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# CONTRIBUTION OF THE $Na^+/H^+$ ANTIPORTER TO THE REGULATION OF INTRACELLULAR pH IN A CRAYFISH STRETCH RECEPTOR NEURONE

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## Summary

Regulation of intracellular pH (pHi) following acidosis induced by $NH_4^+/NH_3$ exposures was re-investigated in a crayfish stretch receptor neurone using $H^+$- and $Na^+$-selective microelectrodes. All experiments were performed in nominally $HCO_3^-/CO_2$-free salines. From studies in $Na^+$-free saline and from electrochemical calculations, we concluded that pHi regulation was dependent on extracellular $Na^+$ concentration ($[Na^+]_o$). The half-maximal rate of pHi recovery had an apparent Michaelis–Menten constant of 21mmol $l^{-1}$ $[Na^+]_o$. The use of this experimental approach and an improved technique enabled us to observe pHi regulation even in $Cl^-$-free saline, in contrast to earlier findings. In addition, amiloride (2mmol $l^{-1}$) inhibited the maximum rate of pHi recovery by about 80%, SITS (1mmol $l^{-1}$) by about 20%.

The results strongly suggest the operation of two separate pHi-regulating mechanisms, a $Na^+$-dependent $HCO_3^-/Cl^-$ antiporter (probably the $Na^+/H^+/HCO_3^-/Cl^-$ antiporter described earlier) and a $Na^+/H^+$ antiporter. Both mechanisms have been described in crayfish ganglion cells and muscle fibres, but the individual contribution to pHi regulation varies considerably in these preparations. Functional aspects of the pHi-regulating mechanisms in relation to ionic changes during the moulting cycle are discussed.

## Introduction

Several preparations from various crayfish species have been investigated to find the ion transporters responsible for regulating intracellular pH (pHi). In crayfish, $Na^+$, $HCO_3^-$ and $Cl^-$ are the ions involved in pHi regulation, *via* a $Na^+/H^+$ and/or a $Na^+$-dependent $HCO_3^-/Cl^-$ exchange mechanism. Although these mechanisms operate separately in crayfish ganglion cells (Moody, 1981) and muscle cells (Galler and Moser, 1986), the pHi-regulating mechanism in the sensory neurone of the stretch receptor (Moser, 1985) seems to consist of a single combined mechanism, involving $Na^+/H^+/HCO_3^-/Cl^-$ exchange or its equivalent.

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Key words: intracellular pH, ion-transport mechanisms, SITS, amiloride, EIPA, sodium, chloride, bicarbonate, ion-selective microelectrode, moult, sensory neurone, *Astacus astacus*, crayfish, stretch receptor.

During our recent investigations (Moser *et al.* 1989; Fresser *et al.* 1991; Mair, 1992) we noticed that pHi was fully regulated in nominally $HCO_3^-$-free salines. Even under such experimental conditions the operation of the $HCO_3^-/Cl^-$ antiporter cannot be excluded since $HCO_3^-$ may be formed from $CO_2$ by the carbonic anhydrase located at the outside of this cell membrane (Kaila *et al.* 1992*b*). Complete pHi recovery and the small alkaline shift of the resting pHi value with time during experiments indicate that, in the stretch receptor neurone, pHi regulation was hardly impaired by the nominal absence of $HCO_3^-$. In addition, experiments in $Cl^-$-free saline (Mair, 1992), supported by the results from Voipio *et al.* (1991), provide further evidence for a minor contribution of the $HCO_3^-/Cl^-$ antiporter to pHi regulation. All these results contradict earlier findings and this caused us to re-investigate pHi regulation in the sensory neurone using a different approach and an improved technique.

In this work we demonstrate the presence of two independent pHi-regulating mechanisms, a $Na^+/H^+$ antiporter and a $Na^+$-dependent $HCO_3^-/Cl^-$ (probably a $Na^+/H^+/HCO_3^-/Cl^-$) antiporter, both known in many other animals (Roos and Boron, 1981; Thomas, 1984; Chesler, 1990; Schwiening and Thomas, 1992). Some aspects of both antiporters were studied in further detail.

Preliminary results have been presented at a scientific meeting (Moser *et al.* 1989).

## Materials and methods

Experiments and calibration of ion-selective microelectrodes were performed at 16°C. *Astacus astacus* L., bought from a hatchery (Keller, Augsburg, Germany), were dissected to obtain the slowly adapting sensory neurone of the stretch receptor, which was mounted in an experimental chamber, connected to a flow-through system, as described earlier (Brown *et al.* 1978; Moser, 1985; Fresser *et al.* 1991; Mair, 1992). The flow exchanged bath volume within about 6s.

### *Chemicals and solutions*

Chemicals of highest purity were obtained from Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) and Fluka (Buchs, Switzerland). The amiloride analogue EIPA (ethylisopropylamiloride) was a present from Ciba-Geigy (Basel, Switzerland). The pH of all solutions was measured with an Orion 8162 (Ross-type) glass electrode. Normal *Astacus* saline (NAS) was modified from that devised by van Harreveld (1936) and had the following composition ($mmol\,l^{-1}$): 207 NaCl; 5.4 KCl; 2.4 $MgCl_2$; 13.5 $CaCl_2$; 10 Hepes, adjusted to pH7.4 with NaOH. In $NH_4^+$-NAS, $NH_4^+$ was substituted for $20mmol\,l^{-1}$ $Na^+$.

*N*-Methyl-D-glucamine (NMDG) was used as an equimolar substitute for $Na^+$, when reduced-$Na^+$ (e.g. 30$Na^+$-NAS), or $Na^+$-free (0-$Na^+$-NAS) salines were applied. In all NMDG-containing solutions, HCl was used to adjust pH to 7.4.

$Cl^-$-free salines (0-$Cl^-$-NAS) had the following composition ($mmol\,l^{-1}$): 207 sodium isethionate; 5.4 potassium gluconate; 9.6 D-gluconic acid hemimagnesium salt; 54 D-gluconic acid hemicalcium salt; 10 Hepes, adjusted to pH7.4 with NaOH. For 0-$Cl^-$-$NH_4$-NAS, $20mmol\,l^{-1}$ sodium isethionate was substituted by $NH_4NO_3$.

Measurements with $Ca^{2+}$ macroelectrodes showed that calcium gluconate concentration had to be increased fourfold to obtain the free $Ca^{2+}$ concentration found in NAS, owing to the substantial chelating action of the $Cl^-$ substitutes used. A similar action was assumed for $Mg^{2+}$, although the amount could have been overestimated. In view of our recent (N. Mair, H. Moser and F. Fresser, unpublished observations) results with varying amounts of $Ca^{2+}$, an overestimation of divalent cation concentration seems less critical than an underestimation.

SITS (1mmol $l^{-1}$), a blocker of the $HCO_3^-/Cl^-$ antiport, was added to NAS, and exposed to ultrasound just before being used. Amiloride and EIPA, blockers of the $Na^+/H^+$ antiport, were dissolved in distilled water by ultrasound and kept light-protected and cooled as a 20 or 10mmol $l^{-1}$ stock solution. Before use, the amounts for the final amiloride or EIPA concentration were taken and diluted to half the final volume with distilled water. This was made up to the final volume with double-concentrated NAS to give the correct concentration after mixing.

### *Electrodes*

Glass for both single- (GC 150) and double-barrelled (TGC 150) microelectrodes was obtained from Clark (Reading, UK) and pulled vertically (model 700C, David Kopf Instruments, USA). Simultaneous intracellular recordings were performed with a double-barrelled microelectrode, monitoring membrane potential ($E_m$; and serving as an internal reference) and $Na^+$ concentration ([$Na^+$]; thus assuming an equal activity coefficient in the neurone and the saline), and a single-barrelled microelectrode, monitoring $H^+$ concentration ([$H^+$]) or pH. Signals from the $E_m$ barrel (filled with a mixture of $K_2SO_4$ and KCl; Deisz and Lux, 1982; Moser, 1985) and from an additional bath microelectrode (filled with 3mol $l^{-1}$ KCl) were measured with an electrometer (WPI 750) against a grounded calomel electrode (Amagruss, Halmstadt-Bargen, Germany) connected to the sink of the bath through a 3mol $l^{-1}$ KCl/agar bridge. The signal of the bath electrode was subtracted from $E_m$, which is only important during exposures in $Cl^-$-free salines, when the (external) reference potential changes.

Ion-selective microelectrodes (ISMs) were made as described by Fresser *et al.* (1991) and Grafe *et al.* (1985), using Fluka no. 71176 and no. 95293 (TDDA) as $Na^+$ and $H^+$ membrane cocktails, respectively. In a few cases, Fluka no. 95297 ($H^+$ cocktail) was used, but this proved to interfere with organic blocker substances.

$Na^+$- and $H^+$-ISMs were calibrated in parallel, using five calibrating solutions (S1–S5) of constant ionic strength (215mmol $l^{-1}$). In the sequence S1–S5 the concentrations (mmol $l^{-1}$) for NaCl (+NaOH) were: 205 (+5), 50, 21, 10, 5; and for KCl (+KOH): 5, 157 (+8), 189 (+5), 191.5 (+13.5), 205 (+5). The buffers used were 10mmol $l^{-1}$: Hepes, Hepes, Hepes, Pipes and Hepes, respectively, which, in combination with the above-mentioned base, resulted in alkaline salines which were adjusted to pH7.4, 8.0, 7.4, 6.4 and 7.4, respectively, by the addition of HCl.

Pre- and post-calibration curves of $Na^+$-ISMs ($N$=50) were not significantly different and showed slopes of 54mV/pNa in the 210–21mmol $l^{-1}$ $Na^+$ range, and 40mV/pNa in the 50–5mmol $l^{-1}$ $Na^+$ range. A difference of 16.1mV between NAS and S1 saline indicated a selectivity value of +1.8 for $Ca^{2+}$, which was higher than values in the

literature (Ammann, 1986). The mean post-calibration potential value of (Fluka no. 95293) $H^+$-ISMs was 30.9mV between pH7.4 and 8.0, and 56.1mV between pH7.4 and 6.4, these being smaller by 1.8mV and by 5.7mV, respectively, than during pre-calibration. Pre- and post-calibrations with Fluka no. 95297 $H^+$-ISMs differed by only about 1mV in the ranges given above.

### *Electrical arrangement, recording and display*

Electrical arrangement and recording were as reported in Fresser *et al.* (1991). In addition, original pen recordings were digitized, using SummaSketch II hardware, and processed with Sigma-Scan V 3.90 software, converting mV to mmol $l^{-1}$ or pH. To present the data, the graphics program Sigma Plot 4.1 (Jandel Scientific) was used. Most of the fine steps in the illustrations are due to limitations of resolution.

### *Measurement of the rate of acid extrusion*

#### *Maximum pHi recovery rate*

In single cells and in most of our results this rate remained constant for about 1.5–4min, during which time pHi recovered by 0.3–0.5 units. For example, the same maximum recovery rate could be obtained if the measurement was taken at a pHi of 6.4 or 6.7. The main criterion for evaluation was that the ranges of maximum pHi recovery rate to be compared should overlap. Under these conditions, the error due to pHi-dependent effects on intracellular buffering capacity and on allosteric modulation of the pHi-regulating transporters should be small.

#### *Normalized maximum rate of pHi recovery*

The maximum rate of pHi recovery from the control is taken as 100%. This type of evaluation had to be used when there was no overlap in the pHi range of maximum recovery (e.g. 'low-$Na^+$'-experiments). This measure is much more subject to experimental error, because of the reasons given above, which may now become important.

The values given are means ± standard deviation (S.D.) from a certain number (*N*) of experiments, unless stated otherwise.

## Results

### *Controls*

All experiments were performed in nominally $HCO_3^-/CO_2$-free solutions. Cells were acidified according to the $NH_4^+/NH_3$ rebound technique (Boron and De Weer, 1976), and the terminology introduced by these authors will be used here.

Controls were usually performed prior to a test. A control run started with exposure to normal *Astacus* saline (NAS) until the variables of interest (pHi, $[Na^+]_i$, $E_m$) had stabilized to a reasonable extent. The cells were then exposed to 20mmol $l^{-1}$ $NH_4^+$-NAS for 3min, followed by a washout in NAS. A test run consisted of a 3-min $NH_4^+$-NAS

exposure with a subsequent 6-min exposure to the test saline (e.g. amiloride-NAS) and a final washout in NAS. Modifications are noted in the text.

Measurements in 231 control exposures gave a mean pHi of 7.36±0.10. During $NH_4^+$-NAS exposures the initial alkalization reached a pHi maximum of 7.70±0.10 at a rate of 0.91±0.28pHunitsmin$^{-1}$. The maximum rate of slow acidification was 0.24±0.05pHunitsmin$^{-1}$, resulting in a final pHi of 7.27±0.10 at the end of the $NH_4^+$-NAS exposure. During washout in NAS, the cells acidified at a maximum rate of 0.96±0.21pHunitsmin$^{-1}$, resulting in a maximum acidification to a pHi of 6.50±0.31, from which pHi finally recovered at a maximum rate of 0.19±0.06pHunitsmin$^{-1}$.

Intracellular $Na^+$ concentration ($[Na^+]_i$) was measured in 178 control experiments. At rest, $[Na^+]_i$ was 18.8±9.4mmol l$^{-1}$. During fast acidification, $[Na^+]_i$ increased at a maximum rate of 8.2±3.1mmol l$^{-1}$ min$^{-1}$, to reach a peak value of 31.1±11.0mmol l$^{-1}$ during the early period of pHi recovery. The maximum rate of $[Na^+]_i$ decrease during pHi recovery was 2.9±1.3mmol l$^{-1}$ min$^{-1}$.

At rest the mean membrane potential ($E_m$) from 231 control experiments was −65.7±5.0mV. At the end of the $NH_4^+$-NAS exposure the cells had depolarized to −55.3±6.1mV.

### *Effect of 0-$Na^+$-NAS on pHi recovery*

In the control exposure shown in Fig. 1, pHi recovered at a maximum rate of 0.23pHunitsmin$^{-1}$ and reached its original value after about 20min. In a subsequent experiment, exposure to 0-$Na^+$-NAS, immediately after $NH_4^+$-NAS, resulted in a block of pHi recovery. Twenty-three similar experiments were performed in 16 different cells, the exposures to 0-$Na^+$-NAS lasting 6 or 12min. Five different types of pHi response were found, as shown in Fig. 2 and these were studied in greater detail. By calculating the (individual) electrochemical equilibrium pHi (pHi = pHe + 0.0174$E_m$, where pHe is extracellular pH), we were able to decide whether the responses were below or above equilibrium. The majority of cells ($N$=7) showed a type 1 response, i.e. a slow increase in pHi even in 0-$Na^+$-NAS. This pHi change generally slowed down with time, revealing a passive movement of $H^+$, until electrochemical equilibrium was achieved. In other cells ($N$=4) we found type 2 and type 3 responses where a stable pHi value was finally obtained. In these cells the measured pHi was close to, or identical with, the calculated electrochemical equilibrium pHi. A type 4 response, also seen in Fig. 1, was found in three additional cells. The measured pHi values were clearly above their electrochemical equilibrium so that the $H^+$ gradient remained inwardly directed. It was characteristic of the type 4 response that acidification in 0-$Na^+$-NAS proceeded with time, but became slower. Only 2 of 23 exposures exhibited a type 5 response, in which some pHi regulation may have taken place in 0-$Na^+$-NAS.

Electrochemical considerations of $H^+$ gradients with respect to the calculated $H^+$ equilibrium are sufficient to explain 21 (out of 23) pHi responses observed during 0-$Na^+$-NAS exposures. The results strongly suggest a dominant role of $Na^+$ in the pHi-regulating mechanism in the sensory neurone. In addition, the type 1 response shown in Fig. 2 suggests either a permeability of the plasma membrane to $H^+$ or $OH^-$ or indicates that some of the pHi-regulating mechanism is driven by the $H^+$ gradient.

Approximately 30% more acidification occurred in 0-$Na^+$-NAS than in controls (Fig. 1). Releasing the block on pHi regulation by addition of NAS resulted in an accelerated rate of pHi recovery compared with controls.

The slower pHi recovery rate in controls could arise from the fact that they still contain intracellular $NH_4^+$ ($[NH_4^+]_i$), which exerts a prolonged acidifying action. However, we exclude this argument since, at the moment of maximum acidification, all $[NH_4^+]_i$ has probably left the cell as $NH_3$ (see Fig. 5 in Fresser *et al.* 1991), which is in line with the Boron and de Weer (1976) model. Instead, the acceleration of the pHi recovery rate on exposure to 0-$Na^+$-NAS might be due partly to a steeper (outwardly directed) $H^+$ gradient resulting from the stronger acidification and partly to a steeper (inwardly directed) $Na^+$

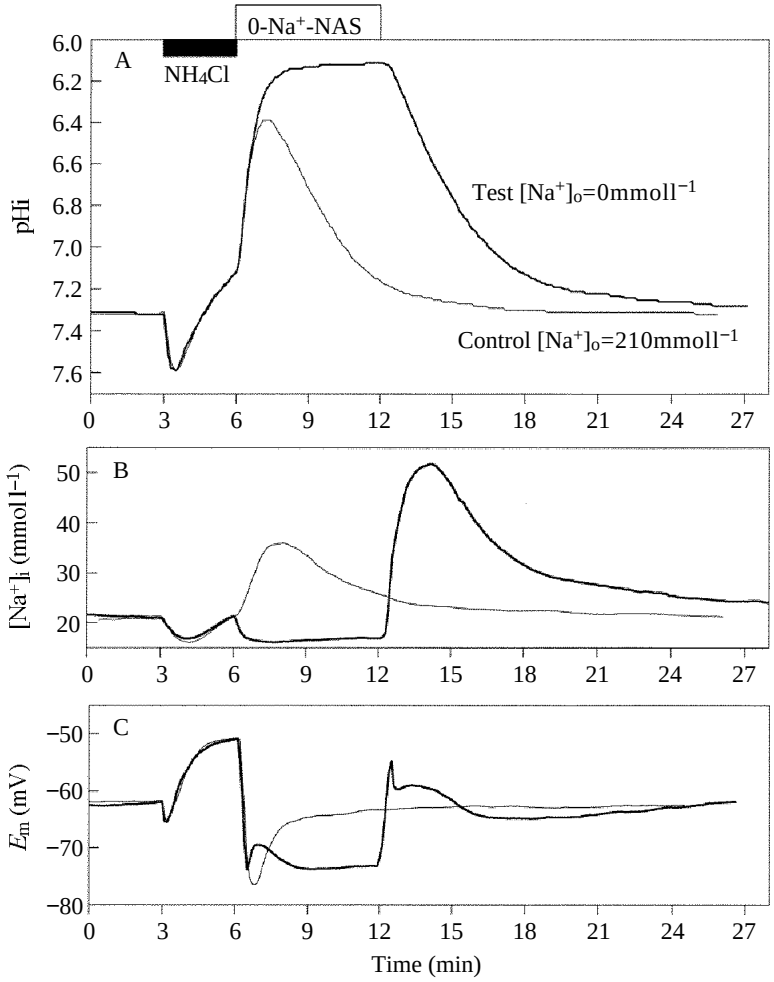


Fig. 1. Simultaneous measurements of pHi (A), intracellular $Na^+$ concentration ($[Na^+]_i$, B) and membrane potential ($E_m$, C). After exposure to $NH_4^+$-NAS (black bar), the cell was washed either in NAS (control) or, for 6min, in 0-$Na^+$-NAS (test). In 0-$Na^+$-NAS, pHi recovery stopped and $[Na^+]_i$ remained at a low level.

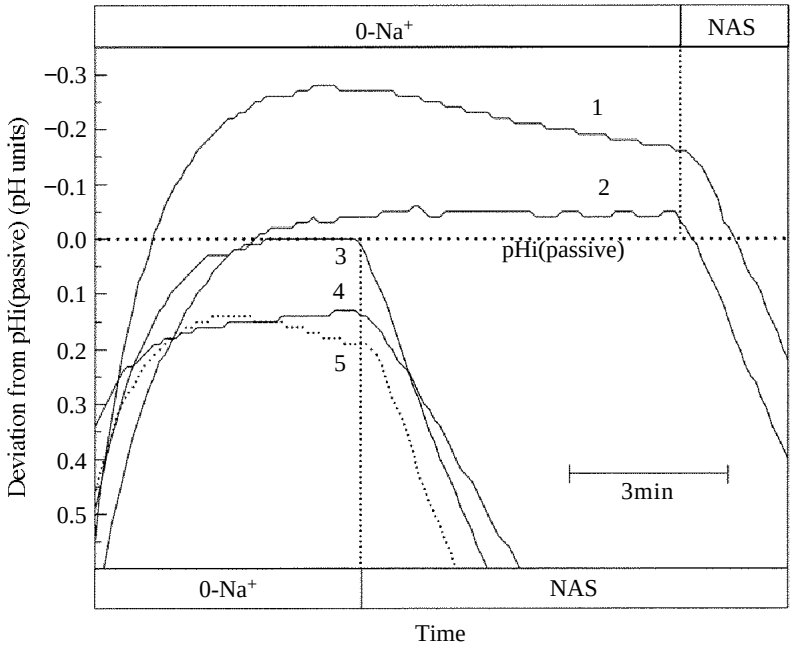


Fig. 2. Measurements of pHi in 0-$Na^+$-NAS after exposure to $NH_4^+$-NAS in different cells (1–5). Individual passive pHi values were calculated and taken as a reference (dotted line) for the actual pHi recordings. pHi responses like those in cells 1–4 can be explained by assuming passive transport mechanisms; in cell 5, some pHi regulation may have occurred, but passive transport cannot be excluded.

gradient caused by a considerable loss of $[Na^+]_i$ in 0-$Na^+$-NAS. Such factors would indicate that a combined $Na^+$ plus $H^+$ gradient could act as the driving force for the operation of the (subsequently identified) $Na^+/H^+$ exchanger. Further results (Moser *et al.* 1989) support this view, since pHi recovered in cells heavily loaded with $Na^+$, in which the transmembrane ratio $[Na^+]_o/[Na^+]_i$ was close to 1, rendering the $Na^+$ gradient negligible. From an electrochemical point of view, the $H^+$ diffusion gradient could have served to drive $H^+$ transport, mimicking some kind of pHi regulation in these cells.

### *Effect of 0-$Cl^-$-NAS on pHi recovery*

There are two major differences in experimental method between this and previous work: (1) while Moser (1985) used $CO_2/HCO_3^-$, we have applied the $NH_4^+/NH_3$ rebound technique (using $NH_4NO_3$ instead of $NH_4Cl$) for intracellular acidification in nominally $CO_2/HCO_3^-$-free salines, thus obtaining larger intracellular acidification; (2) we have compensated for the chelating action of the $Cl^-$ substitutes. As we will show, this approach brought new insight into the mechanisms of pHi regulation.

In order to decrease intracellular $[Cl^-]$, the cells were incubated in 0-$Cl^-$-NAS for a minimum of 6min, but usually for longer. After a 10-min incubation, intracellular $[Cl^-]$ should be very low (Deisz and Lux, 1982; Voipio *et al.* 1991) and be unable to play a role in pHi regulation, if this involves a $HCO_3^-/Cl^-$ exchange mechanism.

Incubation in 0-$Cl^-$-NAS had minor effects on the steady-state variables, generally causing short transient changes in pHi and $[Na^+]_i$, as can be seen in Figs 3 and 4. These might indicate a short reversal in the operation of a $Cl^-$-dependent pHi-regulating mechanism or changes in cell volume. Compared with controls, the maximum intracellular acidification after $NH_4^+$-0-$Cl^-$-NAS exposure in 0-$Cl^-$-NAS was markedly reduced (by about 50%; *N*=15). This phenomenon will be the subject of a separate paper and is not relevant to the pHi regulation process. The main conclusion from Fig. 3 is that pHi recovered even in the absence of $Cl^-$, supporting the findings of Voipio *et al.* (1991). Fig. 4, showing $[Na^+]_i$ measurements, suggests that $Na^+$ was directly involved in pHi regulation even in 0-$Cl^-$-NAS. The operation of a $Na^+/H^+$ exchanger, already assumed by Moser *et al.* (1989), could explain both results.

Similar experiments, performed in salines with uncompensated levels of divalent cations, help to explain results obtained previously (Moser, 1985). Cells placed in 'uncompensated' 0-$Cl^-$-NAS showed a several-fold increase in $[Na^+]_i$ within 3min, which considerably reduced the transmembrane $Na^+$ gradient. This, together with a minor

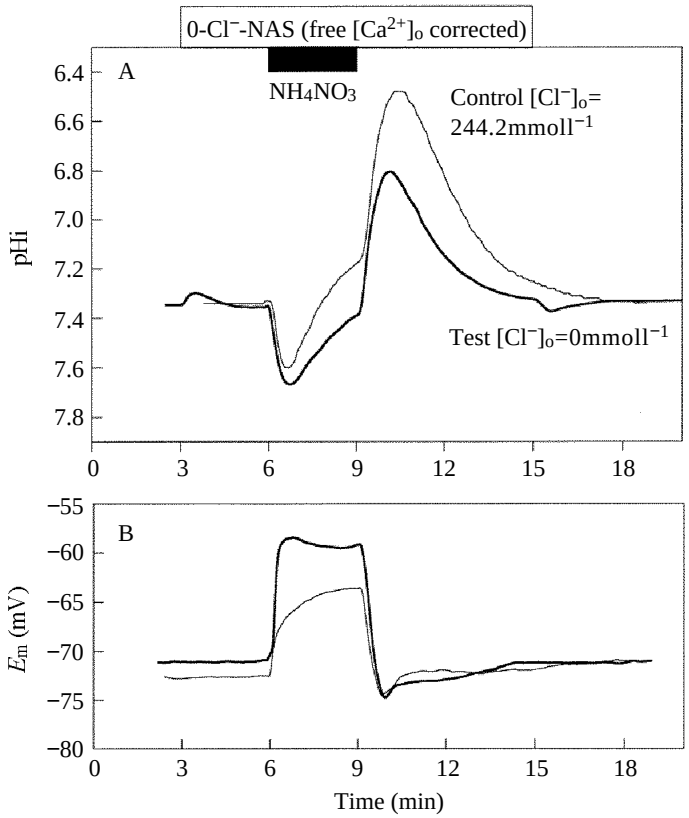


Fig. 3. Simultaneous measurements of pHi (A) and membrane potential ($E_m$, B), under control conditions or in 0-$Cl^-$-NAS, in a cell acid-loaded with 20mmol $l^{-1}$ $NH_4NO_3$-NAS. pHi recovered in the absence of extracellular $Cl^-$, when levels of divalent cations were corrected for the chelating action of the $Cl^-$ substitutes.

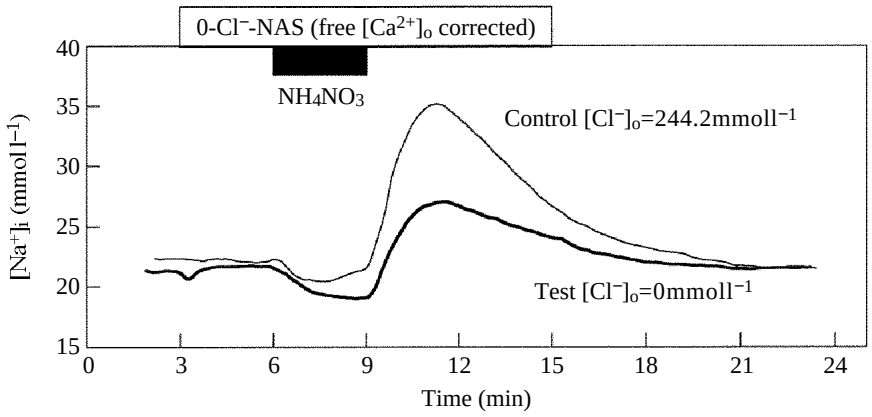


Fig. 4. Measurement of intracellular $Na^+$ concentration, under control conditions and in 0-$Cl^-$-NAS in a cell acid-loaded with 20mmol $l^{-1}$ $NH_4NO_3$-NAS.

acid load caused by $CO_2/HCO_3^-$-exposure, tends to reduce the activity of the $Na^+/H^+$ antiporter and, thus, electrochemical equilibrium conditions with no net flux of these ions could have been achieved.

### *Effects of SITS on pHi regulation*

SITS, a specific blocker of both $Na^+$-dependent and $Na^+$-independent $Cl^-/HCO_3^-$ exchange (Cabantchik *et al.* 1978; Roos and Boron, 1981), was used at a concentration of 1 mmol $l^{-1}$. When SITS was applied immediately after $NH_4^+$-NAS for 6min, the maximum rate of pHi recovery was decreased by 21±9% ($N$=3) compared with controls without SITS. This result can be explained only by assuming the operation of two separate pHi-regulating mechanisms, both of which depend on extracellular $Na^+$, as shown before. We suggest that these two mechanisms are (1) a $Na^+$-dependent $Cl^-/HCO_3^-$ exchange, which is responsible for approximately 20% of total pHi regulation (in nominally $HCO_3^-$-free medium; see Discussion), and (2) a $Na^+/H^+$ exchange, dominating pHi regulation under such conditions. The experiments below were performed in order to obtain further information on the $Na^+/H^+$ exchange mechanism.

### *Effects of amiloride or EIPA on pHi regulation*

Amiloride, a specific blocker of the $Na^+/H^+$ antiport (Gaillard and Rodeau, 1978; Grinstein *et al.* 1989), exerts its blocking action by competing with $Na^+$ (Roos and Boron, 1981). The effects of various amiloride concentrations on pHi, $[Na^+]_i$ and $E_m$ in a single cell are illustrated in Fig. 5. Control and amiloride exposure were alternated but, for clarity, only one control trace is shown.

The dose-dependent effect of amiloride and the amiloride analogue EIPA (Kleyman and Cragoe, 1988) on inhibition of the maximum rate of pHi regulation is shown in Fig. 6. Amiloride had an instant effect, even at low concentrations. In the range $10^{-6}$ to $10^{-4}$ mol $l^{-1}$ both drugs showed a similar potency, with a calculated half-maximal

inhibition at $3\times10^{-5}$ mol $l^{-1}$ amiloride. At the same concentrations, there was no significant difference (at the 0.05% level) between the mean values for amiloride and EIPA (Student's *t*-test).

Compared with amiloride, EIPA showed pronounced side-effects: within 6min, EIPA ($10^{-4}$ mol $l^{-1}$) depolarized the membrane potential by about 30mV, an effect never seen even with higher concentrations of amiloride. During washout in NAS, complete recovery of pHi after EIPA exposures took much longer and was accompanied by a slower recovery of $[Na^+]_i$.

Compared with controls, amiloride inhibited the maximum rate of pHi regulation by 15.0±3.6% ($N$=3) at $10^{-6}$ mol $l^{-1}$, the lowest concentration used. Maximum inhibition of

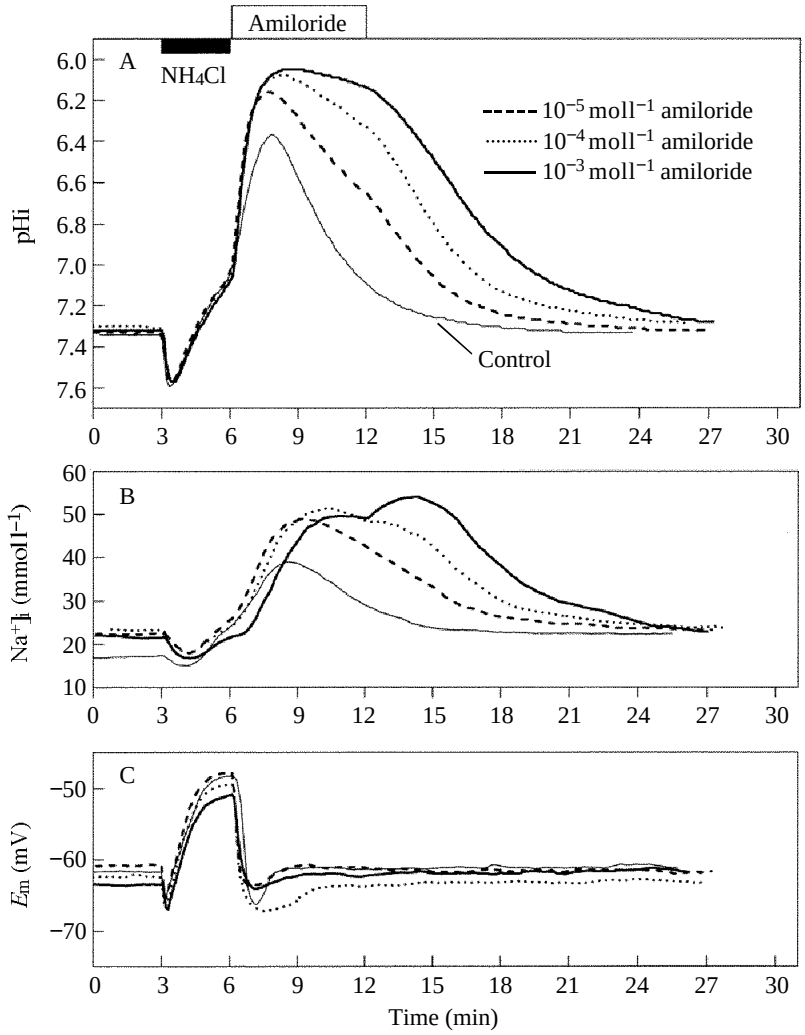


Fig. 5. Simultaneous measurements of pHi (A), intracellular $Na^+$ concentration ($[Na^+]_i$, B) and membrane potential ($E_m$, C). The effects on a single cell of various amiloride concentrations, given after exposure to $NH_4^+$-NAS, are shown.

about 80±5% ($N$=7) was obtained at an amiloride concentration of 2mmol$l^{-1}$. This value did not differ significantly from that obtained at 1mmol$l^{-1}$ (0.05%, unpaired Student's *t*-test).

We conclude that, in nominally $HCO_3^-$-free saline, about 80% of pHi regulation is due to $Na^+/H^+$ antiport and about 20% to $Na^+$-dependent $Cl^-/HCO_3^-$ antiport, this value being

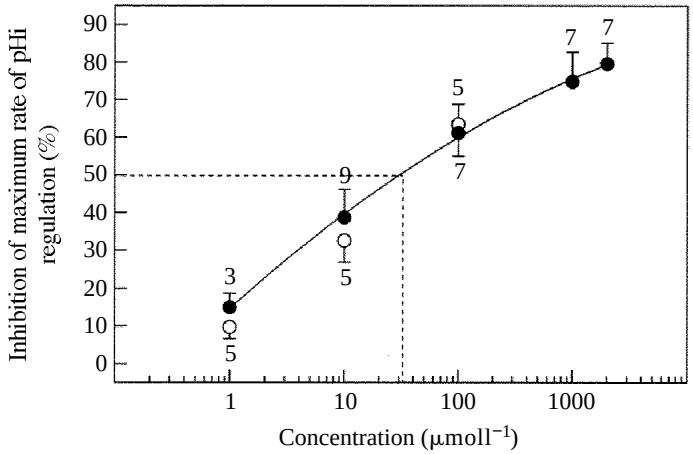


Fig. 6. Dose–response curve for the effect of amiloride (filled circles) and EIPA (open circles) on the inhibition of pHi regulation. Values are mean and standard deviation for the number of cells shown beside the curve.

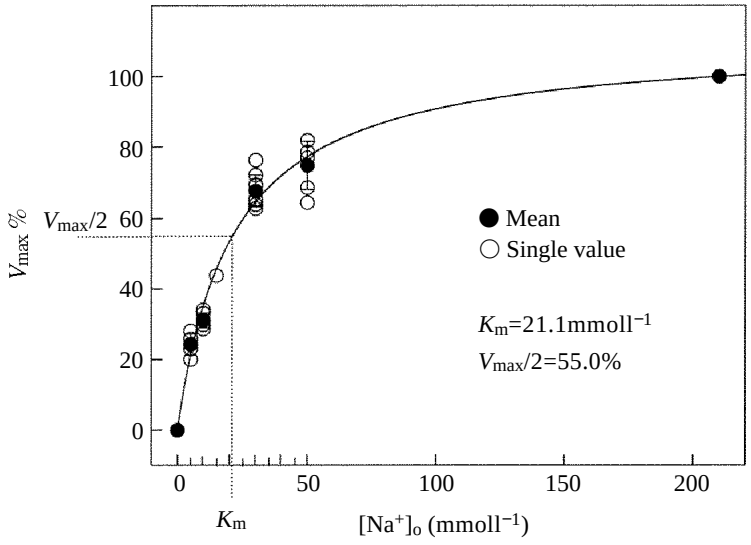


Fig. 7. Plot of normalized maximum pHi regulation rate ($V_{max\%}$) *versus* extracellular $Na^+$ concentration ($[Na^+]_o$). The dependence of the rate constant ($V_{max}$) on $[Na^+]_o$ could be described by Michaelis–Menten kinetics. The data were fitted to a hyperbola according to the equation: $V_{max} = a + V_{max\%}[Na^+]_o/(K_m + [Na^+]_o)$.

close to that determined in SITS. Summarizing, the mean values of inhibition of the maximum rate of pHi regulation obtained either with SITS or with amiloride (2mmol $l^{-1}$) account for complete blockage of pHi regulation.

We tried to demonstrate total inhibition of pHi regulation by blocking both antiporters simultaneously. However, any attempt to combine the blocker of the $Na^+$-dependent $Cl^-/HCO_3^-$ antiport (SITS) with the inhibitor of the $Na^+/H^+$ antiport (amiloride) resulted in precipitation of amiloride, and it appears that this experiment cannot be performed.

The effects of various concentrations of amiloride on $[Na^+]_i$ and on maximum pHi regulation were not as consistent as those found for the maximum rate of pHi regulation. We argue that amiloride, and more clearly EIPA, interacted in a concentration-dependent manner not only with the $Na^+/H^+$ exchanger but also with $Na^+/K^+$-ATPase. Compared with controls, slower rates of $[Na^+]_i$ recovery were seen during and after amiloride exposures in previously acid-loaded cells (Fig. 5). Similar amiloride interactions have been reported by Grinstein *et al.* (1989) for a different preparation (for EIPA interactions, see Kleyman and Cragoe, 1988).

### *Dependence of the operation of the $Na^+/H^+$ and the $Na^+/H^+/HCO_3^-/Cl^-$ antiporter on extracellular $[Na^+]$*

As reported above, pHi regulation was completely inhibited in 0-$Na^+$-NAS. As soon as 5 mmol $l^{-1}$ $Na^+$ was added (5$Na^+$-NAS) acid extrusion started at a normalized maximum rate of pHi recovery of 24±3% ($N$=5). The normalized maximum pHi recovery rate in 10$Na^+$-NAS was 31±2% ($N$=6), in 30$Na^+$-NAS it was 68±4% ($N$=9), in 50$Na^+$-NAS it was 75±7% ($N$=6) and in NAS (equivalent to 210mmol $l^{-1}$ $Na^+$) it was 100%. A plot of these values (Fig. 7) indicates a hyperbolic saturation curve, allowing application of the Michaelis–Menten equation. The calculated apparent Michaelis–Menten constant ($K_m$) was 21.1±0.7mmol $l^{-1}$ (extracellular) $Na^+$.

## Discussion

### *Steady-state values of pHi, $[Na^+]_i$ and* $E_m$

A comparison of the pHi, $[Na^+]_i$ and $E_m$ values, obtained at physiological rest, with previous data (Moser, 1985) shows minor differences. The 0.15 pH units more alkaline pHi found here seems to be caused by several factors, such as a different mode of calibration of the pH electrodes, which accounts for a difference of 0.06 pH units. Additional effects could arise from altered measurements of $E_m$ ($K_2SO_4$/KCl electrode *versus* 3 mol $l^{-1}$ KCl electrode), from seasonal effects (H. Moser, unpublished observation) and from chemical differences in the water in which the animals were kept for several months. We also noted a slow alkaline shift of pHi with time of experimentation. The deviation in mean pHi values cannot be explained by the effects of temperature on pHi as they are inversely related to the observations (Aickin and Thomas, 1977; Rodeau, 1984).

Such arguments could also serve to explain the 4mmol $l^{-1}$ higher $[Na^+]_i$ value previously observed, but the most likely explanation is that we did not wait long enough

until we started the tests. When the cells were penetrated by the double-barrelled electrode we observed a substantial increase in $[Na^+]_i$ from which the cells slowly recovered. Comparing 'early' with 'late' controls in a single cell, we often found that $[Na^+]_i$ had decreased in later controls, a result similar to that of Edman *et al.* (1983, 1986).

The configuration of the double-barrelled electrode may have caused an extra activation of the stretch-activated channels (Erxleben, 1989) or the electrode seal may have been less than perfect, resulting in a 1mV lower mean value for $E_m$.

### *Ion transport involved in pHi regulation in the sensory neurone*

In addition to $NH_4^+$-NAS exposures, we carefully studied pHi changes in 0-$Na^+$-NAS (Figs 1 and 2) and concluded that pHi regulation was $Na^+$-dependent. From further experiments, we concluded that two separate exchange mechanisms existed in the sensory neurone, a $Na^+$-dependent $HCO_3^-/Cl^-$ antiporter and a $Na^+/H^+$ antiporter.

In addition to the observations of Moser (1985), two observations support the existence of a $Na^+$-dependent $HCO_3^-/Cl^-$ antiporter: (1) high concentrations of amiloride (2mmol $l^{-1}$; Figs 5 and 6), a specific blocker of the $Na^+/H^+$ antiporter, never inhibited pHi regulation completely, suggesting that an additional pHi-regulating mechanism exists (with $Na^+$ also being involved); (2) SITS, which blocks the $HCO_3^-/Cl^-$ antiporter, reduced the maximum rate of pHi regulation by about 20%. This reduction in the rate of recovery was approximately equal to the amount needed to cause complete inhibition of pHi regulation in amiloride experiments.

The fact that all salines were nominally $HCO_3^-$-free cannot be taken as an argument against the existence of an $HCO_3^-/Cl^-$ antiporter, as two sources of $HCO_3^-$ are always present: the $CO_2$ of the air and metabolically produced $CO_2$. $CO_2$ can easily diffuse through the plasma membrane and will then be converted to $HCO_3^-$ in a reaction mediated by carbonic anhydrase, known to be located at the outside of plasma membranes even in excitable cells (Chen and Chesler, 1992; Kaila *et al.* 1990, 1992*a*).

From the results presented here, there is no clear evidence to indicate whether $Na^+$ acted indirectly as a modifier of $HCO_3^-/Cl^-$ antiport or whether it was directly transported by a $Na^+/H^+/HCO_3^-/Cl^-$ exchanger or an equivalent. As found by several authors (Aickin *et al.* 1982; Deisz and Lux, 1982; Kaila *et al.* 1992*b*), $E_{Cl}$ is more negative than $E_m$, resulting in an inwardly directed driving force for $Cl^-$. If a $Na^+$-modulated 1:1 $HCO_3^-/Cl^-$ antiport exists, it should export $HCO_3^-$, causing an intracellular acid load. However, an inwardly directed driving force for $HCO_3^-$ is unlikely, since metabolic $CO_2$ production together with both intra- and extracellular carbonic anhydrase (Chen and Chesler, 1992; Kaila *et al.* 1992*a*) will keep $CO_2$ at about the same level on both sides of the plasma membrane. Thus, electrochemical considerations favour the presence of $Na^+/H^+/HCO_3^-/Cl^-$ antiport, as already indicated by Moser (1985).

The existence of $Na^+/H^+$ antiport can be deduced (1) from the effect of external $Na^+$ on pHi regulation and the direct involvement of $Na^+$, as sensed with $Na^+$-ISM (Figs 1 and 7), (2) from experiments with selective blockers of $Na^+/H^+$ antiport, amiloride and EIPA (Figs 5 and 6), which cause a maximum inhibition of pHi regulation of about 80% at a concentration of 1 or 2 mmol $l^{-1}$, and (3) from the observation that pHi was well regulated

even when $Na^+/H^+/HCO_3^-/Cl^-$ antiport was blocked by SITS or by prolonged exposure to 0-$Cl^-$-NAS in nominally $HCO_3^-$-free saline (Fig. 3). During such 0-$Cl^-$-NAS exposures, the cells should have lost most of their internal $Cl^-$ (Deisz and Lux, 1982; Voipio *et al.* 1991), thus retarding the operation of the $HCO_3^-/Cl^-$ exchanger or even stopping it. In 0-$Cl^-$-NAS, we observed considerable changes in $[Na^+]_i$ (Fig. 4), suggesting direct involvement of $Na^+$ in an additional pHi-regulating mechanism, $Na^+/H^+$ antiport.

At physiological pH, the $Na^+/H^+$ antiport is half-maximally inhibited at an amiloride concentration of $3\times10^{-5}$ mol $l^{-1}$. In addition, previous results (Moser *et al.* 1989) indicated that it was the combined $Na^+/H^+$ gradient that fuelled $Na^+/H^+$ antiport (Kinsella and Aronson, 1980; Grinstein and Rothstein, 1986) and that the $Na^+/H^+$ antiport was largely, or totally, unaffected by the transmembrane potential.

At an extracellular pH of 7.4, the normalized maximum rate of pHi recovery depends on $[Na^+]_o$ following a simple Michaelis–Menten relationship, suggesting a single binding site (Fig. 7). We assume that the $K_m$ value is mainly linked to the $Na^+/H^+$ antiport, which carries out about 80% of pHi regulation in our experiments. We found an apparent Michaelis–Menten constant ($K_m$) of 21 mmol $l^{-1}$ $[Na^+]_o$, indicating that the operation of the $Na^+/H^+$ antiporter is unlikely to be limited by physiological $[Na^+]_o$ changes, since $[Na^+]_o$ normally exceeds this value by approximately 10-fold. Even when $[Na^+]_o$ is lowered to about half of its normal value, the action of the $Na^+/H^+$ antiporter is hardly impaired (Fig. 7) and the pHi value will only change a little in the acid direction.

### *The mechanisms of pHi regulation in crayfish preparations*

All three preparations of crayfish, ganglion cell (Moody, 1981), muscle cell (Galler and Moser, 1986) and the sensory neurone of the stretch receptor, have two separate pHi-regulating mechanisms in common, a $Na^+$-dependent $HCO_3^-/Cl^-$ antiport and a $Na^+/H^+$ antiport. Interestingly, the contribution of each mechanism to pHi regulation seems to be different. Muscle fibres had the slowest rate of pHi recovery, the $Na^+$-dependent $HCO_3^-/Cl^-$ antiport contributing 75% and the $Na^+/H^+$ antiport 25% to pHi regulation (Galler and Moser, 1986). The sensory cell showed the fastest pHi recovery, mainly due to $Na^+/H^+$ antiport (by about 80%) and, to a lesser extent (20%), to $Na^+$-dependent $HCO_3^-/Cl^-$ antiport. Between these two extremes, the crayfish neurone (Moody, 1981) exhibited an intermediate rate of pHi recovery, with both mechanisms contributing substantially to pHi regulation under normal conditions. All these observations are in line with the observation that amiloride hardly affects pHi regulation in muscle fibres (Galler, 1985) but has a considerable influence on the sensory cell. The opposite was true for exposure to SITS, 0-$Cl^-$-saline or nominally $HCO_3^-$-free saline, which moderately inhibited pHi recovery in the sensory neurone but had a considerable effect in the muscle fibres.

### *Moulting and pHi regulation*

During the moulting cycle of crustaceans, cuticular $CaCO_3$ mineralization and demineralization is accompanied by changes in $[Ca^{2+}]$, $[HCO_3^-]$ and $[H^+]$ (e.g. Cameron and Wood, 1985; Greenaway, 1985; Roer and Dillaman, 1984; Wheatly and Ignaszewski, 1990). The increase in body size in freshwater crayfish is accomplished by uptake of environmental water. Both processes affect the concentration of ions involved in pHi

regulation. The effects of concentration changes on the mechanisms of pHi regulation are discussed below.

The dependence of the normalized maximum pHi recovery rate on $[Na^+]_o$ (Fig. 7) gives $K_m$ values of 21mmol $l^{-1}$ for the crayfish sensory cell and 39mmol $l^{-1}$ for the neurone (Gaillard and Rodeau, 1987). Although direct comparisons cannot be drawn, because the experimental situations were somewhat different, we assume that pHi remains regulated to a reasonable extent (and may be fully regulated) during the moulting cycle, thus keeping the nervous system functioning, mainly by the operation of the $Na^+/H^+$ antiport.

In crayfish muscle, the functional role of the $Na^+/H^+$ antiport, contributing approximately 25% to pHi regulation (Galler and Moser, 1986), is less clear. Assuming a $K_m$ value in the range 3–50mmol $l^{-1}$ $[Na^+]_o$, as found in many preparations from vertebrates and invertebrates (Aronson, 1985; Moolenaar, 1986), the $Na^+/H^+$ antiport could provide some kind of 'safety mechanism' for pHi regulation with variable importance during the moulting cycle. At moulting, ionic conditions could have slowed or stopped the operation of the $Na^+/H^+/HCO_3^-/Cl^-$ antiport, but the $Na^+/H^+$ antiport, hardly affected by ionic alterations, would remain functional.

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