pulse technique. This method has been described in detail (Boron & DeWeer, 1976). In brief, the preparation was exposed to 10 mM-NH_4^+ for 5–10 min. A rapid intracellular alkalinization resulted as NH_3 rapidly permeated cells. This was followed by a slower acidification, attributed to the permeation of NH_4^+ (Boron & DeWeer, 1976). Wash-out of NH_4^+ from the bath produced a rapid acidification. This was due to the sudden efflux of NH_3, which by mass action, caused the dissociation of NH_4^+ to NH_3 and H^+. The NH_3 so generated readily left the cell; however, the less permeant H^+ was trapped, thereby acidifying the cytoplasm. Cells were also acid loaded by transition from HEPES to HCO_3^- Ringer. In this case, intracellular acidification was due to the rapid permeation of CO_2 and its hydration to form carbonic acid (H_2CO_3) which dissociates into HCO_3^- and H^+ (Jacobs, 1940; Thomas, 1976).

Data analysis. Records of pH_i recovery were fitted to a single exponential using an iterative least-squares algorithm. The intrinsic buffering power of cells (β_i) was estimated by two methods. One set of experiments was carried out in the presence of 1 mM-amiloride in order to block recovery from acid loading (see Results). β_i was calculated based on the decrease of pH_i following wash-out of 10 mM-NH_4^+ from 5 mM-HEPES Ringer. It was assumed that the entire accumulation of intracellular NH_4^+ left the cell as NH_3. Intracellular and extracellular NH_3 concentration were considered equal. β_i was calculated as $-[NH_4^+]_i / \Delta pH_i$, where ΔpH_i is the decrease in pH_i following withdrawal of NH_4^+ and $[NH_4^+]_i$ is the concentration of intracellular NH_4^+ calculated at the time NH_4^+ was withdrawn. An NH_4^+ pK_a of 9·30 (Bates & Pinching, 1950) and a range of extracellular pH in 5 mM-HEPES Ringer (see Results) were used for these calculations. Another set of experiments was performed without amiloride, allowing pH_i to recover from acid loading. To correct for the contribution of acid transport, ΔpH_i was estimated by extrapolating the exponential pH_i recovery to the time NH_4^+ was withdrawn (Aickin & Thomas, 1977*b*).

The rate of net acid extrusion was calculated as $(dpH_i/dt) \times (\beta_i + \beta_b)$, where β_i is the 'intrinsic' or non-HCO_3^--derived buffering power and β_b is the HCO_3^--derived buffering power. A β_i of 16 or 20 mM was used (see Results). Rates were measured by tracing expanded records onto graph paper. Intracellular HCO_3^- concentration ($[HCO_3^-]_i$) was assumed to be zero in HEPES-buffered Ringer. β_b was calculated as $2{\cdot}3 \times [HCO_3^-]_i$ and $[HCO_3^-]_i$ was calculated using the Henderson–Hasselbach equation. Intracellular and extracellular CO_2 tension were considered equal. A CO_2 solubility coefficient of 0·03605 mM/mmHg (Harned & Bonner, 1945) and an apparent first pK_a of carbonic acid of 6·11 (Harned & Davis, 1943) were used.

RESULTS

Extracellular pH

In a number of studies, extracellular pH (pH_o) has been shown to influence pH_i regulatory mechanisms (Boron, McCormick & Roos, 1979; Moody, 1981; Boron & Boulpaep, 1983*a*). Since pH_o may be less than the pH of the bathing solution (Kraig *et al.* 1983; De Hemptinne & Huguenin, 1984) and may vary with the effective buffering power of the Ringer (De Hemptinne & Vanheel, 1984), pH_o was studied in HEPES- and HCO_3^--buffered Ringer. Measurements were made at a tissue depth of 100 μm approximately 100 μm caudal to the large Mauthner neurones (Fig. 1*A*).

In HEPES-buffered Ringer, pH_o was always less than the pH of the bath (pH_b). Fig. 1*B* shows a recording as the electrode was rapidly advanced into the tissue, and then withdrawn. When the electrode was lowered slowly, the greatest fall in pH was recorded within a few hundred micrometres of fluid over the surface (not shown), whereas the pH gradient within the first 100 μm of tissue was relatively small ($< 0{\cdot}10$).

At a given pH_b and concentration of HEPES, pH_o fell within predictable limits. In solutions buffered with 5 mM-HEPES ($pH_b = 7{\cdot}40$–$7{\cdot}50$), the mean pH_o was $7{\cdot}08 \pm 0{\cdot}02$ ($n = 13$ animals, range = 6·90–7·16, all data are $\pm$S.E. of mean). Increasing

pH_b to 7·80 yielded a pH_o between 7·30 and 7·40. In 23 mM-HEPES Ringer (pH_b = 7·60–7·70), the mean pH_o was 7·33 ± 0·02 (n = 10 animals, range 7·22–7·40). In 23 mM-HCO_3^- Ringer (pH_b = 7·35) pH_o ranged between 7·30 and 7·35. Transitions between various Ringer solutions produced shifts in pH_o in accordance with these differences (see below).

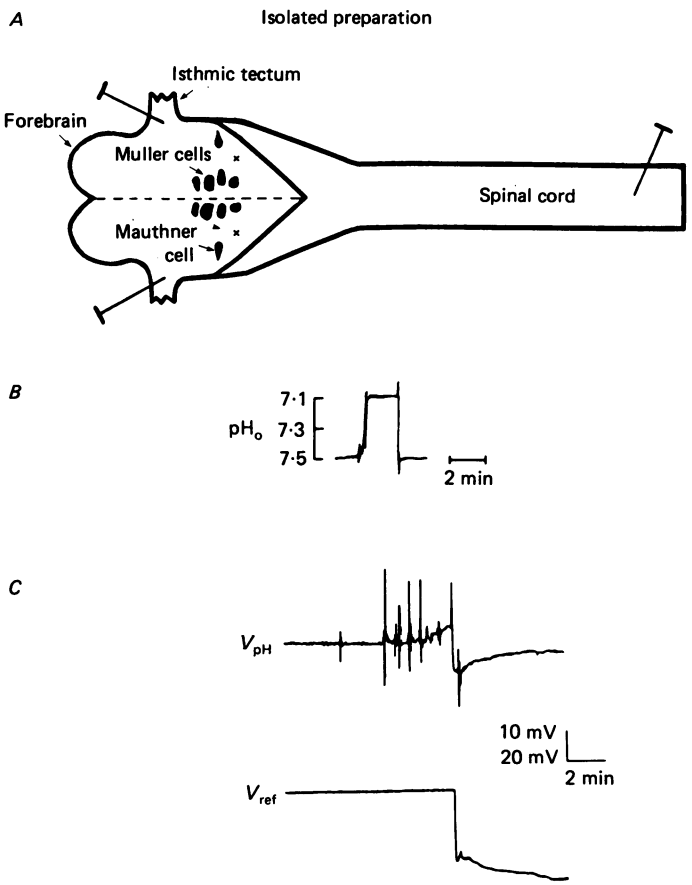

Fig. 1. Schematic diagram of lamprey brain preparation and recording of extracellular and intracellular pH. *A*, location of reticulospinal neurones is shown on the floor of the fourth ventricle. 'X' denotes site of extracellular pH recording, *B*, a step decrease in pH was recorded as the electrode was suddenly lowered from the bath (5 mM-HEPES Ringer, pH_b = 7·50) to the tissue. Note the speed of the electrode response. *C*, recording of a cell penetration. V_{ref} is the potential on the reference barrel and V_{pH} is the potential recorded on the pH-sensitive barrel after subtraction of V_{ref}. Deviations in the acid direction are displayed as upward deflexions.

Regulation of pH_i in HCO_3^--free solutions

The preparation was superfused with HCO_3^--free (HEPES-buffered) Ringer for at least 30 min prior to cell impalement. A recording of a cell penetration is shown in Fig. 1*C*. Soon after impalement an 'injury acidification' often occurred, followed by stabilization or a slow recovery of pH_i. In 5 mM-HEPES Ringer (pH_b = 7·40–7·50)

the mean initial pH_i (within 2 min of impalement) was $7{\cdot}43 \pm 0{\cdot}03$ with a membrane potential of -56 ± 1 mV ($n = 31$). In 23 mM-HEPES Ringer ($pH_b = 7{\cdot}60$–$7{\cdot}70$), initial pH_i was $7{\cdot}47 \pm 0{\cdot}05$ with a membrane potential of -58 ± 2 mV ($n = 12$). Since reference barrels were filled with 0·10 M-K acetate, these determinations employed

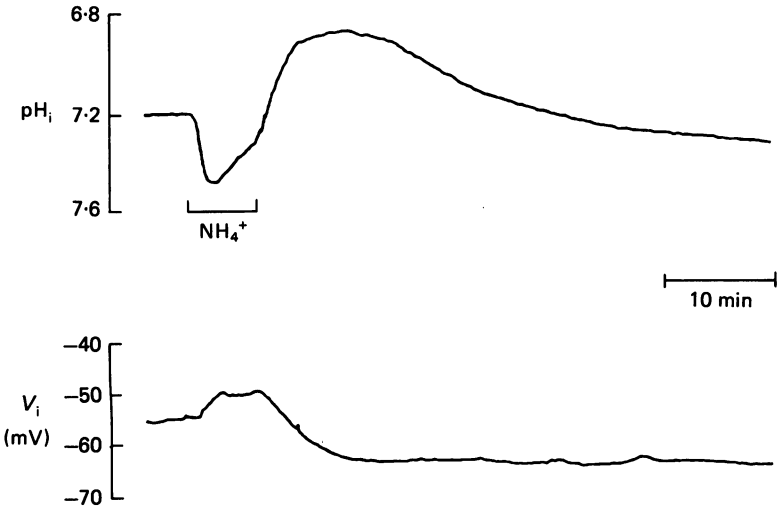

Fig. 2. Recovery from acid loading in HCO_3^--free solution. The cell was acid loaded with a 6 min 10 mM-NH_4Cl pre-pulse in 23 mM-HEPES-buffered Ringer ($pH_b = 7{\cdot}65$).

a 9 mV junction potential correction. In five measurements using 3 M-K or Li acetate and no junction potential correction, the mean initial pH_i was $7{\cdot}41 \pm 0{\cdot}03$ with a membrane potential of 56 ± 2 mV (5 mM-HEPES, $pH_b = 7{\cdot}40$–$7{\cdot}50$).

Assuming a mean pH_o of 7·08 or 7·33, a passive H^+ distribution would predict a pH_i of about 6·2 and 6·5 for 5 and 23 mM-HEPES Ringer, respectively. Intracellular H^+ was therefore maintained against an inward driving potential of at least 70 mV. To study the mechanism of pH_i regulation in HCO_3^--free media, cells were acid loaded with an NH_4^+ pre-pulse (see Methods). As shown in Fig. 2, exposure to HEPES Ringer containing 10 mM-NH_4^+ resulted in a rapid alkalinization followed by a slower acidification. This was usually accompanied by a 5–10 mV depolarization. In squid axon, the slow acidification and depolarizing effect of NH_4^+ have been attributed to permeation of NH_4^+ through K^+ channels (Boron & DeWeer, 1976). Upon wash-out of NH_4^+, the cell acidified rapidly to a pH_i of about 6·9, then recovered with a roughly exponential time course. In 5 mM-HEPES Ringer the mean time constant of recovery was 15 ± 1 min ($n = 5$). In 23 mM-HEPES Ringer (in which pH_o was greater), recovery was somewhat faster (Fig. 2). Acid loading was often associated with a hyperpolarization of 5–10 mV which persisted after pH_i had recovered.

During the course of acid loading and recovery, the electrochemical gradient for H^+ was inward. In the experiment of Fig. 2, passive efflux of H^+ (or influx of OH^-) would have required acidification to a pH_i of 6·5 or less. The recovery of pH_i must therefore be attributed to an energy-dependent process.

The effect of amiloride on pH_i recovery

In vertebrate cells, HCO_3^--independent acid extrusion is mediated by an amiloride-sensitive, net exchange of external Na^+ for intracellular H^+ (Aickin & Thomas, 1977*a*; Deitmer & Ellis, 1980; Boron & Boulpaep, 1983*a*; Moolenaar, Tertoolen & De Laat, 1984; Grinstein, Cohen & Rothstein, 1984). The sensitivity of reticulospinal neurones to amiloride was tested as shown in Fig. 3. An NH_4^+ pre-pulse caused an acidification of 0·34. After 6 min of recovery the cell was exposed to 1 mM-amiloride for 2 min. This immediately halted the recovery and resulted in a slight acidification. The associated depolarization was a common feature of amiloride exposure.

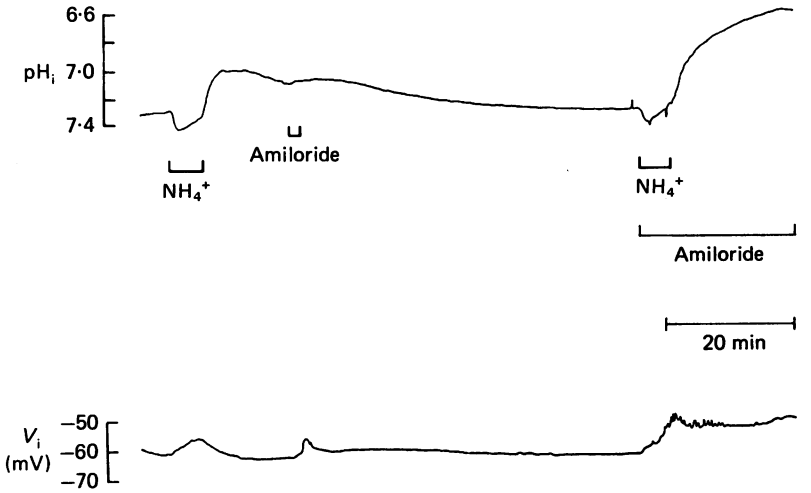

Fig. 3. Effect of amiloride on pH_i recovery. The cell was acid loaded with a 5 min 10 mM-NH_4Cl pre-pulse in 5 mM-HEPES Ringer (pH_b = 7·50). Brief exposure to 1 mM-amiloride caused an immediate, reversible block of recovery. A second NH_4^+ pre-pulse in the presence of 1 mM-amiloride produced a far greater acidification without significant recovery after 16 min. Rapid depolarizing transients on membrane potential record represent filtered synaptic barrages and spikes.

After the drug was washed from the bath, and pH_i was allowed to recover, an NH_4^+ pre-pulse was repeated in the presence of amiloride. This caused an acidification of 0·59, which was 74 % greater than the control. After 16 min there was no significant recovery. At the end of this record, the penetration was suddenly lost.

In eleven experiments, amiloride (1 or 2 mM) blocked the recovery of pH_i in every case (see also Figs. 4 and 10). Usually, a gradual acidification also resulted. This occurred as long as 40 min after an NH_4^+ pre-pulse indicating that these acid sources cannot be attributed to slow wash-out of NH_4^+.

The effect of Na^+-free Ringer

The effect of Na^+ substitution on pH_i is shown in Fig. 4. When Na^+ was completely replaced by choline or BDA^+, the membrane slowly depolarized. This was followed,

at a variable latency, by a slow, accelerating acidification. The mechanism of the depolarization and acid shift was not studied. When Na^+ was returned to the superfusate, these effects rapidly reversed and pH_i and membrane potential slowly recovered. The recovery of pH_i was reversibly inhibited by 1 mM-amiloride; however, in these experiments, membrane potential was unaffected by the drug.

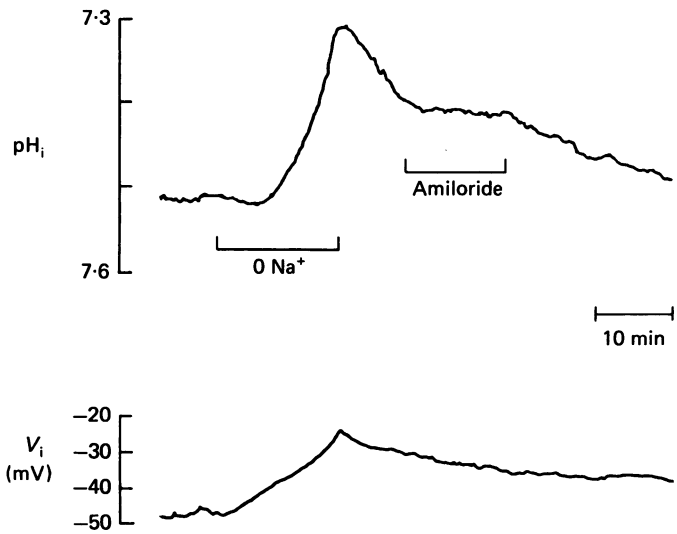

Fig. 4. The effect of Na^+-free solution on pH_i. Replacement of Na^+ with BDA^+ in 5 mM-HEPES Ringer (pH_b = 7·80). A slow accelerating acidification occurred in Na^+-free Ringer, accompanied by a gradual depolarization. Return of Na^+ to the bath caused a rapid reversal of both effects. The recovery of pH_i was reversibly inhibited by 1 mM-amiloride.

The effect of acid loading on intracellular Na^+

Na^+-selective micro-electrodes were used to study the behaviour of $[Na^+]_i$ during acid loading procedures. Base-line $[Na^+]_i$ was estimated to be 15 ± 3 mM ($n = 10$) corresponding to an equilibrium potential for Na^+ of +51 mV. If acid extrusion were mediated by Na–H exchange, activation of acid transport may be expected to cause a rise in $[Na^+]_i$ (Aickin & Thomas, 1977*a*). This was tested by acidifying with an NH_4^+ pre-pulse (Fig. 5). Exposure to NH_4^+ first caused a reduction in $[Na^+]_i$. This is a common response to NH_4^+, first noted in mammalian muscle (Aickin & Thomas, 1977*a*), where it was attributed to transport of NH_4^+ by the Na–K pump. It can be seen that wash-out of NH_4^+ produced an overshooting rise of $[Na^+]_i$. Acid loading with 5% CO_2 resulted in an additional increase. Wash-out of CO_2, which rapidly alkalinizes cells (see below), caused a sudden fall of $[Na^+]_i$. It must be noted, however, that exposure to 5% CO_2 involved the simultaneous introduction of 23 mM-HCO_3^- to the bath. Therefore, activation of Na^+-coupled HCO_3^- transport (Thomas, 1977; Boron & Russel, 1983) could not be ruled out.

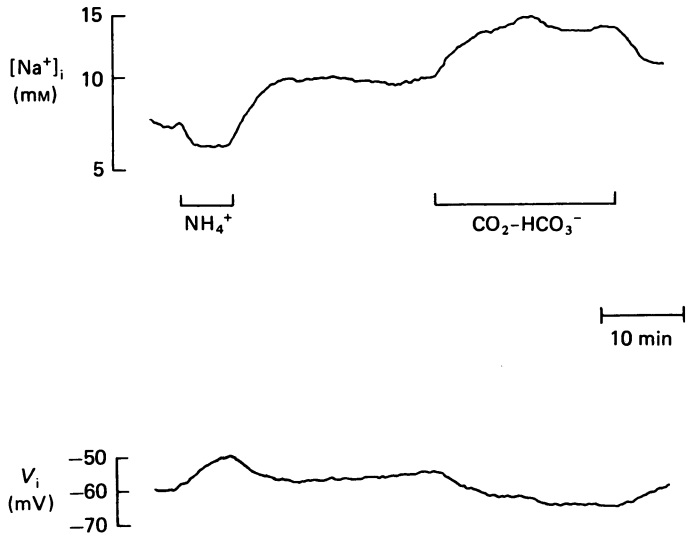

Fig. 5. Effect of acid loading on $[Na^+]_i$. The cell was exposed to a 5 min, 10 mM-NH_4Cl pre-pulse in 5 mM-HEPES Ringer (pH_b = 7·40). After 23 min, the superfusate was changed to 23 mM-HCO_3^- Ringer which contained 5% CO_2 (pH_b = 7·35).

Intracellular buffering power

β_i was estimated by measuring the fall in pH_i following a 10 mM-NH_4Cl pre-pulse in 5 mM-HEPES Ringer (pH_b = 7·40–7·50). In one set of experiments, NH_4^+ was withdrawn in the presence of 1 mM-amiloride thereby blocking any subsequent recovery of pH_i. Using the mean value of pH_o in 5 mM-HEPES Ringer (7·08), β_i was calculated to be 16±3 mM (n = 4). Assuming an upper limit for pH_o of 7·20, the maximum value for β_i would be 20±4 mM. In another set of experiments, amiloride was not used and pH_i was allowed to recover following acid loading. Extrapolation of the exponential recovery to the time NH_4^+ was withdrawn provided a correction for the contribution of acid transport. This method yielded β_i values of 16±2 and 19±3 mM for a pH_o of 7·08 and 7·20 respectively. These values are consistent with β_i in nerve cells of crayfish (21 mM; Moody, 1981) and leech (17 mM; Schlue & Thomas, 1985) but are higher than that of squid axon (9 mM; Boron & DeWeer, 1976) and snail neurones (11 mM; Thomas, 1976).

Regulation of pH_i in HCO_3^--buffered solutions

Transition from 5 mM-HEPES to HCO_3^- buffered Ringer caused a rapid intracellular acidification (Fig. 6). This was due to the influx of CO_2 and its hydration to carbonic acid (Jacobs, 1940; Thomas, 1976). The acid shift was followed by a recovery with a mean time constant of 12±2 min (n = 12). Steady-state pH_i attained in HCO_3^- Ringer was 7·44±0·03 (n = 6). This value is too high to be explained by a passive distribution of H^+, OH^- or HCO_3^-.

Return to HEPES Ringer resulted in a rapid alkalinization, followed by a slow, spontaneous fall of pH_i (Fig. 6). This rebound acidification blunted the preceding

alkaline shift. In some cases the acid shift brought pH_i to a level more acidic than prior to CO_2 exposure. A similar spontaneous acidification was described in smooth muscle cells following wash-out of CO_2–HCO_3^--buffered solutions (Aickin, 1984). The mechanism of this effect was not pursued.

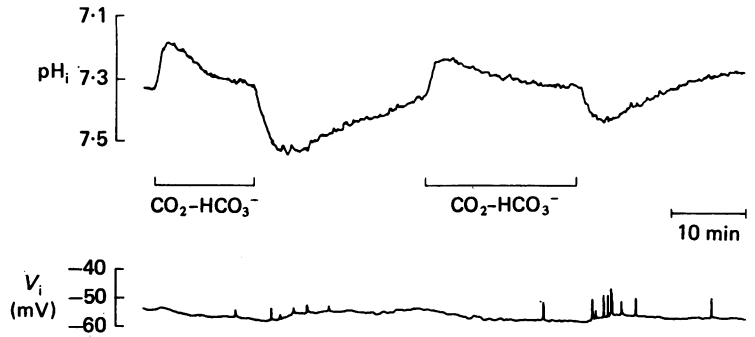

Fig. 6. Application and withdrawal of HCO_3^--buffered Ringer. Superfusate was switched between 5 mM-HEPES Ringer ($pH_b = 7{\cdot}50$) and 23 mM-HCO_3^- Ringer (5% CO_2, $pH_b = 7{\cdot}35$).

In principle it should be possible to calculate β_i based upon the alkalinization which occurs when CO_2 is withdrawn from the bathing media. However, the calculated values using this method were extremely high (50 mM or greater) even when the spontaneous rebound acidification was extrapolated backward. An error in junction potential correction seems unlikely since this would have to amount to 20 mV or more. The basis of this result is uncertain; however, loss of intracellular HCO_3^- across an imperfect electrode seal must certainly occur and may be one factor contributing to an over-estimation of β_i (see Discussion).

To compare the effects of HEPES and HCO_3^- Ringer on acid transport, cells were initially acid loaded in HEPES Ringer. During recovery of pH_i, the bathing solution was switched to HCO_3^- Ringer. The rate of pH_i recovery and the rate of acid extrusion were compared at the same pH_i for each solution.

In these experiments, the effect of solution changes on pH_o was critical. Transition from 5 mM-HEPES ($pH_b = 7{\cdot}40$–$7{\cdot}50$) to 23 mM-HCO_3^- Ringer ($pH_b = 7{\cdot}35$) caused a rise in pH_o of about 0·25 (not shown). This transition also caused a dramatic increase in the rate of pH_i recovery (Fig. 7). However, since pH_o was poorly controlled, the effect of HCO_3^- *per se* was uncertain. In this context, it was noteworthy that transition from 5 to 23 mM-HEPES Ringer (which increases pH_o) also enhanced the recovery rate (Fig. 8, arrow).

To determine whether HCO_3^- could stimulate acid extrusion independent of a rise in pH_o, the buffering power and pH of HEPES Ringer were increased. Transition from 23 mM-HEPES (pH_b 7·60–7·70) to 23 mM-HCO_3^- Ringer caused a decrease in pH_o of about 0·10. The effect on pH_i recovery can be seen in Fig. 8. An initial fall in pH_i (due to CO_2 entry) was followed by a slow recovery. Compared with 23 mM-HEPES Ringer, the rate of recovery in HCO_3^- Ringer was decreased by 30%.

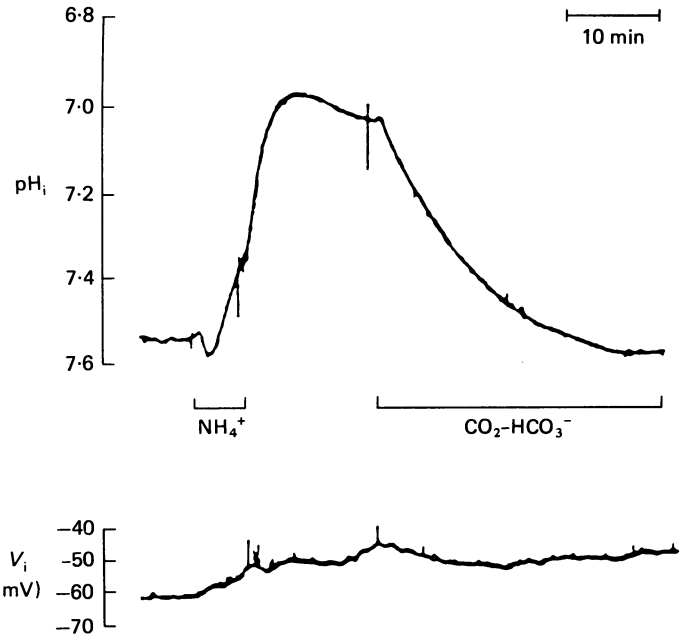

Fig. 7. Transition from HEPES to HCO_3^- Ringer during pH_i recovery. Cell was acid loaded with a 10 mM-NH_4Cl prepulse in 5 mM-HEPES Ringer (pH_b = 7·40). After recovery was established, the superfusate was switched to 23 mM-HCO_3^- Ringer (pH_b = 7·35). pH_o was not measured in this experiment.

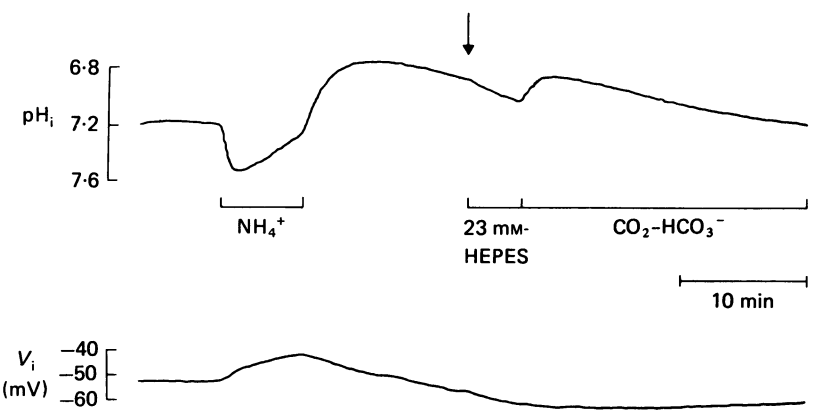

Fig. 8. Transition from 5 mM-HEPES to 23 mM-HEPES to 23 mM-HCO_3^- Ringer during pH_i recovery. Cell was initially acid loaded with a 10 mM-NH_4Cl pre-pulse in 5 mM-HEPES Ringer (pH_b = 7·50). During recovery the superfusate was switched (arrow) from 5 to 23 mM-HEPES Ringer (pH_b = 7·60). This caused a 130% increase in the rate of recovery. The superfusate was then switched to 23 mM-HCO_3^- Ringer (pH_b = 7·35) which decreased the rate of recovery by 30%. After withdrawal from the cell, pH_o in 5 mM-HEPES Ringer was 7·17. Transition to 23 mM-HEPES caused a rise in pH_o to 7·31.

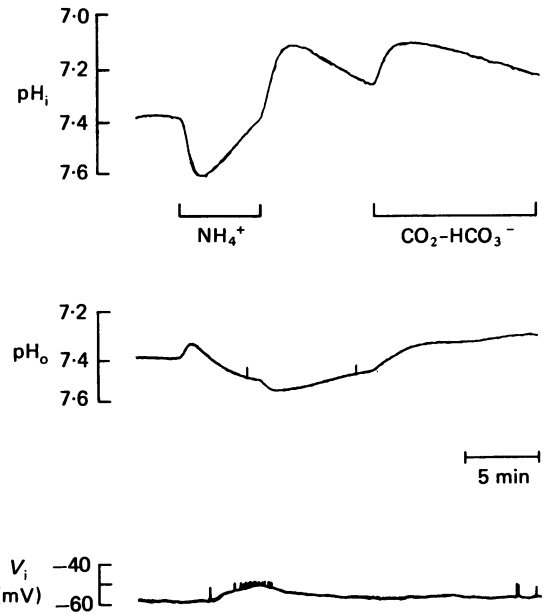

Fig. 9. Simultaneous monitoring of pH_i and pH_o during pH_i recovery. Cell was acid loaded with a 10 mM-NH_4Cl pre-pulse in 23 mM-HEPES Ringer (pH_b = 7·70). During recovery the superfusate was switched to 23 mM-HCO_3^- Ringer (pH_b = 7·35). pH_o was monitored with a separate pH-sensitive micro-electrode placed 100 μm caudal to the cell at a tissue depth of 100 μm.

In this experiment, the effect on pH_o was studied after withdrawal from the cell. The record of Fig. 9 is from one of two experiments in which pH_o and pH_i were monitored simultaneously. In both cases, transition to HCO_3^- Ringer caused a decrease in recovery rate and a fall in pH_o of about 0·10. In a total of six experiments, the mean decrease in recovery rate was 37 ± 6 %.

If Na–H exchange were the only acid extrusion mechanism, transition to HCO_3^- Ringer would be expected to reduce the rate of pH_i recovery. This is because the formation of intracellular HCO_3^- increases the total intracellular buffering power (Thomas, 1976). If this were the only action of HCO_3^- Ringer, the fall in recovery rate would be consistent with the added intracellular buffering, and the calculated rate of acid extrusion would remain unchanged. Assuming a maximum β_i of 20 mM, this was not true for the six experiments cited above. Rather, the calculated acid extrusion rate increased in every case, with a mean rise of 48 ± 8 %. If a lower value for β_i is employed, the proportionate increase in acid extrusion rate would be greater. The results from all six experiments appear in Table 1.

For an over-estimate of base-line pH_i to account for this result, an average error of 18 mV would be required after correcting for the reference junction potential. If a β_i of 16 mM is employed, the required error is 23 mV (see Table 1). Since the standard deviation of the junction potential correction was only 2 mV, an error of the magnitude was highly unlikely.

TABLE 1. Effect of HCO_3^--buffered Ringer on acid extrusion rate

Cell	pH_x	ΔJ % (mM/min) ($\beta_i = 20$)	ΔJ % (mM/min) ($\beta_i = 16$)	E_x (mV) ($\beta_i = 20$)	E_x (mV) ($\beta_i = 16$)	Drift (mV)
05–08–84	7·05	+73 (0·23)	+97 (0·24)	−27	−32	+2·0
07–18–84A	6·88	+46 (0·49)	+64 (0·55)	−23	−28	−0·5
07–18–84B	6·95	+16 (0·07)	+31 (0·11)	−6	−11	+5·0
08–02–84	7·00	+57 (0·39)	+79 (0·43)	−23	−28	−1·0
09–10–84	7·20	+61 (0·36)	+87 (0·42)	−18	−25	−3·0
09–29–84	7·25	+32 (0·23)	+55 (0·31)	−9	−14	+2·0
Mean		+48 (0·30)	+69 (0·34)	−18	−23	

pH_x is the intracellular pH at which acid extrusion rates in HEPES and HCO_3^- Ringer were compared. The percentage difference in calculated acid extrusion rate after transition to HCO_3^- Ringer (ΔJ %) was calculated using a β_i of 20 or 16 mM. Acid extrusion rates were calculated as $(dpH_i/dt) \times (\beta_i + \beta_b)$ where β_b is $2{\cdot}3 \times [HCO_3^-]_i$. Numbers in parentheses indicate the absolute calculated increase in acid extrusion rate in millimoles per litre per minute. E_x is the measurement error required to explain the results assuming that no change in acid extrusion rate occurred. The total drift of the pH micro-electrode during the course of each experiment is also shown. A negative drift would tend to over-estimate ΔJ %. Cells were acid loaded with a 10 mM-NH_4Cl pre-pulse in 23 mM-HEPES Ringer ($pH_b = 7{\cdot}60$–$7{\cdot}70$), except for 05–08–84, where 5 mM-HEPES ($pH_b = 7{\cdot}80$) was used. In the first two preparations (05–08–84 and 07–18–84), pH_o was studied during solution changes at the end of the experiment. Transition from HEPES to HCO_3^- Ringer caused a decrease in pH_o of 0·01 and 0·10 respectively. For 08–02–84, pH_o was not studied during solution changes but was found to be 7·31 in 23 mM-HEPES Ringer at the end of the experiment. For the last two preparations, pH_o and pH_i were monitored simultaneously during the experiment. pH_o was found to decrease by about 0·10 following transition from 23 mM-HEPES to 23 mM-HCO_3^- Ringer.

Transition to HCO_3^- Ringer in the presence of amiloride

In HCO_3^--free solutions, recovery from acid loading did not occur in solutions containing 1–2 mM-amiloride. However, if an independent HCO_3^--dependent acid extrusion mechanism were present, pH_i recovery should be possible in the presence of amiloride (Schlue & Thomas, 1985). This was tested as shown in Fig. 10. After acid loading in 23 mM-HEPES Ringer ($pH_b = 7{\cdot}63$), recovery was blocked with 2 mM-amiloride. This resulted in a slow intracellular acidification. The superfusate was then switched to 23 mM-HCO_3^- Ringer ($pH_b = 7{\cdot}35$) which caused a more rapid acid shift, due to CO_2 influx. A slow recovery of pH_i occurred despite the presence of amiloride.

The effect of DIDS on pH_i recovery

Stilbene derivatives have been shown to block HCO_3^--dependent acid transport in a number of cells (Russel & Boron, 1976; Thomas, 1977; Aickin & Thomas, 1977*a*; Moody, 1981). In lamprey neurones, recovery from acid loading in HCO_3^- media was sensitive to DIDS. The result of a brief exposure to DIDS is shown in Fig. 11*A*. During recovery from an NH_4^+ pre-pulse, the bath was changed from HEPES to HCO_3^- Ringer. This caused a slight acid transient after which pH_i continued to recover. Exposure to 0·1 mM-DIDS for 5 min halted the recovery and resulted in a slight acidification. In other instances, the percentage inhibition by the drug was more clear, as DIDS simply decreased the rate of recovery (Fig. 11*B*). In five such experiments, the mean decrease of pH_i recovery was 65 ± 12 %. When applied briefly,

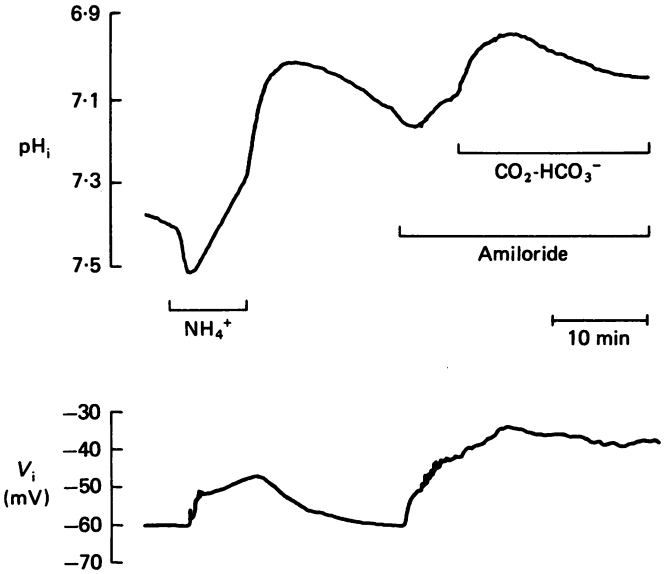

Fig. 10. Recovery of pH_i in the presence of amiloride. Cell was acid loaded with a 10 mM-NH_4Cl pre-pulse in 23 mM-HEPES Ringer (pH_b = 7·63). Recovery was halted by exposure to 2 mM-amiloride. The superfusate was then switched to 23 mM-HCO_3^- Ringer (pH_b = 7·35) containing 2 mM-amiloride.

the effect of the drug was reversible. The result of a more sustained exposure to DIDS is shown in Fig. 11*C*. Transition from 5 mM-HEPES to 23 mM-HCO_3^- Ringer produced a typical transient acidification and recovery (see Fig. 6). When the same solution change was performed twice in the presence of 0·10 mM-DIDS, there was little or no recovery at the same pH_i (dashed line). After a 45 min exposure, wash-out of DIDS did not restore recovery. These observations are consistent with the action of DIDS on erythrocyte membranes, where a high-affinity, reversible ionic interaction with transport sites precedes an irreversible, covalent reaction (Cabantchik, Knauf & Rothstein, 1978).

In nominally HCO_3^--free solutions, the concentration of HCO_3^- in the extracellular space, and the contribution of HCO_3^--dependent transport are presumed to be low, but are not precisely known. The effect of DIDS in HEPES-buffered solutions would help to clarify this issue. Controlled studies utilizing DIDS were not performed in HCO_3^--free media. However, in two experiments, 0·1 mM-DIDS was present in HEPES Ringer during acid loading and subsequent recovery. It is notable that in these instances pH_i recovered in a normal fashion, suggesting that HCO_3^--dependent transport was not critical for pH_i regulation under these conditions.

The effect of Cl^--free Ringer

In nerve cells of snail and squid, recovery from acid loading is blocked in the absence of intracellular Cl^- (Thomas, 1977; Boron & Russell, 1983). To deplete intracellular Cl^- in lamprey neurones, the preparation was superfused with Cl^--free

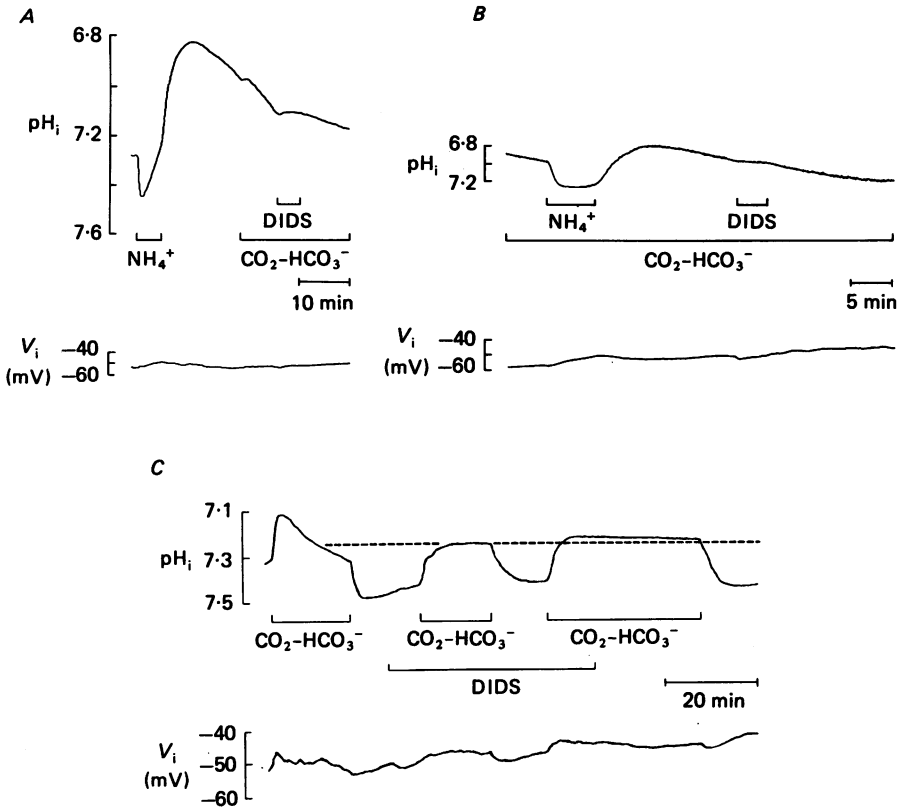

Fig. 11. Effect of DIDS on pH_i recovery. *A*, the cell was acid loaded with a 10 mM-NH_4Cl pre-pulse in 23 mM-HEPES Ringer (pH_b = 7·40). During recovery, the superfusate was switched to 23 mM-HCO_3^- Ringer (pH_b = 7·35). Brief exposure to 0·10 mM-DIDS completely blocked recovery. The effect was partially reversible. *B*, cell was acid loaded with a 20 mM-NH_4Cl pre-pulse in 23 mM-HCO_3^- Ringer. Exposure to 0·10 mM-DIDS reduced the rate of recovery by 67 %. *C*, the cell was acid loaded by transition from 5 mM-HEPES (pH_b = 7·40) to 23 mM-HCO_3^- Ringer (5 % CO_2, pH_b = 7·35). Following recovery of pH_i and wash-out of HCO_3^-, the same solution change was performed twice in the presence of 0·10 mM-DIDS.

HEPES Ringer for 30 min. Cells were then impaled and acid loaded by changing to Cl^--free HCO_3^- Ringer.

In Cl^--free HEPES Ringer, pH_i was apparently low. Using a 9 mV junction potential correction, the mean pH_i was 6·95±0·07 (n = 9). However, since the magnitude of the junction potential shift was not determined for Cl^--free solution, base-line pH_i values were more uncertain. Therefore pH_i recovery rates were compared instead of acid extrusion rates.

Transition to Cl^--free HCO_3^- Ringer (5 % CO_2) caused a rapid acidification followed by a slow recovery. In the experiment of Fig. 12 (isethionate Ringer) the rate of recovery was 0·004/min. After 8 min the bath was changed to 5 mM-HEPES Ringer, thereby washing out HCO_3^- and washing in Cl^- simultaneously. 7 min later the bath was changed to normal HCO_3^- Ringer. This caused a second rapid

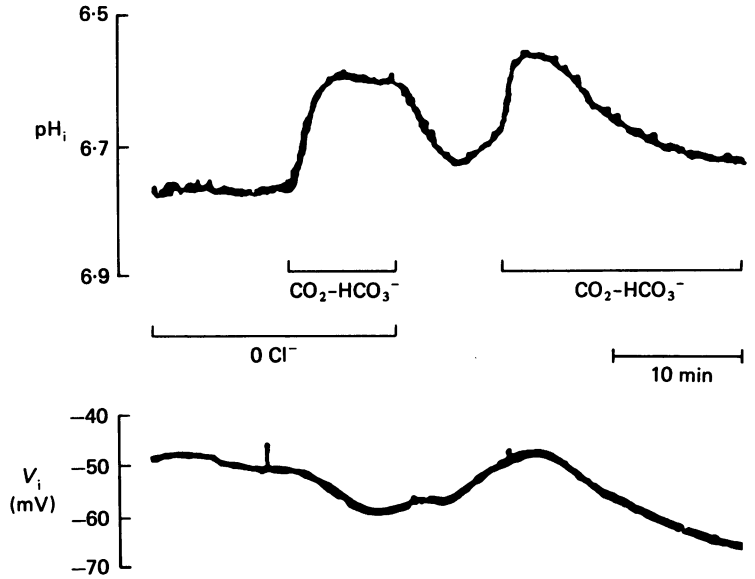

Fig. 12. Effect of Cl^--free Ringer on pH_i recovery. A cell was impaled after a 30 min superfusion in Cl^--free, 5 mM-HEPES Ringer (isethionate was the major Cl^- substitute, pH_b = 7·40). The cell was acid loaded by transition to Cl^--free, 23 mM-HCO_3^- Ringer (5 % CO_2, pH_b = 7·35). After the recovery rate was established, the superfusate was switched to normal 5 mM-HEPES Ringer (pH_b = 7·40), thereby washing out HCO_3^- and returning Cl^- simultaneously. 8 min later the cell was acid loaded a second time by transition to normal 23 mM-HCO_3^- Ringer (5 % CO_2, pH_b = 7·35). Recovery rate was five times faster after exposure to Cl^--containing media. Data photographed directly from chart record. Thickness of trace is due to excessive flow of ink to chart recorder pen.

acidification followed by a recovery of 0·02/min, a fivefold increase in rate compared with Cl^--free solution. Similar results were obtained when methanesulphonate was used as the major Cl^- substitute.

DISCUSSION

Intracellular pH regulation in HCO_3^--free media

The extracellular space of the lamprey brain was consistently more acidic than the superfusion solution. A similar finding was noted in isolated turtle cerebellum (C. Nicholson, unpublished observation), rat cerebellum (Kraig *et al.* 1983) and mammalian skeletal muscle (De Hemptinne & Huguenin, 1984). This gradient was most likely due to lactic acid production. In brains of lamprey larva maintained at 4 °C, Rovainen and colleagues (Rovainen, Lowry & Passonneau, 1969) measured a mean lactate concentration of 1·8 mmol/kg; when maintained at 20 °C, brain lactate levels fell within the same range. A similar acid concentration in the extracellular space may account for the bath–brain pH gradient. Thus, metabolically derived CO_2 would contribute little to the extracellular acidosis. Accordingly, the concentration of extracellular HCO_3^- in nominally HCO_3^--free HEPES Ringer would be extremely low.

The maximum possible HCO_3^- concentration may be estimated by assuming that CO_2 was the only source of extracellular acid. Given a total HEPES concentration of 5 mM, a pH_b of 7·50 and a pH_o of 7·00, 1·4 mM of HEPES acid ($pKa = 7·50$) would be derived from the conjugate base and 1·4 mM of HCO_3^- would be generated from the hydration of CO_2. This concentration of HCO_3^- is less than the Michaelis constant, for HCO_3^- transport in the squid axon (2·3 mM; Boron & Russel, 1983). Thus, at maximum levels, substantial transport of HCO_3^- would require a relatively high-affinity carrier. This possibility cannot be excluded; however, a substantial contribution by such a mechanism appears unlikely inasmuch as the pH_i recovery was completely blocked by amiloride. It was also notable that in two cells pH_i was observed to recover readily when 0·1 mM-DIDS was added to HEPES Ringer. While controlled studies of the effect of DIDS in HEPES Ringer were not performed, the observed behaviour of the pH_i regulatory system in the presence of DIDS stands in sharp contrast with the results of amiloride experiments.

The inhibition of pH_i regulation by amiloride strongly suggests that in HEPES-buffered solutions acid extrusion is accomplished by a Na–H exchange mechanism (Johnson, Epel & Paul, 1976). The rise in $[Na^+]_i$ following an NH_4^+ pre-pulse is also consistent with this mechanism (Aickin & Thomas, 1977*a*). Involvement of Na^+ is supported further by substitution experiments. Replacement of Na^+ with BDA^+ or choline resulted in a slow, accelerating acidification. Return of Na^+ to the bath caused an immediate reversal of this effect.

While these observations, in concert, support the presence of Na–H exchange, alternative interpretations of certain results are possible. It should be stressed that the effect of experimental procedures on factors other than pH_i might also account for the behaviour of $[Na^+]_i$. Reduction of the driving potential for Na^+ entry, due to the depolarizing effect of NH_4^+, could explain the initial fall of $[Na^+]_i$. Its subsequent overshoot upon wash-out of NH_4^+ may therefore be partially due to repolarization of the membrane potential. Similarly, the hyperpolarization upon exposure to HCO_3^- Ringer could cause a concomitant rise in $[Na^+]_i$. The voltage dependence of sodium channel inactivation (Hodgkin & Huxley, 1952) or changes in $[Na^+]_o$ might also contribute to the behaviour of $[Na^+]_i$. Furthermore, in an intact brain stem, changes in Na^+ fluxes could result from effects upon presynaptic elements.

The accelerating intracellular acidification which occurred upon removal of extracellular Na^+ was not studied. However, in separate experiments, replacement of extracellular Na^+ was found to cause an extracellular alkalinization (not shown). The intracellular acid shift was therefore not caused by a fall in pH_o. A reversal of Na–H exchange is consistent with these findings. However, reversal of Na–Ca exchange and a consequent rise in intracellular Ca^{2+} could also have contributed to the fall in pH_i (Vaughan-Jones, Lederer & Eisner, 1983).

A persistent hyperpolarization often accompanied the recovery of pH_i in lamprey neurones. this may be due to activation of an electrogenic Na^+–K^+ transport secondary to increased Na^+ influx (Moolenar *et al.* 1981). A similar hyperpolarization following acid loading of lymphocytes was found to be ouabain sensitive and triggered by Na–H exchange (Grinstein *et al.* 1984). The depolarization caused by amiloride is also consistent with this idea. However, it must again be stressed that in the intact brain stem, effects on a multitude of pre- and post-synaptic processes cannot be excluded.

Intracellular pH regulation in HCO_3^--buffered media

The presence of endogenous acid sources can result in pH_o shifts when solutions of different buffering power are exchanged. De Hemptinne & Vanheel (1984) found that a fall in pH_o, arising from a reduction in buffering power, decreased the rate of acid extrusion in rat muscle. In the present study, replacement of 5 mM-HEPES with 23 mM-HCO_3^- Ringer caused an increase in the rate of pH_i recovery. In the experiment of Fig. 7, transition to HCO_3^- Ringer caused at least a fifteenfold increase in the rate of acid extrusion. This initially suggested the existence of a dominant HCO_3^--dependent acid transport system. However, in experiments where the pH and buffering power of solutions were adjusted to minimize shifts in pH_o, transition to HCO_3^- Ringer caused a decrease in recovery rate. None the less, comparison of the acid extrusion rate in 23 mM-HEPES *versus* 23 mM-HCO_3^- buffers revealed a mean increase of at least 48 %.

This analysis was likely to underestimate the relative effect of HCO_3^- for a number of reasons. First, calculations were based on a maximum estimate of intracellular buffering power (20 mM) which tended to minimize the proportionate increase in acid extrusion rate. In addition, transition from 23 mM-HEPES Ringer to 23 mM-HCO_3^- Ringer was associated with a slight fall in pH_o which may be expected to inhibit acid transport. The rate of metabolic acid accumulation may also have risen throughout the course of pH_i recovery. In barnacle muscle, the rate of spontaneous acidification in SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid)-poisoned fibres increased with the preceding length of time in which acid extrusion occurred (Boron *et al.* 1979). A similar phenomenon is suggested by the rebound acidification which occurred after withdrawal of HCO_3^- Ringer (Fig. 6).

It should also be noted that when a cell is impaled in HCO_3^- Ringer, an additional acid load is imposed, since a fraction of injury current is mediated by an efflux of HCO_3^-. This will act to impede pH_i recovery and an underestimate of HCO_3^--stimulated transport will result. These effects may be particularly important when studying large cells of low input resistance, where membrane damage has the least effect on membrane potential (Taylor & Thomas, 1984).

Leakage of HCO_3^- through an imperfect electrode seal may be particularly pronounced when CO_2 and HCO_3^- are withdrawn from the bathing medium. It is unlikely that sufficient efflux occurs to cause a two or threefold over-estimation of β_i. None the less, as CO_2 and HCO_3^- are washed out, the fraction of injury current carried by HCO_3^- will increase, which may curtail the expected rise in pH_i. Other factors such as a fall in pH_o or endogenous production of HCO_3^- could also contribute to calculation of an exceedingly large β_i.

The sensitivity of pH_i recovery to DIDS provided additional evidence for a HCO_3^--dependent acid extrusion mechanism. It was curious that on two occasions exposure to DIDS caused a slight acidification (Fig. 11*A*). A number of acid sources, as discussed above, might account for this observation. The ability of cells to extrude acid in the presence of 2 mM-amiloride (Fig. 10) was also consistent with a mechanism other than Na–H exchange. The independence of each mechanism could be further supported if the inhibitory action of amiloride and DIDS were shown to be additive (Aickin & Thomas, 1977*a*); however, these experiments could not be successfully performed.

The ionic mechanism for HCO_3^--dependent acid transport is not known. In nerve cells of snail (Thomas, 1977) and squid (Boron & Russel, 1983), HCO_3^--dependent acid extrusion involves the electroneutral net influx of HCO_3^- and Na^+ and the efflux of Cl^- in the ratio 2:1:1. It is unclear whether a similar mechanism operates in lamprey neurones. The response to Na^+-free HCO_3^- Ringer (not shown) and Na^+-free HEPES Ringer (Fig. 4), were qualitatively similar. A slow acidification in the absence of extracellular Na^+ was followed by recovery when Na^+ was returned to the bath. Although the acidification may be explained by a reversal of an Na^+- and HCO_3^--dependent mechanism (Russell, Boron & Brodwick, 1983; Evans & Thomas, 1984), a reversal of Na–H or Na–Ca exchange (Vaughan-Jones *et al.* 1983) could also account for this finding.

Involvement of Cl^- was suggested by the sensitivity of the pH_i recovery to DIDS. Stilbene derivatives block Cl–HCO_3 exchange in erythrocytes (Cabantchik *et al.* 1978) and inhibit a number of HCO_3^--dependent pH_i regulatory mechanisms (Russel & Boron, 1976; Thomas, 1977; Moody, 1981). However, these compounds may also block Cl^--independent transport of HCO_3^- (Boron & Boulpaep, 1983*b*). Sensitivity to DIDS alone is therefore insufficient evidence to link Cl^- fluxes to acid transport. However, pH_i recovery was also slowed in Cl^--free solutions. Taken together, these findings more strongly support a role for Cl^- in acid transport.

In summary, these results suggest the presence of two mechanisms for pH_i regulation in lamprey central neurones. One mechanism is Na–H exchange. The other requires extracellular HCO_3^- and may be Cl^- dependent as well. As such, these cells resemble vertebrate skeletal muscle, where Na–H exchange and a HCO_3^--dependent mechanism also coexist (Aickin & Thomas, 1977*a*; Abercrombie, Putnam & Roos, 1983). They may also be similar to crayfish central neurones (Moody, 1981) and leech Retzius cells (Schlue & Thomas, 1985) where a distinct Na^+ and HCO_3^--dependent mechanism operates in addition to Na–H exchange. However, it must be stressed that the data available for lamprey neurones neither support nor exclude a role for Na^+ in the HCO_3^--dependent system.

The importance of studying pH_i regulation in vertebrate central neurones may lie in a better understanding of brain pathology. In the vertebrate C.N.S., ischaemia is associated with a rapid collapse of the Na^+ gradient (Hansen & Zeuthen, 1981) and a prolonged extracellular acidosis (Kraig *et al.* 1983). The results on reticulospinal neurones and a large number of diverse cell types (Thomas, 1984), indicate that a total shutdown of pH_i regulation would occur under these conditions. Disruption of pH_i homeostasis must therefore be weighed as a factor in the pathophysiology of brain ischaemia.

Amiloride was a gift of Merck, Sharp & Dohme. H^+ exchanger was kindly provided by Professor W. Simon, Swiss Federal Institute of Technology. I wish to thank Charles Nicholson for his unfailing advice and encouragement and Walter Boron for many helpful discussions. Brian MacVicar and Joe Ayers provided invaluable advice on the location, capture and maintenance of lamprey. Technical assistance was provided by C. Cronin. This work was supported by NINCDS Grant NS-13741. M. Chesler was an MSTP fellow suppported by NIGMS Grant 5 T32 Gm-07308.

REFERENCES

ABERCROMBIE, R. F., PUTNAM, R. W. & ROOS, A. (1983). the intracellular pH of frog skeletal muscle: its regulation in isotonic solutions. *Journal of Physiology* **345**, 175–187.

AICKIN, C. C. (1984). Direct measurement of intracellular pH and buffering power in smooth muscle cells of guinea-pig vas deferens. *Journal of Physiology* **349**, 571–585.

AICKIN, C. C. & THOMAS, R. C. (1977*a*). An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *Journal of Physiology* **273**, 295–316.

AICKIN, C. C. & THOMAS, R. C. (1977*b*). Micro-electrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibres. *Journal of Physiology* **267**, 791–810.

AMMANN, D., LANTER, F., STEINER, R. A., SCHULTHESS, P., SHIJO, Y. & SIMON, W. (1981). Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular studies. *Analytical Chemistry* **53**, 2267–2269.

BATES, R. G. & PINCHING, G. D. (1950). Dissociation constant of aqueous ammonia at 0 to 50° from e.m.f. studies of the ammonium salt of a weak acid. *Journal of the American Chemical Society* **72**, 1393–1396.

BORON, W. F. & BOULPAEP, E. L. (1983*a*). Intracellular pH regulation in the renal proximal tubule of the salamander. Na–H exchange. *Journal of General Physiology* **81**, 29–52.

BORON, W. F. & BOULPAEP, E. L. (1983*b*). Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. *Journal of General Physiology* **81**, 52–94.

BORON, W. F. & DEWEER, P. (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *Journal of General Physiology* **67**, 91–112.

BORON, W. F. & RUSSELL, J. M. (1983). Stoichiometry and ion dependencies of the intracellular-pH-regulation mechanism in squid giant axons. *Journal of General Physiology* **81**, 373–399.

BORON, W. F., MCCORMICK, W. C. & ROOS, A. (1979). pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. *American Journal of Physiology* **237**, C185–193.

CABANTCHIK, Z. I., KNAUF, P. A. & ROTHSTEIN, A. (1978). The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of 'probes'. *Biochimica et biophysica acta* **515**, 239–302.

CALDWELL, P. C. (1968). Liquid junction potentials and their effect on potential measurements in biological systems. *International Review of Cytology* **24**, 345–371.

CHESLER, M. & NICHOLSON, C. (1984). Regulation of pH in lamprey central neurons. *Society for Neuroscience Abstracts* **10**, 116.

CHESLER, M. & NICHOLSON, C. (1985). Regulation of intracellular pH in vertebrate central neurons. *Brain Research* **325**, 313–316.

CHRISTOFFERSON, G. R. J. & SKIBSTED, L. H. (1975). Calcium ion activity in physiological salt solutions: influence of anions substituted for chloride. *Comparative Biochemistry and Physiology* **52A**, 317–322.

DE HEMPTINNE, A. & HUGUENIN, F. (1984). The influence of muscle respiration and glycolysis on surface and intracellular pH in fibres of the rat soleus. *Journal of Physiology* **347**, 581–592.

DE HEMPTINNE, A. & VANHEEL, B. (1984). Influence of external buffering and surface pH on intracellular pH and its regulation in rat and mouse soleus muscle. *Journal of Physiology* **346**, 73*P*.

DEAN, J. A. (1979). *Lange's Handbook of Chemistry*, 12th ed. New York: McGraw-Hill.

DEITMER, J. W. & ELLIS, D. (1980). Interactions between the regulation of the intracellular pH and sodium activity of sheep cardiac purkinje fibres. *Journal of Physiology* **304**, 471–488.

EISENMAN, G. (1961). Cation selective glass electrodes and their mode of operation. *Biophysical Journal* **2** (part 2), 259–323.

EVANS, M. G. & THOMAS, R. C. (1984). Acid influx into snail neurones caused by reversal of the normal pH_i-regulating system. *Journal of Physiology* **346**, 143–154.

GOLD, M. R. & MARTIN, A. R. (1982). Intracellular Cl^- accumulation reduces Cl^- conductance in inhibitory synaptic channels. *Nature* **299**, 828–830.

GRINSTEIN, S., COHEN, S. & ROTHSTEIN, A. (1984). Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *Journal of General Physiology* **83**, 341–369.

HANSEN, A. J. & ZEUTHEN, T. (1981). Extracellular ion concentration during spreading depression and ischemia in the rat brain cortex. *Acta physiologica scandinavica* **113**, 437–445.

HARNED, H. S. & BONNER, F. T. (1945). The first ionization of carbonic acid in aqueous solutions of sodium chloride. *Journal of the American Chemical Society* **67**, 1026.

HARNED, H. S. & DAVIS JR, R. (1943). The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50°. *Journal of the American Chemical Society* **65**, 2030–2037.

HODGKIN, A. L. & HUXLEY, A. F. (1952). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *Journal of Physiology* **116**, 497–506.

JACOBS, M. H. (1940). Some aspects of cell permeability to weak electrolytes. *Cold Spring Harbor Symposia on Quantitative Biology* **8**, 30.

JOHNSON, J. D., EPEL, D. & PAUL. M. (1976). Intracellular pH and activation of sea urchin eggs after fertilization. *Nature* **262**, 661–664.

KENYON, J. L. & GIBBONS, W. R. (1977). Effects of low chloride on action potentials of sheep cardiac purkinje cells. *Journal of General Physiology* **70**, 635–660.

KRAIG, R. P., FERREIRA-FILHO, C. R. & NICHOLSON, C. (1983). Alkaline and acid transients in cerebellar microenvironment. *Journal of Neurophysiology* **49**, 831–850.

MOODY, W. J. (1981). The ionic mechanism of intracellular pH regulation in crayfish neurones. *Journal of Physiology* **316**, 293–308.

MOOLENAAR, W. H., BOONSTRA, J., VAN DER SAAG, P. T. & DE LAAT, S. W. (1981). Sodium/proton exchange in mouse neuroblastoma cells. *Journal of Biological Chemistry* **256**, 12883–12887.

MOOLENAAR, W. H., TERTOOLEN, L. F. J. & DE LAAT, S. W. (1984). The regulation of cytoplasm pH in human fibroblasts. *Journal of Biological Chemistry* **259**, 7563–7569.

MOSER, H. (1985). Intracellular pH regulation in the sensory neurone of the stretch receptor of the crayfish (*Astacus fluviatilis*). *Journal of Physiology* **362**, 23–38.

ROVAINEN, C. M. (1979). Neurobiology of lampreys. *Physiological Review* **59**, 1007–1077.

ROVAINEN, C. M., LOWRY, O. H. & PASSONNEAU, J. V. (1969). Levels of metabolites and production of glucose in the lamprey brain. *Journal of Neurochemistry* **16** 1451–1458.

RUSSELL, J. M. & BORON, W. F. (1976). Role of chloride transport in regulation of intracellular pH. *Nature* **264**, 73–74.

RUSSELL, J. M., BORON, W. F. & BRODWICK, M. S. (1983). Intracellular pH and Na fluxes in barnacle muscle with evidence for reversal of the ionic mechanism of intracellular pH regulation. *Journal of General Physiology* **82**, 47–78.

SCHULE, W. R. & THOMAS, R. C. (1985). A dual mechanism for intracellular pH regulation by leech neurones. *Journal of Physiology* **364**, 327–338.

STEINER, R. A., OEHME, M., AMMANN, D. & SIMON, W. (1979). Neutral carrier sodium ion-selective microelectrode for intracellular studies. *Analytical Chemistry* **51**, 351–353.

TAYLOR, P. S. & THOMAS, R. C. (1984). The effect of leakage on microelectrode measurements of intracellular sodium activity in crab muscle fibres. *Journal of Physiology* **352**, 539–550.

THOMAS, R. C. (1976). The effect of CO_2 on the intracellular pH and buffering power of snail neurones. *Journal of Physiology* **255**, 715–735.

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *Journal of Physiology* **273**, 317–338.

THOMAS, R. C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. *Journal of Physiology* **354**, 3–22*P*.

VANHEEL, B., DE HEMPTINNE, A. & LEUSEN, I. (1984). Analysis of Cl^-–HCO_3^- exchange during recovery from intracellular acidosis in cardiac Purkinje strands. *American Journal of Physiology* **246**, C391–400.

VAUGHAN-JONES, R. (1982). Chloride–bicarbonate exchange in the sheep cardiac purkinje fibre. In *Intracellular pH: its Measurement, Regulation and Utilization in Cellular Functions*, ed., NUCCITELLI, R. & DEAMER, D. W. pp 239–252. New York: Alan R. Liss.

VAUGHAN-JONES, R. D., LEDERER, V. J. & EISNER, D. A. (1983). Ca^{2+} ions can affect intracellular pH in mammalian cardiac muscle. *Nature* **301**, 522–524.