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THE IONIC MECHANISM OF INTRACELLULAR pH REGULATION IN CRAYFISH NEURONES

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SUMMARY

1. Intracellular pH (pH_i) regulation in crayfish neurones was studied using pH-, Na^+-, and Cl^--sensitive micro-electrodes. Neuronal pH regulation has previously been studied only in molluscs.

2. The average resting pH_i of crayfish neurones was $7{\cdot}12 \pm 0{\cdot}09$, which is 1 pH unit more alkaline than that predicted were H^+ ions distributed in equilibrium with the membrane potential.

3. When the cytoplasm was acidified (by NH_4Cl loading, CO_2 application, or HCl injection), pH_i recovered towards its resting value.

4. Removal of Na^+ from the external solution inhibited pH_i recovery from an acid load by more than 90 %. pH_i recovery resumed immediately when external Na^+ was reintroduced.

5. The resting intracellular Na^+ concentration ($[Na^+]_i$) of crayfish neurones was 15–25 mM. During pH_i recovery from an acid load, $[Na^+]_i$ increased by 10–50 mM.

6. Reducing the external HCO_3^- concentration from 5 mM to 0 mM slowed pH_i recovery by an average of about 45 %. This slowing was appreciable even in cells in which Na^+ removal almost totally blocked pH_i recovery.

7. The resting intracellular Cl^- concentration ($[Cl^-]_i$) was 30–40 mM, indicating that these cells actively accumulate Cl^-. During pH_i recovery from an acid load, $[Cl^-]_i$ decreased by 3–5 mM.

8. In the presence of the anion exchange inhibitor SITS (4-acetamide-4′-isothiocyanostilbene-2,2′-disulphonic acid), pH_i recovery was slowed to the rate which was normally seen in HCO_3^--free Ringer solution. SITS abolished the dependence of pH_i recovery on the external HCO_3^- concentration.

9. It is concluded that pH_i regulation in crayfish neurones involves two separate mechanisms: a Na^+-dependent, HCO_3^--independent acid extrusion process, and a Cl^-–HCO_3^- exchange which is probably also Na^+-dependent.

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INTRODUCTION

Cytoplasmic H^+ activity is an important variable affecting cell function. Changes in intracellular pH (pH_i) have been shown to participate in early post-fertilization events in sea urchin eggs (Grainger, Winkler, Shen & Steinhardt, 1979), to alter electrical coupling between cells of *Xenopus* embryos (Turin & Warner, 1980), and to increase the amplitude of Ca^{2+} action potentials in crustacean muscle fibres (Moody, 1980).

In most cells, cytoplasmic pH is maintained at a value higher than that predicted were H^+ ions distributed across the membrane in equilibrium with the membrane potential (Boron, 1980; but see Shen & Steinhardt, 1978). The mechanisms of active acid extrusion required to maintain high cytoplasmic pH in the face of passive leakage of H^+ into the cell and metabolic H^+ production have been studied in several cells. In snail neurones, pH_i regulation involves a single Na^+-dependent Cl^-–HCO_3^- exchange mechanism (Thomas, 1977). A similar system appears to operate in barnacle muscle fibres (Boron, 1977; Boron & Roos, 1978), and in the squid giant axon (Russell & Boron, 1979). On the other hand, in mouse muscle fibres (Aickin & Thomas, 1977) a Na^+-dependent mechanism of acid extrusion operates separately from $Cl^- - HCO_3^-$ exchange.

It is difficult to draw conclusions about the distribution of pH_i regulating mechanisms among different cells from the above studies. pH_i regulation in neurones has been studied only in molluscs, and the results obtained there seem to hold for invertebrate, but not mammalian muscle cells. It is possible that all neurones share the single mechanism of acid extrusion found in snail neurones, or that vertebrate and invertebrate neurones differ in this regard, or that, even among invertebrate neurones, differences in pH_i regulation exist, brought about by factors not yet discovered. To determine whether the pH_i regulating system described in molluscs applies to other invertebrate neurones, I have studied pH_i regulation in neurones of crayfish abdominal ganglia using ion-sensitive micro-electrodes. Acidifications were imposed on the cytoplasm and the active recovery of pH_i was monitored. Three methods were used to cause intracellular acidification: NH_4Cl acid loading (Boron & deWeer, 1976*a*), application of CO_2, and direct ionophoretic injection of HCl into the cell.

Following cytoplasmic acidification by any of these three methods, pH_i recovered to its normal resting value. I have investigated the effect of the ionic composition of the external solution on the rate of this pH_i recovery, and measured the changes in intracellular ionic concentrations which accompany pH_i recovery. The results indicate that unlike snail neurones, crayfish neurones have two separate mechanisms of acid extrusion: $Cl^- - HCO_3^-$ exchange, which appears to require external Na^+ (as in snail neurones), and a separate Na^+-dependent mechanism which does not involve HCO_3^-.

Some of these experiments have been reported in abstract form (Moody, 1979).

METHODS

Recordings were made from neurones in isolated abdominal ganglia of adult crayfish (*P. clarkii*). In most experiments the soma of the motor giant neurone (Takeda & Kennedy, 1964) was used, but occasionally experiments were done on other large cells in the ganglia. No differences were noted between the cells, although no systematic study of this point was made. Desheathed ganglia were pinned in a chamber with a solution volume of 0·3–0·5 ml. Ringer solution continuously superfused the preparation. Solutions were changed with minimal disturbance using a twelve-way rotary tap (Partridge & Thomas, 1975). Reservoirs containing CO_2 solutions were connected to the experimental bath by stainless steel tubing to prevent loss of CO_2 to the air. All experiments were done at room temperature.

Solutions

Normal crayfish Ringer solution had the following composition (mM): NaCl, 195; KCl, 5·4; $MgCl_2$, 5; $CaCl_2$, 13·5; $NaHCO_3$, 5; HEPES, 15. The Ringer solution was bubbled continuously with 0·5 % CO_2 (99·5 % O_2) and had a pH of 7·4. In HCO_3^--free Ringer solution, additional HEPES replaced $NaHCO_3^-$. Ringer solution equilibrated with 2·5 % and 5 % CO_2 contained 25 mM and 50 mM-HCO_3^-, respectively, replacing Cl^-, and 0·1 mM-phosphate to prevent the precipitation of $CaCO_3$. NH_4Cl, when used, replaced NaCl and was added to the Ringer solution as a solid immediately before use. NH_4Cl Ringer solution was also HCO_3^--free, since bubbling with CO_2 would tend to drive off NH_3 from the solution and thus reduce the concentration of NH_4Cl. In Na^+-free Ringer solution, BDAC (bis 2-hydroxyethyl dimethylammonium chloride) replaced NaCl, except in a few experiments in which the effects of Li^+ as a Na^+ substitute were tested. In experiments in which intracellular Cl^- was measured during CO_2 application (see Fig. 7), the normal Ringer solution contained 45 mM-HEPES instead of 20 mM, replacing Cl^-. Thus, in 5 % CO_2 the additional 45 mM-HCO_3^- replaced HEPES and the external Cl^- concentration could be held constant throughout the experiment.

Micro-electrodes

Conventional micro-electrodes were filled with 4 M-K acetate (NH_4Cl and CO_2 experiments) or 3 M-KCl (H^+ injection experiments), and had resistances of 10–40 MΩ. The membrane potential recording was referenced to an extracellular micro-electrode filled with 3 M-KCl and broken to a resistance of less than 1 MΩ. Injection of HCl was accomplished by passing current between two intracellular micro-electrodes, one containing 1 M-HCl and the other 3 M-KCl (Thomas, 1977).

pH-sensitive micro-electrodes were constructed according to Thomas (1978*b*), except that 750 lb/in^2 (50 bar) N_2 pressure was applied to the inner pipette to make the glass–glass seal. The voltage response of these micro-electrodes was 57–59 mV/pH unit, and the response was 90 % complete in 5–45 sec. Na^+- and Cl^--sensitive micro-electrodes were constructed according to Thomas (1978*b*). Na^+ electrodes gave a 56–58 mV response to a ten-fold change in Na^+ concentration (Li^+ substitution), with 90 % response times of 45–60 sec. Cl^- electrodes employed the Corning liquid exchange resin and gave a 54–57 mV response to a ten-fold change in Cl^- concentration (glucuronate substitution). Responses of Cl^- electrodes were complete within 5 sec, and were probably limited by the exchange time of the solution in the experimental chamber.

Electrical arrangements

Signals from the ion-sensitive micro-electrodes were recorded with varactor diode amplifiers (Analog Devices 311J). The difference signal between an ion-sensitive electrode and the membrane potential electrode was displayed on a chart recorder. The resistance of the membrane potential electrode was monitored continuously during the experiment.

Results from a given experiment were discarded if the resistance of the membrane potential electrode increased by more than a few MΩ on penetration or during the experiment, or if calibrations of the ion-sensitive micro-electrodes at the beginning and end of the experiment disagreed by more than 5 mV. All Figures are direct photographs of the chart records.

RESULTS

The normal pH_i of crayfish neurones and the application of an acid load

The penetration of a crayfish neurone by conventional and pH-sensitive micro-electrodes is shown in Fig. 1. The pH electrode was inserted into the cell first; a negative potential was recorded which represents the sum of the membrane potential and a potential proportional to pH_i. When the conventional micro-electrode was inserted the membrane potential was subtracted from the pH electrode signal and the lower trace began to record pH_i. After recovery from penetration, pH_i stabilized

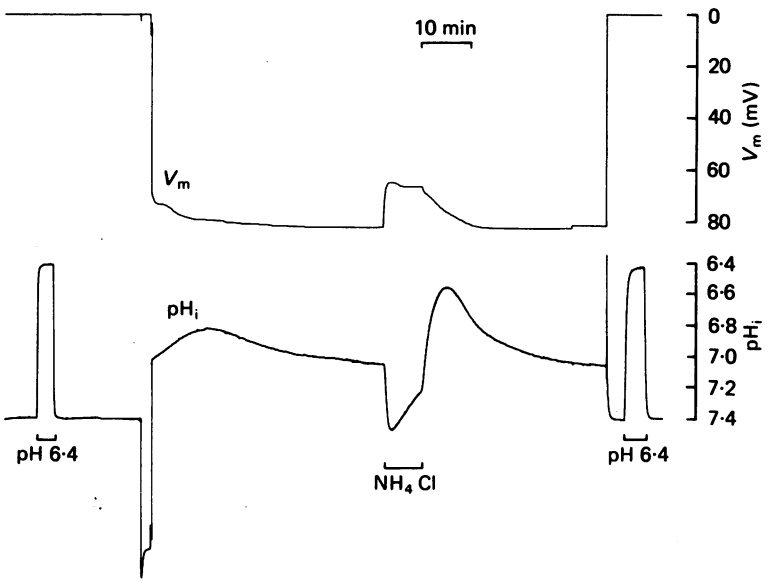

Fig. 1. Pen recording of an experiment showing penetration of a crayfish neurone with membrane-potential (upper) and pH-sensitive (lower) micro-electrodes, and the application of an NH_4Cl acid load. After calibration of the pH electrode in pH 6·4 Ringer solution, both electrodes were inserted into the cell, and when pH_i and membrane potential (V_m) had stabilized, the cell was exposed to 20 mM-NH_4Cl for 7 min. When NH_4Cl was removed, pH_i decreased to 6·47 and then recovered to its normal value. At the end of the experiment both electrodes were withdrawn and the pH electrode recalibrated. The Ringer solution contained 5 mM-HCO_3^-, except during exposure of the cell to NH_4Cl. The effects of NH_4Cl on V_m are similar to, but somewhat larger than, those seen under similar condition in squid axons (see Boron & deWeer, 1976, for discussion).

at 7·03 and the membrane potential at −80 mV. The average resting pH_i recorded in these experiments was $7{\cdot}12 \pm 0{\cdot}09$ (mean ± S.D.; $n = 32$), and the average resting potential $-76 \pm 7{\cdot}4$ mV ($n = 32$). pH_i in crayfish neurones is thus maintained approximately 1 unit more alkaline than that predicted from an equilibrium distribution of H^+ ions across the cell membrane.

The cell in Fig. 1 showed a pronounced acid injury on penetration. pH_i was 7·03 initially, but decreased steadily during the next 10 min to 6·8. pH_i then began to recover, but did not return to its initial value of 7·03 until almost 45 min after

penetration. Many of the neurones showed acid injury on penetration, but it was usually less pronounced than in Fig. 1. Acid injury was exacerbated if HCO_3^- was omitted from the Ringer solution, which normally contained 5 mM-HCO_3^-. This is probably due to the decreased intracellular buffering power in HCO_3^--free Ringer solution (Thomas, 1976), and to the slowing of pH_i regulation in the absence of external HCO_3^- (see below).

Fig. 1 also illustrates the NH_4Cl acid loading procedure. The cell was exposed to 20 mM-NH_4Cl Ringer solution for 7 min, during which time there was a rapid alkalinization of the cytoplasm followed by a slower acidification. The rapid alkalinization is caused by the entry of NH_3 into the cell and its association with cytoplasmic H^+. The slower acidification occurs as NH_4^+ ions enter the cell down their electrochemical gradient, probably through K^+ channels (Boron & deWeer, 1976*a*). When NH_4Cl is removed from the external solution, virtually all intracellular NH_4^+ ions dissociate and leave the cell as NH_3, since NH_3 is the more permeant species and its efflux is not retarded by the potential gradient across the membrane. This results in the net addition of H^+ ions to the cytoplasm, seen as a decrease in pH_i following NH_4Cl removal. In the cell in Fig. 1, pH_i fell to 6·47 following NH_4Cl removal.

Recovery from the acid load, which in this cell took about 20 min, represents the movement of H^+, or its equivalent, against an energy gradient. In the experiments shown in Figs. 2, 3 and 4, recovery from an NH_4Cl acid load is taken as a measure of the pH_i regulating system of the cell.

Effect on pH_i *recovery of removal of external* Na^+

In several cell types, pH_i recovery is linked to the influx of Na^+ ions from the external medium (Johnson, Epel & Paul, 1976; Aickin & Thomas, 1977; Thomas, 1977). To see whether this is also the case for crayfish neurones, pH_i recovery from an NH_4Cl acid load was recorded in the presence and absence of external Na^+. The results of one such experiment are shown in Fig. 2. In this cell the first exposure to NH_4Cl was followed by a period in normal Ringer solution, to show the control rate of pH_i recovery. The second acid load was carried out in Ringer solution in which all Na^+ had been replaced by BDAC. When NH_4Cl was removed this time, there was little or no recovery of pH_i. pH_i returned rapidly to its normal level when Na^+ was readmitted to the external solution. Thus Na^+-free Ringer solution blocks pH_i recovery almost completely. Results similar to those in Fig. 2 were obtained in twelve other cells, using both NH_4Cl removal and 5 % CO_2 application to produce acid loads.

To see whether pH_i recovery which had already begun could be stopped by removal of external Na^+, a third NH_4Cl acid load was made, initially in Na^+-free Ringer solution (Fig. 2). When pH_i had stabilized at 6·55, the cell was given a brief (2 min) exposure to Na^+-containing Ringer solution. pH_i recovered from 6·55 to 6·9 during the 2 min in Na^+-containing Ringer solution, but stopped abruptly when Na^+ was again removed. After 5 min in Na^+ Ringer solution, Na^+ was readmitted, and pH_i recovery resumed, bringing pH_i back to its normal resting value of 7·2.

Several experiments were carried out using Li^+ instead of BDAC as a Na^+ substitute. Na^+-free (Li^+) Ringer solution was about 80–90 % as effective in blocking pH_i recovery as Na^+-free (BDAC) Ringer solution, suggesting that Li^+ can substitute somewhat for Na^+ in supporting acid extrusion.

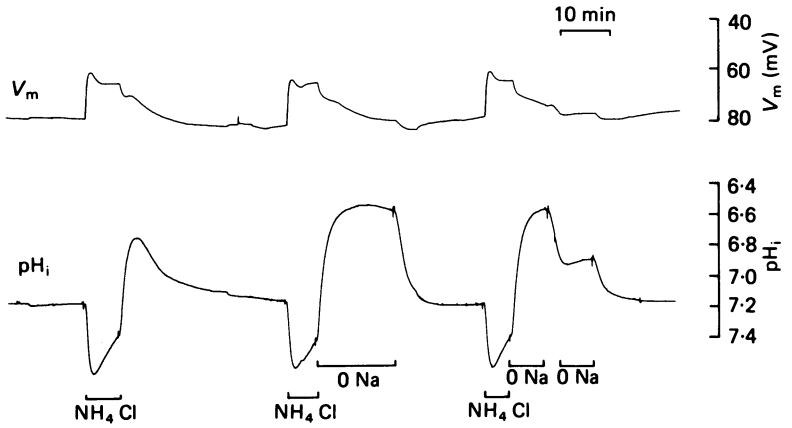

Fig. 2. Experiment showing the effect of removal of external Na^+ on pH_i recovery from an acid load. After recovery from penetration (not shown), the cell was exposed to 20 mM-NH_4Cl for 8 min. pH_i was allowed to recover from this acid load in normal Ringer solution. When pH_i had recovered completely, the cell was again exposed to NH_4Cl. This time, acid loading was done in Na^+-free Ringer solution (0 Na), and very little pH_i recovery occurred. Upon return to Na^+-containing Ringer solution, pH_i recovered rapidly to its normal value. A third NH_4Cl exposure was followed by Na^+-free Ringer solution (0 Na). When pH_i reached its lowest value (6·55) the cell was exposed to Na^+-containing Ringer solution for 2 min. This rapidly stimulated pH_i recovery. Na^+ was then again removed from the Ringer solution and pH_i recovery immediately stopped. After 7 min in Na^+-free Ringer solution, Na^+ was readmitted and pH_i recovered to its normal value. Ringer solution contained 5 mM-HCO_3^- throughout, except during the NH_4Cl exposures.

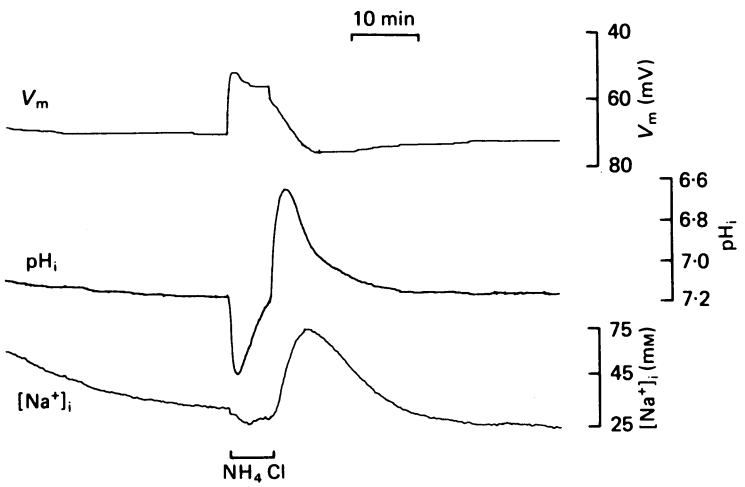

Fig. 3. Experiment measuring pH_i and $[Na^+]_i$ in the same neurone during application and recovery from an acid load. The slow decrease in $[Na^+]_i$ from the beginning of the record to the NH_4Cl application was recovery from penetration injury. Note that there was little sign of injury in either the pH_i or V_m records. When NH_4Cl was applied, there was little change in $[Na^+]_i$, Upon removal of NH_4Cl, pH_i decreased to 6·6 and then recovered. During intracellular acidification and recovery, $[Na^+]_i$ increased by nearly 50 mM. Following the end of this record the NH_4Cl application was repeated with the same result. The Ringer solution contained 5 mM-HCO_3^- throughout, except during the NH_4Cl exposure.

Effect of an acid load on $[Na^+]_i$

The fact that pH_i recovery is inhibited by Na^+-free Ringer solution does not prove that Na^+ influx is an obligatory part of pH_i regulation. The pH_i regulating system could simply require the binding of Na^+ to some external site, without necessarily transporting Na^+ ions. However, if Na^+ influx occurs, then an increase in $[Na^+]_i$ should be detected during pH_i recovery, presuming that Na^+ entry is large enough to overwhelm briefly the Na^+ pump. To test this prediction, several experiments were carried out in which pH_i and $[Na^+]_i$ were recorded simultaneously in the same neurone. Fig. 3 illustrates one such experiment. In this cell, after recovery from penetration, pH_i stabilized at 7·15 and $[Na^+]_i$ at 32·5 mM (by the end of the experiment $[Na^+]_i$ had decreased further to 25 mM). The cell was then exposed to 20 mM-NH_4Cl for 6 min. When NH_4Cl was removed, pH_i decreased to 6·62 and then recovered to its resting level. During the acidification and recovery, $[Na^+]_i$ increased from 32·5 mM to 75 mM, reaching its peak concentration at about the time of 50% pH_i recovery, and then slowly returned to somewhat below its previous level.

The increase in $[Na^+]_i$ during an acid load could be caused by the decreased pH_i itself rather than the processes governing its recovery. In Fig. 3, $[Na^+]_i$ did in fact start to increase immediately upon removal of NH_4Cl, well before actual pH_i recovery began. However, the pH_i regulating system probably begins to operate as soon as pH_i falls, and works against the post-NH_4Cl acidification while pH_i is still decreasing. An indication of this can be seen in Fig. 2. When pH_i regulation was inhibited by Na^+-free Ringer solution following the second NH_4Cl exposure, the acidification was almost 50% larger than in normal Ringer solution, indicating that the pH_i regulating system is activated before the lowest pH_i value is reached. The experiment shown in Fig. 4 provides evidence that at least part of the increase in $[Na^+]_i$ following NH_4Cl removal is caused by pH_i recovery, not the low pH_i itself. Here pH_i recovery was partially blocked by low external pH (I have found no pharmacological agent which blocks recovery completely), and the resulting changes in $[Na^+]_i$ were measured. The first acid load was carried out at an external pH of 7·4 and the following two at an external pH of 5·9. During the first acid load there was a transient increase in $[Na^+]_i$. During the second two, pH_i recovered only to 6·74, and a somewhat smaller overshoot of $[Na^+]_i$ occurred. In both cases, when the external pH was returned to 7·4, pH_i recovery resumed and a further increase in $[Na^+]_i$ occurred. It is apparent from Fig. 4 that at least part of the increase in $[Na^+]_i$ following NH_4Cl removal must coincide with pH_i recovery.

The facts that pH_i recovery requires external Na^+ and that $[Na^+]_i$ increases during pH_i recovery, indicate that Na^+ influx is a necessary part of the pH_i regulating system in crayfish neurones.

Effect of external HCO_3^- *concentration on* pH_i *recovery*

Cl^--HCO_3^- exchange participates in pH_i regulation in snail neurones (Thomas, 1977), mouse muscle fibres (Aickin & Thomas, 1977) and barnacle muscle fibres (Boron, 1977). If Cl^--HCO_3^- exchange is also involved in pH_i regulation in crayfish neurones then the rate of pH_i recovery from an acid load should be dependent on the external HCO_3^- concentration. To test this, pH_i was lowered by direct injection

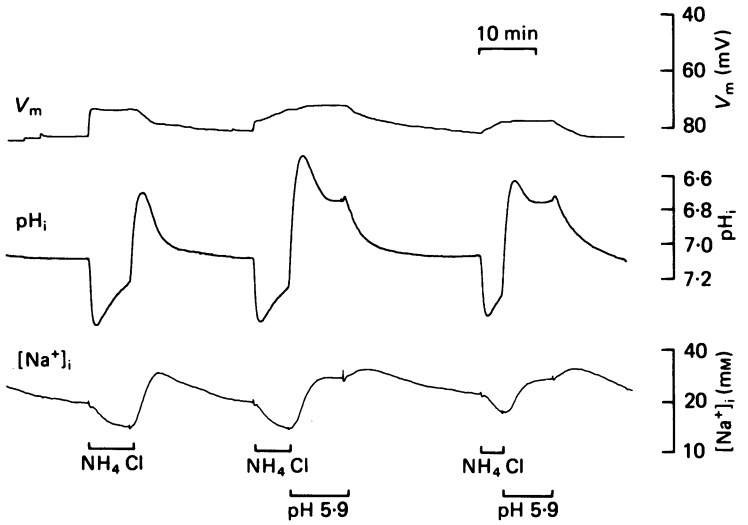

Fig. 4. Experiment measuring pH_i and $[Na^+]_i$ when pH_i recovery from an acid load was partially blocked by low external pH. In this cell, as in about half those studied, $[Na^+]_i$ decreased during exposure to NH_4Cl; this may be caused by stimulation of the Na^+ pump by NH_4^+ ions (see Aickin & Thomas, 1977). During the first acid load and recovery, $[Na^+]_i$ increased. When pH_i and $[Na^+]_i$ had returned to their resting levels a second NH_4Cl exposure was made and the external pH upon removal of NH_4Cl changed to 5·9. Only partial recovery of pH_i occurred in this solution, and the increase in $[Na^+]_i$ was smaller than after the first NH_4Cl exposure. When the external pH was returned to 7·4, pH_i recovered and a further increase in $[Na^+]_i$ occurred. The third acid load was also carried out initially at an external pH of 5·9, with the same result. The Ringer solution was HCO_3^--free throughout.

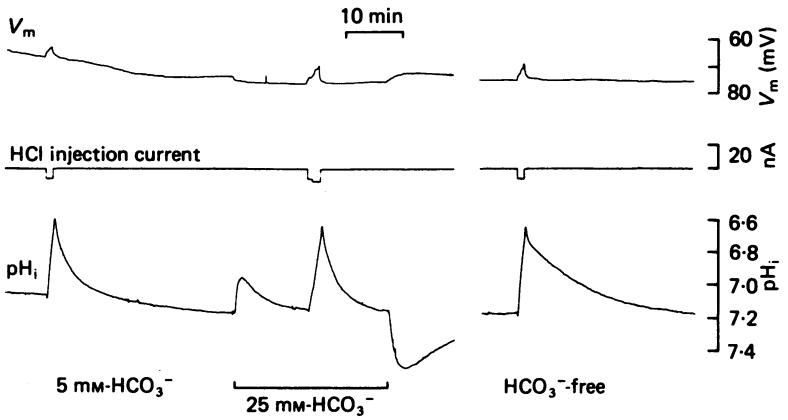

Fig. 5. Experiment showing the effect of external HCO_3^- concentration on pH_i recovery. pH_i was lowered by ionophoretic injection of HCl; the middle trace shows the injection current, which was passed between two intracellular micro-electrodes, one containing 1 M-HCl and the other 3 M-KCl. The first injection was made with the cell exposed to 5 mM-HCO_3^- Ringer solution. When pH_i had recovered, the HCO_3^- concentration of the Ringer solution was raised to 25 mM. This produced a transient acidification as CO_2 entered the cell. A second injection was then made to show the recovery of pH_i in 25 mM-HCO_3^-. When pH_i had recovered, HCO_3^- was removed from the Ringer solution. This produced a transient alkalinization as CO_2 left the cell, and the record is interrupted at this point for 20 min, during which pH_i returned to its normal value. Finally a third injection was made to show pH_i recovery in HCO_3^--free Ringer solution.

of HCl and recovery measured in Ringer solutions containing 0 mM-, 5 mM- and 25 mM-HCO_3^- (CO_2-free, 0·5% and 2·5% CO_2, respectively).

Fig. 5 illustrates one such experiment. The first HCl injection was made with the cell exposed to 5 mM-HCO_3^- Ringer solution. When pH_i had recovered, the external HCO_3^- was increase to 25 mM, producing a transient acidification as CO_2 entered the cell. When pH_i had returned to normal, a second HCl injection was made to measure the rate of pH_i recovery in 25 mM-HCO_3^-. Following removal of HCO_3^- and

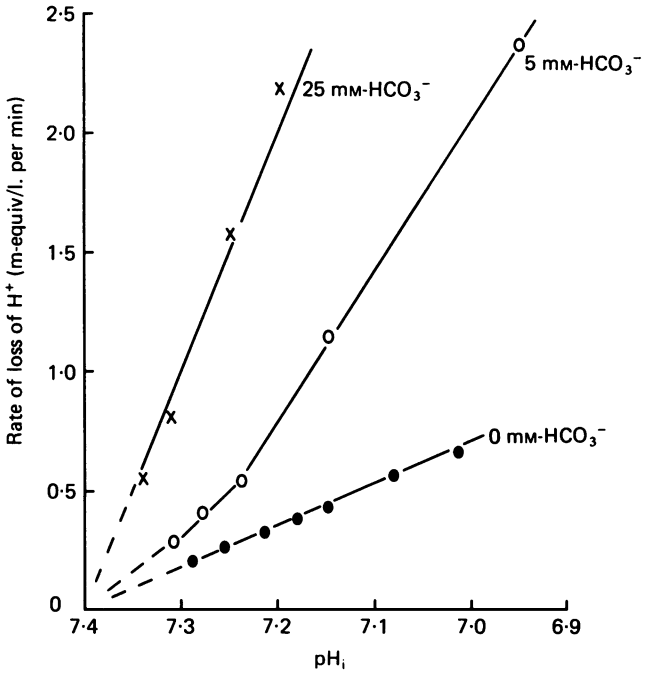

Fig. 6. Effect of HCO_3^- on the rate of H^+ extrusion. Graphs taken from the records in Fig. 5. The calculated rate of H^+ loss is plotted against pH_i for three different HCO_3^- concentrations. See text for details.

stabilization of pH_i, a third injection of HCl was made and pH_i recovery recorded in HCO_3^--free Ringer solution. It is clear from Fig. 5 that pH_i recovery was substantially slowed by removal of HCO_3^- from the Ringer solution, but was little affected by increasing the external HCO_3^- concentration from 5 mM to 25 mM. However, the intracellular buffering power is greatly increased in 25 mM-HCO_3^- Ringer solution (Thomas, 1976), so that a given amount of acid extrusion results in a much smaller change in pH_i than occurs in 5 mM-HCO_3^-. Therefore the similarity of pH_i recovery rates in 5 mM- and 25 mM-HCO_3^- indicates that the actual rate of acid extrusion is greater in 25 mM-HCO_3^-.

The rate of acid extrusion as a function of external HCO_3^- concentration is expressed more quantitatively in Fig. 6. Here, the records of Fig. 5 have been corrected for changes in intracellular buffering power (see below). These graphs show that the rate of acid extrusion increased as extracellular HCO_3^- concentration was raised. Acid extrusion was accelerated by a factor of 2·5 in 5 mM-HCO_3^-, and by

6·5 in 25 mM-HCO_3^-, as compared with the rate of HCO_3^--free Ringer solution. For comparison, raising the external HCO_3^- concentration from 0 mM to 25 mM in snail neurones increases the rate of acid extrusion by a factor of 14.

The plots in Fig. 6 were made as follows. From the chart records in Fig. 5, pH_i was measured at 2 min intervals during each of the three recovery curves. For each interval, $\Delta pH_i/\Delta t$ was calculated. Using the mean pH_i of each interval, the intracellular HCO_3^- concentration was calculated, assuming the P_{CO_2} values of Ringer solution and cytoplasm to be equal. The additional intracellular buffering power due to HCO_3^- was calculated for each pH_i value as 2·3 $[HCO_3^-]$ (Woodbury, 1965). This figure was added to the intrinsic buffering power of the cell, calculated to be 21 m-equiv/l.-pH from the pH_i change produced by the removal of 2·5% CO_2 (see Thomas, 1977). Thus the total intracellular buffering power, β, was determind for each pH_i value during the three recovery curves in Fig. 5. The rate of loss of H^+ from the cell at each point was taken to be $\beta \times \Delta pH_i/\Delta t$. This number is plotted against pH_i in Fig. 6. These results can be compared directly with those in snail neurones by referring to Fig. 3 in Thomas (1977).

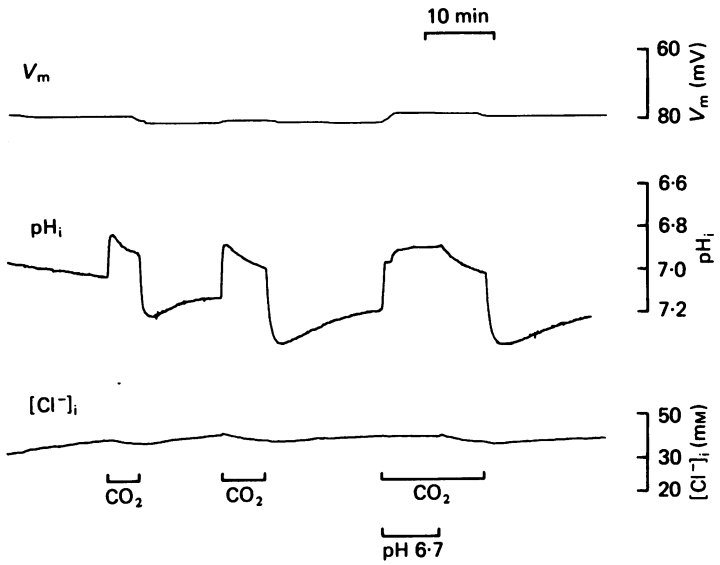

Fig. 7. Experiment recording pH_i and $[Cl^-]_i$ in the same neurone during acid loads produced by 5% CO_2. The V_m electrode was filled with 4 M-K acetate. During the first application of 5% CO_2, pH_i fell rapidly as CO_2 entered the cell, and then recovered towards its normal level. During pH_i recovery, $[Cl^-]_i$ decreased by about 3·5 mM. The second application of 5% CO_2 gave the same result. The third application of 5% CO_2 began at an external pH of 6·7, at which there was little recovery of pH_i. No decrease in $[Cl^-]_i$ occurred. When the external pH was returned to 7·4 with 5% CO_2 still present, pH_i recovery was stimulated and a decrease in $[Cl^-]_i$ occurred. The Ringer solution contained 5 mM-HCO_3^- at all times when 5% CO_2 was not present. External Cl^- concentration was held constant throughout the experiment.

Effect of an acid load on intracellular Cl^- concentration

If the sensitivity of pH_i recovery to external HCO_3^- reflects the operation Cl^-–HCO_3^- exchange, then a decrease in $[Cl^-]_i$ should occur following an acid load. To investigate this, pH_i and $[Cl^-]_i$ were measured simultaneously in four experiments, one of which is illustrated in Fig. 7. In this cell, the resting $[Cl^-]_i$ was 35 mM; this

value is 24 mM higher than that predicted by an equilibrium distribution of Cl^-. Similarly high values of $[Cl^-]_i$ were recorded in each of the other experiments.

The application of NH_4Cl in the external solution itself caused a rapid decrease in $[Cl^-]_i$, perhaps by interfering with active Cl^- transport (see Lux, 1971). It was therefore necessary to use 5 % CO_2 to stimulate acid extrusion when measuring $[Cl^-]_i$. Fig. 7 shows that a small but reproducible decrease in $[Cl^-]_i$ occurred during acid extrusion; during each CO_2 application $[Cl^-]_i$ fell by about 3·5 mM. The third application of CO_2 in Fig. 7 was begun at an external pH of 6·7, at which no pH_i recovery occurred. No decrease in $[Cl^-]_i$ was recorded until the external pH was returned to 7·4 and pH_i recovery began. This indicates that it was the recovery of pH_i and not its initial decrease that caused the decrease in $[Cl^-]_i$.

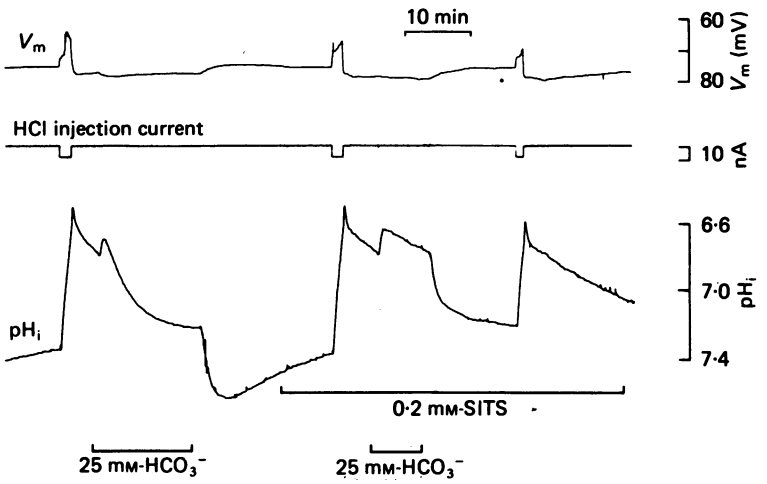

Fig. 8. Experiment showing the effects of external HCO_3^- concentration on pH_i recovery in the presence and absence of SITS. pH_i was decreased by ionophoretic injection of HCl; the middle trace shows the injection current. The first HCl injection was made in 5 mM-HCO_3^- Ringer solution. When pH_i had recovered by about 0·2 unit, the external HCO_3^- concentration was increased to 25 mM. This caused a small acidification, as CO_2 entered the cell, and a substantial increase in the rate of pH_i recovery. When pH_i had recovered completely, HCO_3^- was removed from the Ringer solution, causing a transient alkalinization. SITS (0·2 mM) was then added to the Ringer solution, and remained in all solutions for the rest of the experiment. About 10 min after the addition of SITS a second HCl injection was made. As before, when pH_i had recovered by about 0·2 unit, the external HCO_3^- concentration was increased to 25 mM. A small acidification occurred, but this time pH_i recovery was not accelerated. After 10 min HCO_3^- was removed from the Ringer solution, pH_i allowed to recover, and a third HCl injection made to show more completely pH_i recovery in HCO_3^--free Ringer solution containing SITS.

pH_i *recovery in the presence of SITS*

The above results indicate that pH_i regulation in crayfish neurones involves both Cl^-–HCO_3^- exchange and the influx of Na^+ ions. To test whether there are two acid extrusion mechanisms – Cl^-–HCO_3^- exchange and a HCO_3^--independent system which uses Na^+ ions (Aickin & Thomas, 1977) – or a single Na^+-dependent Cl^-–HCO_3^- exchange (Thomas, 1977), I investigated the effects on pH_i recovery of the Cl^-–HCO_3^-

exchange inhibitor SITS (4-acetmide-4′-isothiocyanostilbene-2,2′-disulphonic acid: Knauf & Rothstein, 1971; see Boron, 1980).

If there are two separate systems, then SITS should not completely block pH_i recovery, but the dependence of the rate of recovery on external HCO_3^- should be eliminated. If only one, HCO_3^--dependent system governs pH_i recovery, then SITS should either block pH_i recovery completely, or, if the block is incomplete, the dependence on external HCO_3^- should be retained.

Fig. 8 shows an experiment designed to test these predictions; pH_i was lowered by injection of HCl. The first HCl injection was made with the cell exposed to HCO_3^--free Ringer solution. During recovery of pH_i, the Ringer solution was changed to one containing 25 mM-HCO_3^-. After a transient acidification, caused by the entry of CO_2 into the cell, pH_i recovery was accelerated substantially. When recovery was complete, HCO_3^- was removed from the Ringer solution and 0·2 mM-SITS was added. A second injection of HCl was made, and during the course of pH_i recovery the Ringer solution was again changed to one containing 25 mM-HCO_3^-. This time, in the presence of SITS, 25 mM-HCO_3^- caused a small acidification but did not accelerate pH_i recovery. HCO_3^- was then removed and, finally, a third injection of HCl was made to show more completely the pH_i recovery in HCO_3^--free Ringer solution containing SITS.

The results in Fig. 8 were corrected for changes in intracellular buffering power and analysed as in Fig. 6. The calculations showed that changing from 0 mM- to 25 mM-HCO_3^- in the absence of SITS accelerated acid extrusion by a factor of 7. In the presence of SITS the same increase in HCO_3^- concentration had no effect on the rate of acid extrusion, the slight slowing apparent in Fig. 8 being entirely accounted for by the increase in intracellular buffering power. The rate of pH_i recovery in HCO_3^--free Ringer solution was slightly slowed by SITS. When acid extrusion rates were compared for Ringer solution containing 0 mM-HCO_3^- with and without SITS, 5 mM-HCO_3^- (not shown in Fig. 8, but determined for this cell), and 25 mM-HCO_3^-, this slowing could be accounted for by assuming that nominally HCO_3^--free Ringer solution contained about 1 mM-HCO_3^- (0·1 % CO_2). This is not an unreasonable figure, considering that room air contains 0·04 % CO_2 and the preparation itself probably generates some CO_2.

The results in Fig. 8 show that in the presence of SITS, pH_i regulation proceeds at approximately the rate expected for HCO_3^--free Ringer solution, regardless of the actual HCO_3^- concentration. This indicates that pH_i regulation in crayfish neurones involves two separate mechanisms, only one of which is HCO_3^--dependent and SITS-sensitive. As discussed below, the mechanisms may each have a dependence on external Na^+.

DISCUSSION

The ionic mechanism of pH_i *regulation in crayfish neurones*

The above results show that intracellular pH in crayfish neurones is maintained at a value about 1 unit higher than that predicted from an equilibrium distribution of H^+ ions across the plasma membrane. The mechanisms by which pH_i is regulated were studied by imposing acidifications on the cytoplasm and monitoring the active recovery of pH_i. Two separate mechanisms of acid extrusion were found to operate during pH_i recovery.

The first mechanism depends on the presence of Na^+ in the external solution (Fig. 2), and involves the entry of Na^+ into the cell (Figs. 3 and 4). It is independent of the concentration of HCO_3^- in the external solution and is not blocked by the anion exchange inhibitor SITS (Fig. 8). This component of pH_i regulation is probably identical to the Na^+–H^+ exchange described in mouse muscle fibres (Aickin & Thomas, 1977) and sea urchin eggs (Johnson *et al.* 1976).

The second mechanism of acid extrusion is sensitive to the external HCO_3^- concentration (Figs. 5 and 6), is blocked completely by SITS (Fig. 8), and involves the efflux of Cl^- from the cell (Fig. 7). This second component of pH_i regulation appears also to be Na^+-sensitive, since pH_i recovery is almost totally blocked by Na^+-free Ringer solution, even in cells in which the contribution of the HCO_3^--sensitive component is appreciable. For example, in the cell in Fig. 2, pH_i recovery was slowed by almost 50 % when the external HCO_3^- concentration was reduced from 5 mM to 0 mM (not shown). In this same cell, the removal of external Na^+ slowed pH_i recovery by more than 95 % in 5 mM-HCO_3^- Ringer solution, indicating that both the HCO_3^--sensitive and HCO_3^- insensitive components had been inhibited. In several separate experiments the addition of SITS to Na^+-free Ringer solution was shown to produce no appreciable further inhibition of pH_i recovery. This second component of pH_i regulation resembles the Na^+-dependent Cl^-–HCO_3^- exchange which accounts for all of the pH_i recovery in snail neurones (Thomas, 1977), barnacle muscle (Boron, 1977; Boron & Roos, 1978) and squid axon (Russell & Boron, 1979).

The relative contributions of the above two mechanisms to the overall rate of pH_i recovery following an acid load can be estimated from the effect of HCO_3^- removal or SITS application. For example, in the cell of Fig. 5, HCO_3^--sensitive acid extrusion accounted for about 60 % of pH_i recovery in 5 mM-HCO_3^- Ringer solution and about 85 % in 25 mM-HCO_3^-. There was considerable variability between cells in these figures, but on average, the HCO_3^--sensitive component accounted for about 45 % of pH_i recovery in 5 mM-HCO_3^-.

Comparison with pH_i *regulation in other cells*

pH_i regulation in crayfish neurones most closely resembles that in mouse muscle fibres, in which two similar independent mechanisms have been reported (Aickin & Thomas, 1977). In this preparation, Na^+–H^+ exchange formed about 80 % of acid extrusion – considerably more than in crayfish. The second, HCO_3^--dependent component in mouse muscle was not reported to require external Na^+, as it apparently does in crayfish. However, these authors were unable for technical reasons to examine the effect of a complete replacement of external Na^+ with an inert cation like BDAC. It is therefore possible that the pH_i regulating mechanisms in mouse muscle and crayfish neurones are the same. These results eliminate the possibility that pH_i regulation is accomplished by two separate mechanisms only in vertebrate cells.

In snail neurones (Thomas, 1977), pH_i regulation is accomplished by a single mechanism: a Na^+-dependent Cl^--HCO_3^- exchange which is similar to the second component in crayfish neurones. pH_i recovery in snail neurones is completely blocked by SITS, and thus there is apparently no HCO_3^--insensitive component (Thomas, 1977). The difference in pH_i regulation in crayfish and snail neurones may be related to the fact that in crayfish neurones $[Cl^-]_i$ is maintained higher than its equilibrium value (see above), whereas in snail neurones it is lower (Thomas, 1977). The single

pH_i regulating system in snail neurones obligatorily couples Cl^- efflux to acid extrusion, and thus helps to maintain low $[Cl^-]_i$. In crayfish, Cl^- efflux works against the maintainance of high resting $[Cl^-]_i$ and thus the addition of a separate acid extrusion system which does not require Cl^- efflux would be advantageous. The relative contribution of Cl^--HCO_3^- exchange to acid extrusion could be varied under different circumstances to meet the requirements of $[Cl^-]_i$ regulation.

The energy source for pH_i *regulation*

The source of energy for pH_i regulation is not entirely clear. In snail neurones the influx of Na^+ down its electrochemical gradient could in theory provide sufficient energy for the movement of the other ions involved in acid extrusion (Thomas, 1977). Reduction of ATP to levels sufficiently low to reduce substantially the activity of the Na^+ pump does not block pH_i recovery (Thomas, 1976). In squid axon, on the other hand, ATP is required for acid extrusion (Boron & deWeer, 1976*b*), even though the system also involves the influx of Na^+ down a large electrochemical gradient (Russell & Boron, 1979). Either ATP or Na^+ could provide the energy, with the other serving a catalytic role (Boron, 1980).

Two observations suggest that in crayfish neurones the HCO_3^--independent component of pH_i regulation, at least, is driven by Na^+ influx and operates at a rate proportional to the difference between the electrochemical gradient favouring Na^+ entry and that retarding H^+ exit:

(1) When acid loading was done at low extracellular pH to increase the energy gradient against which acid extrusion occurred, recovery stopped short of returning pH_i to its normal value and pH_i stabilized at a value at which the inward driving forces on Na^+ and H^+ were equal. For example, in the experiment in Fig. 4, which was carried out in HCO^-_3-free Ringer solution to minimize the contribution of the HCO_3^--dependent component, pH_i recovery from the second acid load stopped when pH_i and $[Na^+]_i$ reached values of 6·74 and 28 mM respectively. At these values the inward driving force on H^+ was 121·5 mV and for Na^+, 122·3 mV. Evidently, pH_i recovery stopped because the Na^+ electrochemical gradient became insufficient to drive H^+ efflux. (The resting pH_i under normal circumstances, however, is not determined by the Na^+ gradient. For example, at the values of pH_i and $[Na^+]_i$ just before the first NH_4Cl exposure in Fig. 4, the inward driving force on Na^+ exceeds that on H^+ by more than 70 mV.)

(2) When acid loading was done in Na^+-free Ringer solution, pH_i recovery when Na^+ was readmitted to the external solution was considerably faster than if the entire acid load had been carried out at normal external Na^+ concentration (Fig. 2). A period in Na^+-free Ringer solution causes $[Na^+]_i$ to decrease substantially (personal observation; see Thomas, 1972), and when Na^+ is reintroduced, the Na^+ gradient is larger than had Na^+ been present during the acid loading. Comparing the first two acid loads in Fig. 2, it is apparent that at the same values of pH_i, external pH and external $[Na^+]$, pH_i recovery was faster after a period in Na^+-free Ringer solution, when $[Na^+]_i$ was low. This again suggests that the Na^+ gradient drives pH_i recovery.

A requirement for the influx of Na^+ down its electrochemical gradient is a common feature of the transport processes regulating the internal concentrations of several ions. These include, in addition to H^+, Ca^{2+} (Baker, 1972), Mg^{2+} (Caldwell-Violich

& Requena, 1979), and in some cases Cl^- (Russell, 1979). The result of this may be that under physiological conditions cells maintain a fairly constant internal ionic environment at the expense of allowing $[Na^+]_i$ to vary. Thus experimental procedures designed to change the intracellular concentration of a particular ion may result in increased $[Na^+]_i$ instead of or in addition to their intended effect.

Many electrophysiological experiments involve changing the extracellular concentration of one ion and examining the effects on electrical events recorded intracellularly. The assumption is often implicit that the results of such experiments can be predicted from the change in the transmembrane concentration gradient of the ion involved. The data presented above emphasize that the effects on the internal ionic composition of such changes in the extracellular solution may be complex and often unpredictable. For example, replacement of external Na^+ caused a decrease in $[Na^+]_i$ and inhibited pH_i regulation. The rate of cytoplasmic acidification under such circumstances would depend on the rates of both passive H^+ entry into the cell and metabolic H^+ production. Addition of a small amount of NH_4^+ to the external solution caused a decrease in both $[Na^+]_i$ and $[Cl^-]_i$, even if the external Na^+ concentration was held constant, and a transient increase in pH_i. Upon removal of NH_4^+, there was a transient decrease in pH_i and a slower increase in $[Na^+]_i$. Clearly, detailed information about the mechanisms of regulation of internal ion levels is required to interpret experiments in which the external ionic composition is altered. Direct measurements are needed to determine the magnitude and specificity of action of procedures designed to alter the internal ionic composition of cells.

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