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THE ROLE OF BICARBONATE, CHLORIDE AND SODIUM IONS IN THE REGULATION OF INTRACELLULAR pH IN SNAIL NEURONES

BY R. C. THOMAS

From the Department of Physiology, University of Bristol, Bristol BS8 1TD

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SUMMARY

1. Intracellular pH (pH_i), Cl^- and Na^+ levels were recorded in snail neurones using ion-sensitive micro-electrodes, and the mechanism of the pH_i recovery from internal acidification investigated.

2. Reducing the external HCO_3^- concentration greatly inhibited the rate of pH_i recovery from HCl injection.

3. Reducing external Cl^- did not inhibit pH_i recovery, but reducing internal Cl^-, by exposing the cell to sulphate Ringer, inhibited pH_i recovery from CO_2 application.

4. During pH_i recovery from CO_2 application the internal Cl^- concentration decreased. The measured fall in internal Cl^- concentration averaged about 25% of the calculated increase in internal HCO_3^-.

5. Removal of external Na inhibited the pH_i recovery from either CO_2 application or HCl injection.

6. During the pH_i recovery from acidification there was an increase in the internal Na^+ concentration ($[Na^+]_i$). The increase was larger than that occurring when the Na pump was inhibited by K-free Ringer.

7. The increase in $[Na^+]_i$ that occurred during pH_i recovery from an injection of HCl was about half of that produced by a similar injection of NaCl.

8. The inhibitory effects of Na-free Ringer and of the anion exchange inhibitor SITS on pH_i recovery after HCl injection were not additive.

9. It is concluded that the pH_i regulating system involves tightly linked Cl^--HCO_3^- and Na^+-H^+ exchange, with Na entry down its concentration gradient probably providing the energy to drive the movement inwards of HCO_3^- and the movement outward of Cl^- and H^+ ions.

INTRODUCTION

The way in which intracellular pH (pH_i) is regulated is almost unknown; indeed it is only recently that it has become clear that it is regulated at all (for references see Aickin & Thomas, 1977*b*). The values of pH_i found in various tissues are such that there needs to be some mechanism to balance the predicted net passive influx of H^+ ions, or the equivalent net passive efflux of HCO_3^- or OH^- ions. Such a mechanism, or pH_i regulating system, could involve the active transport of H^+, HCO_3^- or OH^- ions, or possibly some kind of acid consumption by the cell (Cohen & Iles, 1975). The large capacity of the system in snail neurones, however, makes the latter scheme unlikely.

It has recently been found that the pH_i regulating system in snail neurones and squid giant axons is inhibited by the removal of HCO_3^- ions (Thomas, 1976*a*; Boron & De Weer, 1976) suggesting that part of the system may involve HCO_3^- influx against its electrochemical gradient. There is also evidence that Cl^- ions are involved, since the anion-exchange inhibitor SITS blocks pH_i recovery in both snail neurones and squid axons (Thomas, 1976*c*; Russell & Boron, 1976). Russell & Boron (1976) have also shown a linkage of Cl^- efflux to pH_i recovery from internal acidification in squid axons. In sheep heart Purkinje fibres and mouse soleus muscle, however, SITS has only a small effect on pH_i recovery (Ellis & Thomas, 1976; Aickin & Thomas, 1977*b*).

As well as Cl^--HCO_3^- exchange, a number of other mechanisms have been proposed by which pH_i might be regulated. These include K^+-H^+ exchange, Na^+-H^+ exchange and coupled H^+ and Cl^- extrusion. In this paper I describe experiments designed to establish which, if any, of the proposed schemes can best explain the way in which the pH_i of snail neurones is regulated. I have tested the pH_i regulating system by acidifying the cell interior, and then observing the recovery from this pH_i decrease under a variety of conditions. I have not investigated the recovery from intracellular alkalinization, partly because it is rather slow and could be due simply to passive ion fluxes.

Two methods have been used to acidify the cell interior; these were iontophoretic HCl injection and CO_2 application. Acid injection has the advantage that it is repeatable at relatively short intervals, and involves no change in external HCO_3^- concentration. On the other hand it requires two extra intracellular electrodes. The application of CO_2 is technically simpler and has an important advantage when investigating the role of anions: the pH_i can be displaced without anion injection. But before an application can be repeated the CO_2 must be removed, and recovery of the pH_i from CO_2 removal can take 30–60 min.

The role of HCO_3^-, Cl^- and Na^+ ions in the pH_i recovery from acidification has been investigated by changing the external concentration of all three ions and the internal concentration of Cl^-, and by recording changes in internal Cl^- and Na^+ concentrations. The results suggest that in snail neurones pH_i is regulated by a system involving tightly linked Na^+-H^+ and Cl^--HCO_3^- exchange, with Na^+ and HCO_3^- ions entering and H^+ and Cl^- ions leaving the cell. A preliminary report of some of this work has been communicated to the Physiological Society (Thomas, 1976*d*).

METHODS

These were similar to those described previously (Thomas, 1974, 1976*a*) except that in some experiments a Cl^- or Na^+ sensitive micro-electrode was used to record $[Cl^-]_i$ or $[Na^+]_i$ at the same time as pH_i.

General. Experiments were done on the largest nerve cell at the rear of the right pallial ganglion of *Helix aspersa*, as previously described (Thomas, 1976*a*). The brain was removed from dormant snails which had been previously starved in the laboratory for at least a week. The brain was mounted on a P.T.F.E. block and the outer connective tissue over the suboesophageal ganglia removed. The block was then placed in the experimental bath and the suboesophageal ganglia superfused with CO_2 snail Ringer. The thin inner connective tissue covering the right pallial ganglion was then torn with a tungsten hook. At least 1 hr was then allowed for the preparation to stabilize before the cell was penetrated.

If undamaged during the dissection, the cell was penetrated with up to five micro-electrodes, one by one. The ion-sensitive micro-electrodes were usually inserted first perpendicularly, but the (sharper) conventional micro-electrodes were inserted sideways by a horizontal movement of the vertically mounted micro-electrodes.

Solutions. Throughout the experiments the preparation was continuously superfused with a snail Ringer solution. The various experimental solutions were kept in glass bottles mounted about 50 cm above the bath, to which they were connected via a multiway tap (Partridge & Thomas, 1975). Tubing used for the CO_2 solutions was stainless steel, for the others, polyethylene.

The normal CO_2 Ringer was equilibrated with 2·3% CO_2 in O_2, had a pH of 7·5 and the following composition (mM): KCl, 4; NaCl, 80; $CaCl_2$, 7; $MgCl_2$, 5; $NaHCO_3$, 21; Na phosphate, 0·1. Other CO_2 Ringers (0·5, 0·9, 2·5 and 4·6%) were made with the same ratio of $NaHCO_3$: CO_2 to keep the pH at 7·5, and had either added Na HEPES (2-*N*-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid) or reduced NaCl to maintain tonicity. The CO_2-free Ringer was equilibrated with air, had a pH of 7·5, and the same composition except for 20 mM-Na HEPES instead of $NaHCO_3$, and the omission of phosphate. Cl-free solutions were made by substituting isotonic amounts of sulphates for chlorides, and Na-free solutions by substituting bis (2-hydroxyethyl) dimethylammonium chloride (BDAC, obtained from Eastman Organic Chemicals) for NaCl, and Tris-bicarbonate for $NaHCO_3$.

SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid) was obtained from British Drug Houses Chemicals Ltd, and made up as a 1 mM stock solution in distilled water.

Conventional micro-electrodes. These were made from filamented 2 mm borosilicate glass tubing and filled with 2·5 M-KCl or 0·6 M-K_2SO_4 for voltage recording, and 2·5 M-KCl, 2 M-HCl, or 2 M-NaCl for iontophoretic injection. The K_2SO_4-filled micro-electrodes used (25–40 MΩ) were found to record a lower membrane potential

than a KCl-filled electrode in the same cell. Experiments in which K_2SO_4 electrodes were used were therefore corrected to allow for this, by measuring the voltage difference between the K_2SO_4 and a KCl micro-electrode in the same cell at the end of each experiment.

pH and Na^+-sensitive micro-electrodes. These were made as previously described (Thomas, 1976*b*) except that the compressed gas used in the final seal of the pH electrode was supplied at a higher pressure than previously. Gas at a pressure of about 10 bar was taken from a cylinder via a nylon tube to a stainless steel tube sealed into the top of the pH glass micro-pipette with wax. The higher pressure

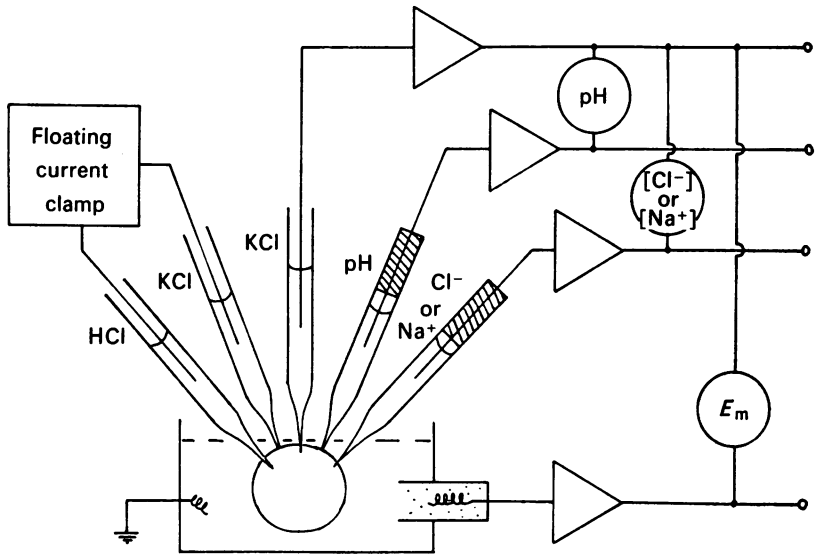

Fig. 1. Diagram showing the basic experimental arrangement. Only one of the experiments described used all five micro-electrodes illustrated. For details see text.

allowed the glass–glass seal to be made closer to the tip of the pH-sensitive glass, (giving a smaller recess volume and a faster response time) than possible previously.

The pH-sensitive micro-electrodes were calibated by running a pH 6·5 Ringer through the bath. The Na^+-sensitive micro-electrodes were calibrated in a series of Ringer solutions with constant ionic composition except for lowered Na and correspondingly elevated K concentrations.

Cl^--sensitive liquid-ion-exchanger micro-electrodes. These were made from siliconized borosilicate glass micropipettes as described by Ascher, Kunze & Neild (1976). For reasons described in the Results section, they were calibrated in a series of solutions in which HEPES was the main anion. The Cl-free calibration solution had the following composition (mM): K salt of HEPES (pH 7·5), 125 and Na_2SO_4, 10. To make a range of calibrating solutions 1 M-KCl was added to give 1, 2, 5, 10 and 20 mM-Cl^- concentrations. (Although the KCl addition made the solutions hypertonic, this did not significantly affect the response of the electrode.)

Electrical arrangements. These are illustrated in Fig. 1; although most experiments were performed with only two or three of the electrodes shown. The two current-passing micro-electrodes on the left were connected to a 'floating current clamp'

(Thomas, 1975) which allowed pre-determined currents to be passed between the two electrodes or between one electrode and the bath. Between injections a backing current of 5 nA was passed in the reverse direction. The central electrode was for recording E_m and the two electrodes on the right for recording pH and $[Cl^-]_i$ or $[Na^+]_i$. The E_m and bath electrodes were connected to unity-gain FET operational amplifiers, and the two ion-sensitive electrodes to varactor diode operational amplifiers (Analog Devices AD311J) set up to give a unity gain. Voltages from the electrodes were recorded on a multi-channel potentiometric pen-recorder as follows: E_m was the difference between the outputs of bath electrode and E_m micro-electrode amplifiers, pH the difference between the low-pass filtered output of the E_m amplifier and the pH amplifier, and Cl^- or Na^+ the difference between the filtered E_m output and the Cl^- or Na^+ amplifier. The voltage from the E_m electrode and one of the current-passing micro-electrodes was also displayed on an oscilloscope.

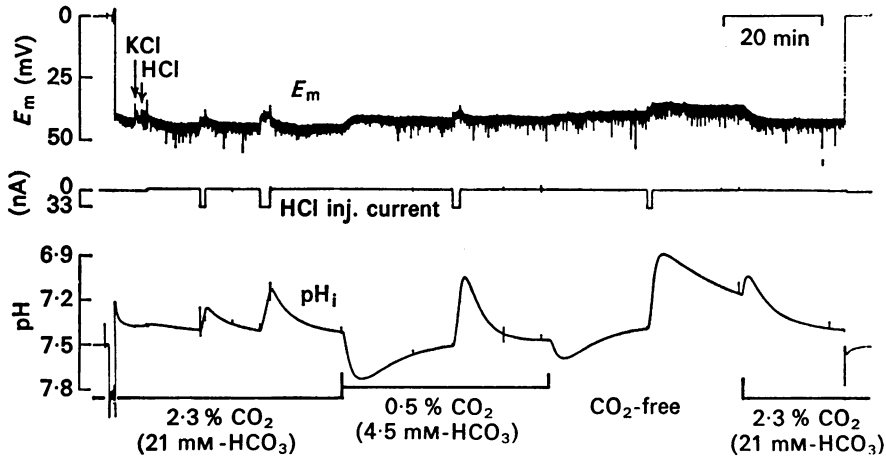

Fig. 2. Pen-recording of an experiment to show the effects of different levels of CO_2 and bicarbonate on the pH_i recovery from HCl injection. The voltage recorded by the KCl-filled membrane potential electrode (E_m) is shown at the top, the current passed between the two injection electrodes is shown in the middle, and the voltage recorded by the pH-sensitive micro-electrode is shown at the bottom. The points at which the two injection micro-electrodes were inserted into the cell are indicated above the E_m record. The CO_2 and bicarbonate concentration of the superfusing Ringer is indicated below the pH_i record. The long time constant of the pen-recorder has reduced the recorded size of the spontaneously occurring action potential to only a few millivolts. External pH was 7·5 throughout in all experiments illustrated except that shown in Fig. 9.

RESULTS

The effect on the pH_i recovery from an acid injection of reducing external bicarbonate

Fig. 2 illustrates an experiment in which HCl was injected into a snail neurone four times. For the first two injections the cell was superfused with 2·3 % CO_2 Ringer containing 21 mM-HCO_3^-. The pH_i fell during the

injection, and then recovered. The solution was then changed to one containing 0·5 % CO_2 and 4·5 mM-HCO_3^-. After the third injection of HCl the pH_i again recovered rapidly. Then CO_2-free Ringer was applied, and the fourth injection made. This time the pH_i recovered much more slowly than before. Finally the 2·3 % CO_2 Ringer was replaced, the pH_i transiently fell as CO_2 entered the cell, and then rapidly recovered.

After each acidification, the pH_i recovered exponentially, as shown for the 2nd, 3rd and 4th injections in Fig. 3*A*. The time constants were 4·26, 4·45, and 21·35 min respectively. The similarity of the time constants for pH_i recovery in 21 and 4·5 mM-HCO_3^- suggests that the recovery process was not affected by HCO_3^- changes in this range, but this similarity is deceptive as the different intracellular buffering powers in the two solutions need to be taken into account. It is quite clear, however, that HCO_3^- removal greatly slows pH_i recovery.

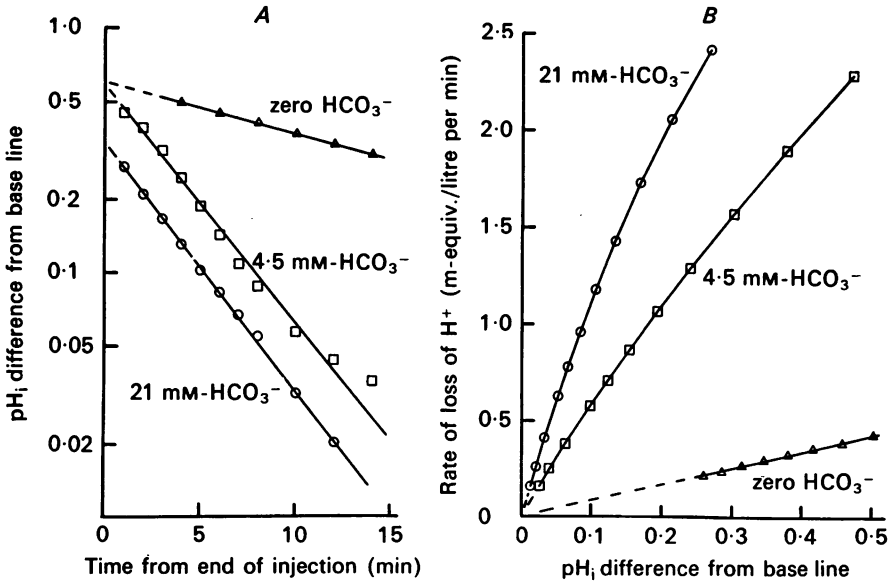

Fig. 3. Graphs of data taken from Fig. 2. *A*, the pH_i difference from its base line level during recovery from the second, third and fourth injections plotted on a logarithmic scale against time from the end of the injection. *B*, the calculated rate of loss of H^+ ions for the same three pH_i recoveries plotted against pH_i difference from base line level.

As shown previously, (Thomas, 1976*a*) increasing external HCO_3^- at constant pH greatly increases the internal buffering power. The higher the buffering power the more H^+ ions will have to be transported out of a cell (or otherwise neutralized) to achieve a given pH_i change. The *relative* buffering powers in the three solutions used in the experiment shown in Fig. 2 can be calculated from the ratio of the amount of HCl injected (given by the charge) and the pH_i change (the latter was

determined by extrapolating a graph of pH_i against time back to the end of the injection as described by Aickin & Thomas, 1977*a*). To determine the *absolute* buffering powers, the buffering power in CO_2-free conditions was first calculated. This was done by measuring the pH_i change caused by CO_2 addition or removal. When 0·5 % CO_2 was removed, the pH_i change extrapolated back to one minute after CO_2 removal was 0·23, and the calculated internal HCO_3^- before CO_2 removal was 4·15 mM. Assuming that all the HCO_3^- ions left the cell as CO_2, in effect leaving behind 4·15 mM-OH^-, the buffering power was 4·15/0·23, or 18·1 m-equiv H^+/pH unit per litre. Similarly the buffering power from the pH change observed on addition of 2·3 % CO_2 (extrapolated back to the time of application, since pH_i recovers much faster from acidification than from alkalinization) gave a buffering power of 18·5. Taking the non-CO_2 buffering power as 18·3, the acid injected during the 4th injection can be calculated from the pH_i change to be 10·6 m-equiv/l. cell volume. Knowing the relative size of the other injections, it is simple to calculate the corresponding buffering powers. They were 62·3 and 63·5 from the first two injections, and 26·6 from the third. The buffer values calculated from the non-CO_2 buffering power, plus a value for the contribution from intracellular HCO_3^-, were 55·1 and 28·6 in 2·3 % and 0·5 % CO_2 respectively. But in CO_2, the buffering power is not constant: it varies with $[HCO_3^-]_i$ and thus pH_i (Thomas, 1976*a*).

To convert the graphs of pH_i against time into some measure of the rate of H^+ extrusion, the gradient must be multiplied by the buffering power. Since in CO_2 the buffering power varies, a separate value for each pH_i point is required. This was calculated from the non-CO_2 buffering power plus the calculated HCO_3^- concentration multiplied by 2·3, and the values were used to plot the points shown in Fig. 3*B*. This shows the effect of changing $[HCO_3^-]_o$ much more clearly than Fig. 3*A*. For a displacement from the base-line pH_i of 0·1 pH units, the rates of H^+ extrusion in CO_2-free, 0·5 and 2·3 % CO_2 were respectively 0·08, 0·59 and 1·13 m-equiv H^+/l. cell volume per min. Putting it another way, the H^+ extrusion process is 14 times faster in 2·3 % CO_2 than in CO_2-free Ringer (another experiment showing the inhibitory effect of CO_2 removal is illustrated in Fig. 7).

The inhibitory effect of HCO_3^- removal on pH_i recovery in squid giant axons and snail neurones has been reported previously (Boron & De Weer, 1976; Thomas 1976*a*), but only in qualitative terms. It should be noted that in the present experiments the CO_2-free solution was only nominally free of HCO_3^-, since it was equilibrated with air. Presumably the preparation itself was also producing some CO_2.

The effect on the pH_i recovery of reducing external chloride

If external HCO_3^- is involved in the pH_i recovery system, it seems unlikely that external Cl^- is too. This is confirmed by experiments such as that illustrated in Fig. 4. Acid was injected three times, and for the second injection the cell was superfused with a Cl^--free Ringer in which sulphate was the principal anion. The anion injected with the H^+ was Cl^-, and the E_m was recorded with a KCl-filled micro-electrode, so $[Cl^-]_i$ was probably higher than normal in spite of the absence of external Cl^-.

It is clear that pH_i recovery is not inhibited by the removal of external Cl^-: if anything the recovery is accelerated. The change in E_m is probably

largely an artefact, since the bath reference electrode gives a potential change when Cl^- is changed.

The effect on pH_i recovery of reducing internal chloride

If part of the pH_i recovery from intracellular acidification involves neutralization of the excess H^+ by a HCO_3^- influx, then it seems probable that the entering HCO_3^- is exchanged for internal Cl^- ions. If so, a reduction in the internal Cl^- ion concentration ($[Cl^-]_i$) should slow pH_i recovery. An experiment to test this is illustrated in Fig. 5.

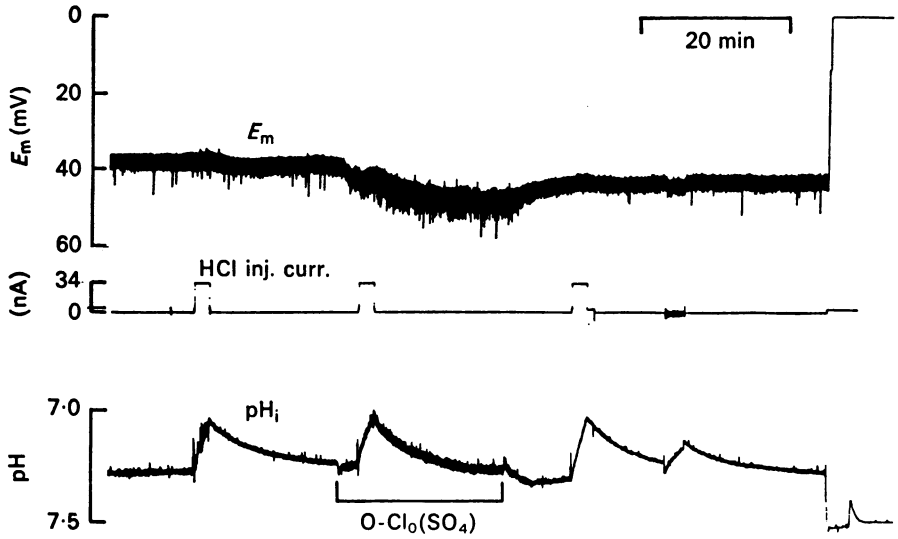

Fig. 4. Pen-recording from experiment showing the effects of removal of external Cl^- on the pH_i recovery after HCl injection. For the period indicated the cell was exposed to Cl^--free sulphate Ringer. About 10 min after the third injection there was a fault in the injection circuit, causing an unintentional HCl injection. E_m was recorded with a KCl-filled microelectrode.

To avoid raising $[Cl^-]_i$, E_m was recorded with a K_2SO_4-filled microelectrode, and both pH_i and $[Cl^-]_i$ were recorded with ion-sensitive microelectrodes. Initially the preparation was superfused with CO_2-free Ringer. About 10 min after the beginning of the experiment the Ringer was changed to one equilibrated with 2·3 % CO_2. As CO_2 entered the cell, pH_i fell, and then recovered. As pH_i recovered, $[Cl^-]_i$ decreased. When the pH_i had recovered to its previous level, external CO_2 was removed, leading to a rapid rise in pH_i as previously described (Thomas, 1976*a*). Before pH_i had completely recovered, the normal CO_2-free Ringer was replaced by a CO_2-free Ringer in which 90 % of the chloride had been replaced

by sulphate. This caused a decrease in $[Cl^-]_i$, as Cl^- ions presumably left the cell. When $[Cl^-]_i$ had fallen to about 2 mM, CO_2 was again applied, but with external Cl still kept at 10 % of its normal level. As CO_2 entered the cell, pH_i again fell. But this time it only recovered slightly. After 8 min the CO_2 was removed and pH_i allowed to stabilize, still in 10 % external Cl. Then the cell was penetrated with an 18 MΩ KCl-filled micro-electrode. Leakage from this electrode caused $[Cl^-]_i$ to rise only slowly.

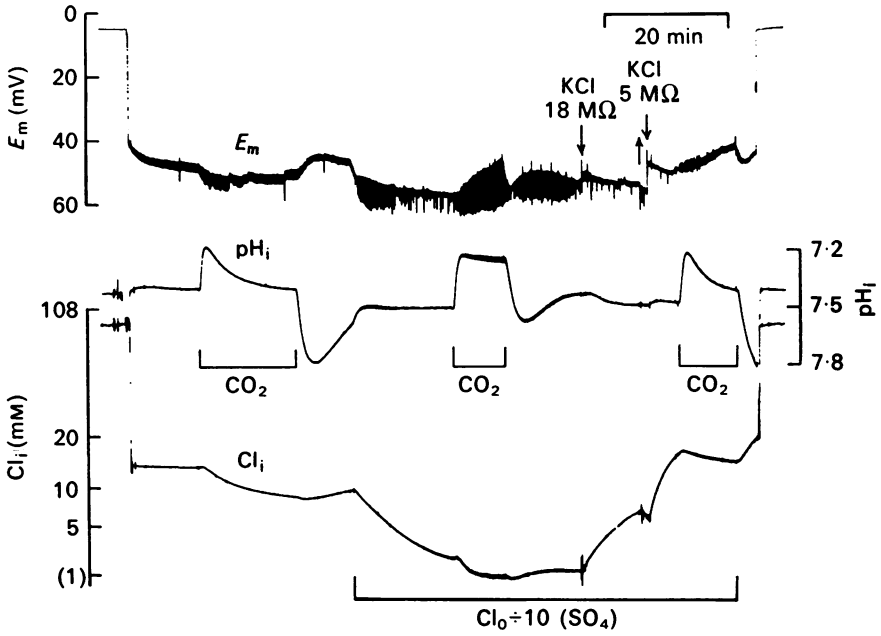

Fig. 5. Experiment showing the effects of CO_2 application on E_m, pH_i and $[Cl^-]_i$, and the effects on the pH_i recovery from CO_2 application of exposing the cell to low Cl^- Ringer (a solution in which 90 % of the Cl^- was replaced by SO_4^{2-}). E_m was recorded with a K_2SO_4-filled micro-electrode. At the points indicated an 18 MΩ, KCl-filled micro-electrode was pushed into the cell, withdrawn, and re-inserted after the resistance had been reduced to 5 MΩ.

The KCl electrode was therefore removed, broken to a resistance of 5 MΩ, and re-inserted. This caused $[Cl^-]_i$ to increase relatively rapidly. When $[Cl^-]_i$ had reached about the same level as at the beginning of the experiment, CO_2 Ringer was applied for a third time, with external Cl still being kept at 10 % of its normal level. Again pH_i fell as CO_2 entered the cell, but this time the pH_i recovered as rapidly as during the first exposure to CO_2.

This experiment thus not only shows that $[Cl^-]_i$ decreased as pH_i recovered from acidification by CO_2 application, but also that pH_i

recovery was blocked by depleting the cell interior of Cl. The pH_i recovery was not affected by the reduction in external Cl, as shown by the response to KCl injection.

The effect of pH_i recovery on the internal Cl^-

During the recovery of the pH_i from the first application of CO_2 shown in Fig. 5, $[Cl^-]_i$ decreased in parallel with the increase in pH_i. Another experiment showing this relationship is illustrated in Fig. 6. Three different levels of CO_2 were applied at intervals, each causing a transient intracellular acidification, the extent of the acidification being related to the concentration of CO_2. As the pH_i recovered during the exposures to

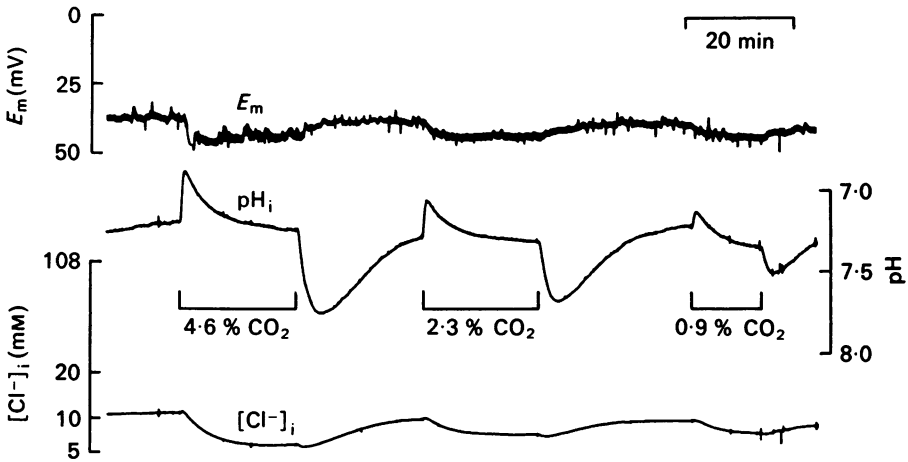

Fig. 6. Part of an experiment showing the effect on E_m, pH_i and $[Cl^-]_i$ of three different levels of CO_2. E_m was recorded with a K_2SO_4-filled microelectrode.

CO_2, there was a corresponding decrease in $[Cl^-]_i$. The change in $[Cl^-]_i$ from its value before CO_2 application to its value *at the point when pH_i had recovered to its pre-CO_2 value* was 5·7 mM for 4·6 % CO_2, 2·6 mM for 2·3 % CO_2 and 1·1 mM for 0·9 % CO_2. From the values of pH_i at these times, the calculated $[HCO_3^-]_i$ values were 23, 14·2 and 5·0 mM respectively. (For description of the calculation of $[HCO_3^-]_i$ from pH_i see Thomas, 1976*a*). Since these levels of HCO_3^- were accumulated with no net pH_i change, they are equivalent to the total HCO_3^- and OH^- uptake and H^+ extrusion that had occurred by that point in response to CO_2 application. Thus the ratios of Cl^- lost to HCO_3^- accumulated were 0·25, 0·18 and 0·22 respectively. For a total of seven similar measurements of the fall in $[Cl^-]_i$ during pH_i recovery from CO_2 application, the mean $Cl^-:HCO_3^-$ ratio was $0·25 \pm 0·05$ (S.D.).

The method used to calibrate the Cl^--sensitive micro-electrodes was rather unorthodox, and perhaps needs explanation. The liquid-ion exchanger used in these electrodes is sensitive to a wide variety of anions as well as to Cl^- (Walker, 1970). Some of these anions are present intracellularly in unknown amounts. Thus when a snail neurone is depleted of Cl^- by a long exposure to Cl^--free, sulphate Ringer, the apparent intracellular Cl^- activity does not fall to zero on a scale obtained by calibrating the electrode in pure KCl solutions. After 30 min, $[Cl^-]_i$ apparently stabilizes at a value of about 4 mM. In the experiments described here it was therefore assumed that $[Cl^-]_i$ did in fact reach a level close to zero during a long exposure to Cl-free Ringer and it was assumed that the Cl^--sensitive electrode's failure to record an apparent zero internal Cl^- activity was because of interference from unidentified intracellular anions. One of these anions is presumably sulphate, which would enter the cell both by leakage from the K_2SO_4-filled conventional micro-electrode and across the cell membrane.

To make calibrating solutions for the Cl^--sensitive micro-electrodes the following trial and error approach was therefore used. A cell was depleted of internal Cl^- by exposing it for 30 min to a Cl-free sulphate Ringer. The Cl-sensitive electrode voltage difference between inside and outside the cell, (E_m subtracted) was then measured, giving a value of about 90 mV. A series of approximately isotonic, Cl-free solutions were then made up, containing roughly intracellular levels of K and Na, and their effect on the potential of the Cl^--sensitive micro-electrode determined. The only Cl^- substitute tested that gave a potential near that found in a Cl^--depleted cell was HEPES. A solution containing 100 mM-HEPES adjusted to pH 7·5 with KOH and 10 mM-Na_2SO_4 gave a potential change (normal Ringer being the reference solution) of 85 mV, but its osmolarity was rather low. It was found that 125 mM-K-HEPES, with 10 mM-Na_2SO_4, had an osmolarity close to that of normal CO_2-free Ringer and gave a potential change of 87 mV. This solution was therefore chosen to give the zero Cl^- point on the calibrating curve. Calibrating solutions for 1, 2, 5, 10 and 20 mM-$[Cl^-]$ were obtained by adding the appropriate volume of 1 M-KCl to the zero chloride solution.

The effect on the pH_i recovery of the removal of external Na

The preceding results confirm the earlier suggestion that the pH_i regulating system in snail neurones involves Cl^--HCO_3^- exchange (Thomas, 1976*c*). Such an exchange involves the movement of both ions against their concentration gradients, and therefore consumes energy. In the squid giant axon, Cl^--HCO_3^- exchange requires intracellular ATP (Russell & Boron, 1976) but in snail neurones preliminary experiments with metabolic inhibitors suggest that ATP is not required (R. C. Thomas, unpublished observations). A possible alternative energy source is provided by the large transmembrane Na gradient.

Fig. 7 illustrates an experiment in which three injections of HCl were made into a snail neurone initially superfused with CO_2 Ringer. A KCl-filled micro-electrode was used to record E_m. After the first control injection, pH_i recovered exponentially in the normal way. The cell was then exposed to a CO_2 Ringer in which all the NaCl had been replaced by bis (2-hydroxyethyl) dimethylammonium chloride (BDAC). This caused a small depolarization and inhibition of the spontaneous action potentials,

but there was little effect on the pH_i. HCl was then injected with the same charge as used for the first injection. This caused a larger fall in pH_i than before, and the pH_i recovery was almost completely blocked. When the normal Ringer was replaced, pH_i rapidly recovered. The replacement of external Na by Li also inhibited pH_i recovery from an acid injection but less effectively than when Na was replaced by BDAC (see Fig. 1 of Thomas, 1976*d*, which also shows that K-free Ringer does not inhibit pH_i recovery after acid injection).

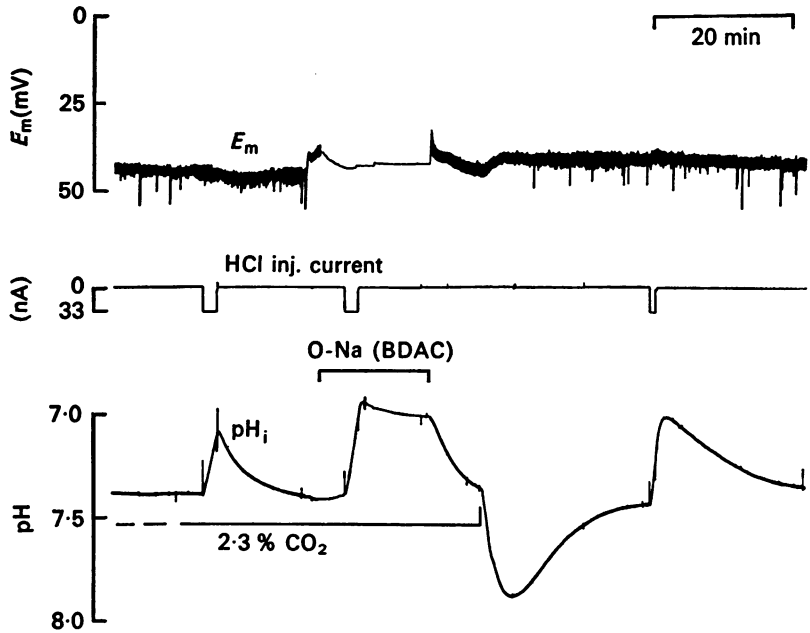

Fig. 7. Part of an experiment showing the effect on E_m and pH_i of three injections of HCl. The first injection was made with the cell in normal CO_2 Ringer, the second with the cell in Na-free CO_2 Ringer, and the third into the cell in normal CO_2-free Ringer. E_m was recorded with a KCl-filled micro-electrode.

The effects of pH_i recovery on the internal Na^+ concentration

The inhibitory effects of Na-free solutions on pH_i recovery suggest that Na^+-H^+ exchange may play a part in the pH_i regulating system. This would most probably involve external Na^+ ions entering the cell in exchange for internal H^+ ions. Since there is a large inward electrochemical gradient favouring Na entry, there is always a passive influx of Na^+ ions into the cell, normally balanced by the Na pump. If pH_i recovery from an intracellular acidification causes an extra Na influx, it should cause a transient rise in $[Na^+]_i$.

Fig. 8 illustrates an experiment designed to observe such a rise in $[Na^+]_i$. The cell was initially in CO_2-free Ringer, and was penetrated with Na^+-sensitive, pH-sensitive and K_2SO_4-filled conventional micro-electrodes. The injury to the cell caused by these penetrations led to a large rise in $[Na^+]_i$ and a fall in pH_i. When $[Na^+]_i$ had begun to recover, and pH_i had stopped falling, the cell was exposed to 2·5 % CO_2 Ringer. As usual, pH_i fell briefly and then recovered exponentially, in this case to a value higher than before CO_2 application. As pH_i recovered there was a

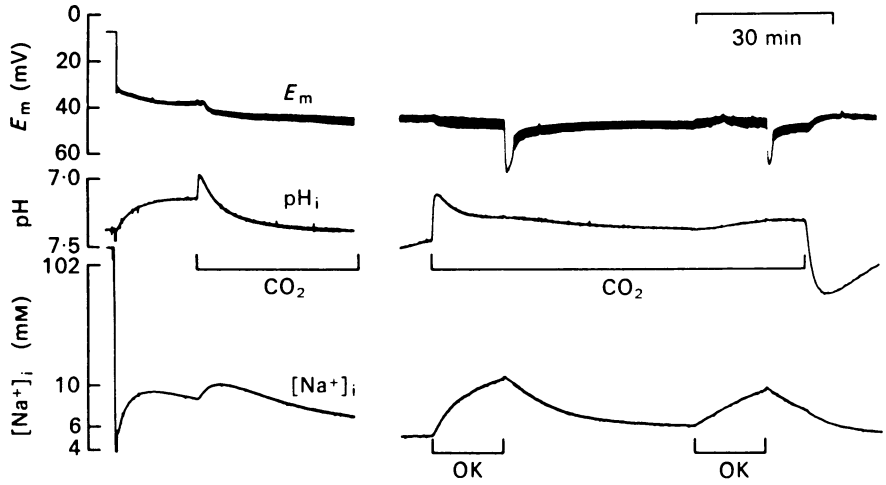

Fig. 8. Experiment showing the effects of CO_2 application and the effects of removal of external K on E_m, pH_i and $[Na^+]_i$. E_m was recorded with a K_2SO_4-filled micro-electrode.

transient rise in $[Na^+]_i$. External CO_2 was then removed, pH_i rose and $[Na^+]_i$ decreased slightly (not shown). Then CO_2 was reapplied, this time in a K-free Ringer. As before, pH_i fell and then recovered, and $[Na^+]_i$ rose. Since the Na pump was inhibited, $[Na^+]_i$ kept rising until K_0 was replaced. When this was done by applying the normal CO_2 Ringer there was a large, brief increase in E_m, due to the electrogenic nature of the Na pump. There was also a further increase in pH_i and a complete recovery of $[Na^+]_i$. Finally the cell was exposed to the K-free Ringer for a further 15 min, causing a slow rise in $[Na^+]_i$ and a small decrease in pH_i.

These results strongly suggest that Na^+-H^+ exchange does play a significant part in the pH_i recovery process. The rise in $[Na^+]_i$ seen on the first exposure to CO_2 was much faster than seen when the Na pump was inhibited by the removal of external K. This rules out the possibility that the CO_2-induced rise in $[Na^+]_i$ was due to an inhibition of the Na pump which might occur during pH_i recovery. Inhibition of the Na pump

at the same time as CO_2 application caused a faster rise in $[Na^+]_i$ than does pump inhibition alone.

Another experiment showing the relationship between pH_i recovery and $[Na^+]_i$ is shown in Fig. 9. On this occasion E_m was recorded by a KCl-filled micro-electrode, and the cell was initially superfused with CO_2 Ringer. There was apparently much less damage on electrode penetration than in the previous experiment, and pH_i and $[Na^+]_i$ were both relatively stable. When the external CO_2 was removed, pH_i rose and slowly returned

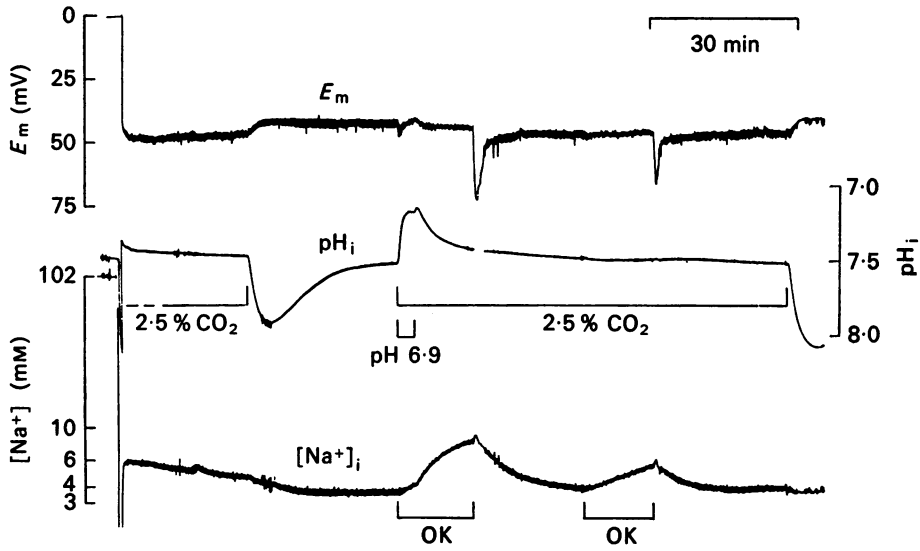

Fig. 9. Experiment showing the effects of CO_2 and removal of external K on E_m, pH_i and $[Na^+]_i$. E_m was recorded with a KCl-filled micro-electrode, and except where indicated the external pH was 7·5.

to its previous level, while $[Na^+]_i$ stabilized at about 4 mM. About 30 min later a K-free CO_2 Ringer was applied, initially at a pH of 6·9 (at such an acid pH_o, there is little recovery of pH_i from the CO_2-induced acidification). The pH_i fell and $[Na^+]_i$ started to increase. Three minutes later a K-free CO_2 Ringer at pH 7·5 was applied, and pH_i rapidly increased towards it normal level. As pH_i recovered, there was an accelerated increase in $[Na^+]_i$. When K was replaced, $[Na^+]_i$ recovered and pH_i increased slightly. About 20 min after the return to normal K_o, the cell was again exposed to K-free CO_2 Ringer and $[Na^+]_i$ increased linearly until K was replaced.

This experiment confirms that the pH_i recovery process causes an extra increase in $[Na^+]_i$ on top of that occurring when the Na pump is inhibited. It also shows that intracellular acidification itself does not cause an extra

Na influx; only when pH_i was recovering was there a significant additional rise in $[Na^+]_i$.

It is possible to estimate how much Na entered the cell by way of pH_i recovery by assuming that the Na influx through pathways other than Na^+-H^+ exchange was the same whether or not the pH_i recovery system was activated by CO_2 application. In the experiment shown in Fig. 8, at the end of the first 14 min period in K-free Ringer $[Na^+]_i$ had increased by 8·2 mM. At the end of the second period in K-free Ringer the increase in $[Na^+]_i$ was 4·6 mM. Thus the pH_i recovery (to 7·28) that

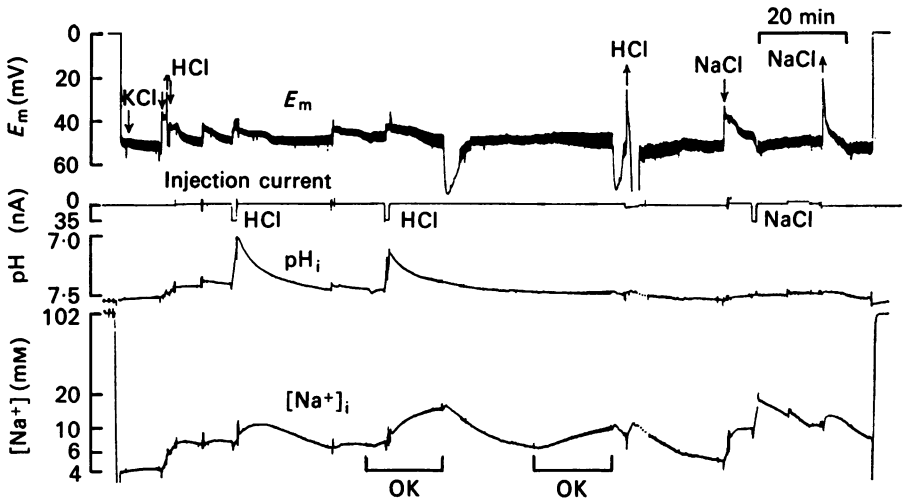

Fig. 10. Experiment showing the effects of two injections of HCl and one of NaCl, and of two periods of K-free Ringer on E_m, pH_i and $[Na^+]_i$. The points at which the various injection electrodes were inserted and removed are indicated above the E_m record. E_m was recorded with a KCl-filled micro-electrode, and the cell was superfused with CO_2 Ringer throughout the experiment. For a short period after the withdrawal of the HCl injection electrode the backing current was inadvertently passed across the cell membrane.

occurred during the first exposure to K-free Ringer caused an additional rise in $[Na^+]_i$ of 3·6 mM. The $[HCO_3^-]_i$ accumulated during this pH_i recovery, calculated from the pH_i, was 12·1 mM. Taking HCO_3^- accumulation as a measure of the total H^+ extrusion and HCO_3^- influx that occurred during the pH_i recovery, the Na^+-H^+ ratio was 3·6/12·1 or 0·30. In the experiment of Fig. 9, and in another similar experiment not illustrated, the calculated Na^+-H^+ ratios were 0·14 and 0·28 respectively.

Comparison of the effects of HCl and NaCl injection on $[Na^+]_i$

A more direct determination of the ratio between the extra Na influx occurring via the pH_i recovery process and the total H^+ extruded (or otherwise neutralized by OH^- or HCO_3^- influx) is possible if HCl and NaCl can be injected into the same cell. Fig. 10 shows the only experiment where this was done successfully. The cell was in CO_2 Ringer throughout, and was penetrated with a KCl-filled E_m electrode, pH and Na^+-sensitive micro-electrodes, and KCl and HCl current-passing micro-electrodes. The first attempt to insert the HCl electrode was unsuccessful, so it was withdrawn and re-inserted, as shown by the three arrows. This procedure caused a fall in E_m and a rise in $[Na^+]_i$, presumably because of damage to the cell membrane. When pH_i and $[Na^+]_i$ had almost stabilized, two injections of HCl were made, the second during a period of exposure to K-free Ringer. After about 20 min in normal Ringer, the cell was again exposed to K-free Ringer, for the same time as before, but without acid injection.

During the first period in K-free Ringer $[Na^+]_i$ rose by 7·9 mM, during the second it rose by 3·3 mM. Thus the additional Na influx during pH_i recovery caused $[Na^+]_i$ to rise by an extra 4·6 mM.

For the last part of the experiment the HCl-filled micro-electrode was withdrawn from the cell, and, after 20 min, replaced with one filled with NaCl. Impaling the cell with this micro-electrode again caused some damage, and a rise in $[Na^+]_i$. There may also have been some leakage of NaCl from the electrode. When $[Na^+]_i$ had stabilized at 9·8 mM, NaCl was injected with the same charge as used for the second HCl injection. At the end of the injection $[Na^+]_i$ had increased by 8·5 mM.

Since HCl and NaCl were injected by the same charge, and the transport numbers for both injections were similar (Thomas 1976*a* and, unpublished observations) the amount of NaCl injected was probably the same as the amount of HCl injected during the second injection. Thus, if all the H^+ injected during the second injection was exchanged for Na^+, there should have been an extra rise in $[Na^+]_i$ during the pH_i recovery of 8·5 mM. But the observed extra rise in $[Na^+]_i$ was only 4·6 mM. Thus the Na^+-H^+ ratio of the pH_i recovery system was 4·6 : 8·5, or 0·54.

It must be pointed out, however, that the pH_i did not recover fully from the second HCl injection until some time after external K was replaced. Also in measuring the rise in $[Na^+]_i$ that occurred during the NaCl injection, no allowance was made for the fact that the Na pump was not inhibited during the injection. But errors from these two points will tend to cancel each other.

The effect of SITS and external Na removal on pH_i recovery

The experiments described so far suggest that both Cl^--HCO_3^- and Na^+-H^+ exchange play an important role in the pH_i regulating system of snail neurones. When external HCO_3^- or internal Cl^- are reduced, pH_i recovery is inhibited. When external Na is removed pH_i recovery is again inhibited. But the rate of change of pH_i with a totally inhibited pH_i regulating system is unknown. Perhaps inhibition of Cl^--HCO_3^- or

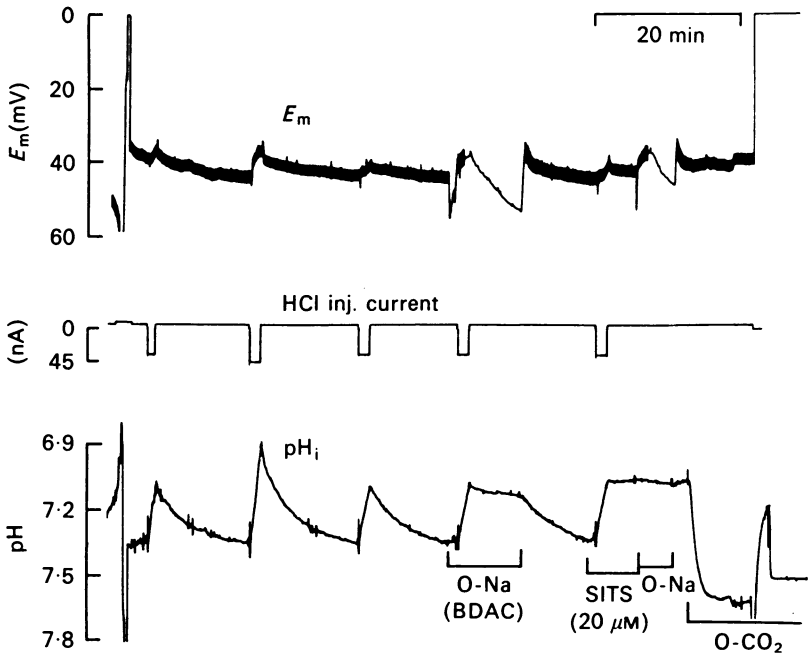

Fig. 11. The effects of removal of external Na and of the application of SITS on the E_m and pH_i recovery from HCl injection. The cell was in CO_2 Ringer except for the period indicated at the end of the experiment. E_m was recorded with a KCl-filled micro-electrode.

Na^+-H^+ exchange alone still leaves half the pH_i regulating system unaffected. If so, simultaneous inhibition of the hypothetical separate Cl^--HCO_3^- and Na^+-H^+ systems will have a much larger effect than inhibition of only one of the exchange systems. The experiment illustrated in Fig. 11 was designed to test this possibility.

The hypothetical separate Na^+-H^+ exchange system was inhibited reversibly by removal of Na, using BDAC as the substitute cation. The Cl^--HCO_3^- exchange was inhibited by SITS, which blocks anion exchange in red blood cells and has already been shown to inhibit irreversibly pH_i

recovery in snail neurones (Thomas, 1976*c*) and squid axons (Russell & Boron, 1976). The cell was penetrated by four electrodes, and HCl injected at intervals. The first three injections were in the normal CO_2 Ringer, and the pH_i recovered in the normal way after each injection. Then the Ringer was changed to Na-free CO_2 Ringer, and a fourth HCl injection made. As shown before in Fig. 7, pH_i recovered very little until external Na was replaced. Before the last HCl injection, the cell was exposed to CO_2 Ringer containing 20 μM-SITS. After this HCl injection, pH_i did not recover at all. The cell was again exposed to Na-free Ringer. The pH_i remained almost constant. Finally the cell was exposed to CO_2-free Ringer, and pH_i rapidly increased as CO_2 left the cell, showing that the pH-sensitive micro-electrode was still working.

Thus, the effects of SITS and Na-free Ringer are clearly not additive, and both treatments are therefore presumably inhibiting the same process.

DISCUSSION

The results show that the pH_i regulating system in snail neurones is inhibited by removing external Na^+ or HCO_3^-, or by reducing internal Cl^-. During pH_i recovery from intracellular acidification there is a fall in $[Cl^-]_i$ and a rise in $[Na^+]_i$. These observations suggest that the pH_i regulating system involves Na^+ and HCO_3^- influx and Cl^- efflux. But the pH_i regulating system is apparently not electrogenic: its operation involves no net transfer of charge across the cell membrane. This can be seen by comparing the effects on E_m in Figs. 8 and 9 of stimulating the pH_i regulating system by CO_2 application with those of stimulating the Na pump by replacing external K.

Since, as already mentioned, the effect of complete inhibition of the pH_i regulating system is unknown, it is hard to assess the degree of inhibition produced in the experiments described above. But if the removal of external Na and the application of SITS produce virtually complete inhibition when applied together as in Fig. 11, then they also produce almost complete inhibition when applied separately. Thus the simplest way to explain all the observations is to propose that the pH_i regulating system in snail neurones involves Na^+ and HCO_3^- influx coupled to H^+ and Cl^- efflux, as shown in Fig. 12, with equal movements of each ion: that is, with a stoichiometry of 1 Na^+:1 H^+:1 HCO_3^-:1 Cl^-.

While there is good evidence for the movement of Na^+ and Cl^- ions in the directions indicated, and good evidence for the participation of HCO_3^- ions, there is no evidence for the movement of H^+ ions. The pH_i regulating system could alternatively involve the influx of 2 HCO_3^- ions for each Na^+ entering or Cl^- leaving, or the entry of OH^- instead of the exit of H^+.

The present results do not allow any distinction between these alternatives; the scheme proposed in Fig. 12 is preferred largely on the grounds of symmetry, since it has equal numbers of ions entering and leaving the cell.

To what extent do the measured changes in $[Cl^-]_i$ fit in with this scheme? In CO_2, H^+ efflux has the effect of converting CO_2 to intracellular HCO_3^-. Thus, according to the proposed scheme, of the HCO_3^- accumulated during exposure to CO_2, half will come from external HCO_3^- exchanged

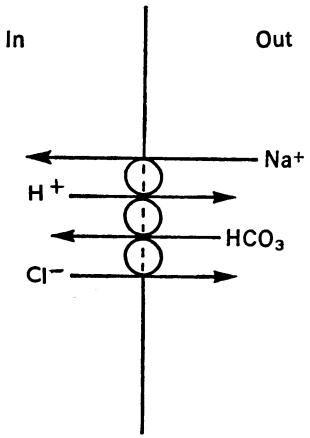

Fig. 12. Diagram illustrating the suggested ionic mechanism of the pH_i regulating system in snail neurones.

for internal Cl^-, and half will come from CO_2; the CO_2 being converted to H^+ and HCO_3^- ions and the H^+ ions then being extruded in exchange for Na^+. Thus the ratio of HCO_3^- accumulated to Cl^- lost during pH_i recovery from CO_2 application, when there was no net pH_i change, should be 2:1. But the average ratio of concentration change found was closer to 4:1. This discrepancy can be explained in a number of ways, since there is little evidence that the movement of equal numbers of Cl^- and HCO_3^- ions across the cell membrane causes equal changes in intracellular concentration. Indeed this would only occur if both the intracellular activity coefficients for, and the volume of the cell occupied by, Cl^- and HCO_3^- ions were the same.

Whilst it is probable that the intracellular activity coefficients are similar, it is unlikely that the effective cell volume is the same for the two ions. Intracellular organelles that had relatively acid interiors would tend to exclude HCO_3^-, but might well take up Cl^-. Thus the discrepancy between theoretical and practical HCO_3^--Cl^- ratios could be explained if HCO_3^- ions were excluded from about half the cell volume available to Cl^-.

A similar suggestion could explain the discrepancy between HCO_3^- and Na^+ accumulation during the recovery from CO_2 application. The proposed scheme requires the accumulation of one extra Na^+ for each 2 HCO_3^- ions. But the average ratio found was also close to 4 HCO_3^-: 1 Na^+. Again, this can be explained if only about half of the cell volume that is available to Na^+ is available to HCO_3^-.

Only one experiment was performed (because of technical difficulties) which avoided the problems of different effective cell volumes for different ions. In this the increase in $[Na^+]_i$ occurring during the recovery from an HCl injection was compared to the increase arising from the direct injection of NaCl into the same cell. The ratio of HCO_3^-:Na^+ (assuming all H^+ converted to HCO_3^-) was close to the expected 2:1.

The electrochemical gradient for Na^+ across the cell membrane is very large; adding E_m and the Nernst potential for Na^+ ions gives a figure of over 100 mV. The corresponding gradients for HCO_3^-, H^+ and Cl^- are all much smaller, about 30, 30 and 10 mV respectively. Thus, the proposed Na influx occurring by way of the pH_i regulating system is down a large electrochemical gradient, and the proposed movements of HCO_3^-, H^+ and Cl^- are all against relatively small gradients. Thus the downhill Na entry could perhaps provide all the energy needed to drive the other ion movements.

There is no evidence for the involvement of ATP in the pH_i recovery process in snail neurones. Preliminary experiments with metabolic inhibitors show that the pH_i regulating system is not inhibited by poisons applied at concentrations which completely block the Na pump. But the Na pump may simply require higher ATP:ADP ratios than are required by the pH_i regulating system.

The pH_i regulating system has so far only been investigated in a small number of preparations, but even so there appear to be three different ionic mechanisms. In squid giant axons pH_i regulation probably involves an ATP-driven Cl^--HCO_3^- exchange, as shown by Boron & De Weer (1976) and Russell & Boron (1976). A similar system seems to operate in barnacle muscle (Boron, 1977). But in mouse skeletal muscle most of the pH_i recovery process seems to involve Na^+:H^+ exchange, with a small but separate involvement of Cl^-:HCO_3^- exchange (Aickin & Thomas, 1977*b*). The one common feature seems to be Cl^-:HCO_3^- exchange which is probably ATP driven in all the preparations except snail neurones. The Na^+:H^+ exchange believed to occur in mouse skeletal muscle, and probably also in sheep heart Purkinje fibres (Ellis & Thomas, 1976) as well as snail neurones, appears to be driven by the Na^+ gradient. There is no obvious reason for this variety of mechanisms: the Na^+ gradient is almost certainly large enough in all the preparations to drive pH_i

regulation in the same way as in snail neurones. Perhaps the relatively high Cl^- permeability of muscle is important.

While the need for a highly active Na pump is well established, it is not clear why, in snail neurones at least, the pH_i regulating system appears to be able to restore pH_i faster than the Na pump can restore $[Na^+]_i$. This does not seem to be because fewer ions need to be transported to restore pH_i: equal injection of HCl and NaCl cause very similar changes in H^+ and Na^+ equilibrium potentials (see Fig. 10, or compare Figs. 2 and 3 of Thomas, 1976*c*). Perhaps the difference between the rates of restoration of $[Na^+]_i$ and pH_i is related to the relative net passive fluxes occurring *in vivo*. Under the conditions of my experiments the cell was superfused with a Ringer completely free of all organic compounds (other than CO_2 and HCO_3^-). But snail blood presumably contains a wide variety of weak acids and bases which may well considerably increase the cell membrane's effective H^+ permeability.

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