

## INTRA- AND EXTRACELLULAR USE AND EVALUATION OF AMMONIUM-SELECTIVE MICROELECTRODES

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### Summary

1. An  $\text{NH}_4^+$ -selective membrane for microelectrodes ( $\text{NH}_4\text{ISM}$ s) was tested under 'biological conditions' in normal *Astacus* saline (NAS), two simulated intracellular salines (SIS) and the sensory neurone of the crayfish stretch receptor.

2. The effects of several physiological variables on intracellular  $\text{NH}_4^+$  measurements were tested *in vitro*. Changes in the background  $\text{K}^+$  and  $\text{Na}^+$  concentrations, the ratio  $\text{K}^+/\text{Na}^+$ , pH, ionic strength, osmotic pressure and volume were examined.

3. Phenomena specific for  $\text{NH}_4\text{ISM}$ s, such as a positive potential shift, an undershoot and a difference between pre- and postcalibration curves are described and discussed. We propose to consider the values of intracellular  $\text{NH}_4^+$  concentration as apparent.

4. The detection limit of the  $\text{NH}_4\text{ISM}$  is closely related to background  $\text{K}^+$  concentration. It is in the region of  $0.1 \text{ mmol l}^{-1} \text{ NH}_4^+$  in NAS (at  $5.4 \text{ mmol l}^{-1} \text{ K}^+$ ) and about  $5 \text{ mmol l}^{-1}$  in SIS (at  $194 \text{ mmol l}^{-1} \text{ K}^+$ ).

5. When comparing levels of intracellular  $\text{NH}_4^+$ , either measured directly by  $\text{NH}_4\text{ISM}$  or calculated, according to Boron and de Weer (*J. gen. Physiol.* 67, 91–112), from simultaneously recorded  $\text{pHi}$ , we found that  $[\text{NH}_4^+]_i$  obtained by direct measurement differed quantitatively from that of the Boron and de Weer model, but that some of the qualitative and temporal aspects of the model agreed with our results. The quantitative difference in  $[\text{NH}_4^+]_i$  determined by the two methods cannot be attributed to temporal and/or quantitative limitations of the  $\text{NH}_4\text{ISM}$ .

### Introduction

One of the latest developments in ion-selective membranes for microelectrodes is an  $\text{NH}_4^+$ -selective membrane, which is based on a mixture of the antibiotics nonactin and monactin (Bührer *et al.* 1988; Ammann, 1986). From calibrations in defined chemical salines it is apparent that potassium interference is considerable, as selectivity coefficients of  $-0.6$  were obtained. This means that about four

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potassium ions produce the same change in potential as one ammonium ion. So far, this cocktail has not been tested under biological conditions. From the calibration curves it is not clear whether this membrane can be used in invertebrate cells, especially the crayfish stretch receptor neurone (SR) which has a (mean) intracellular  $K^+$  activity ( $a_K^i$ ) of about  $135 \text{ mmol l}^{-1}$  (Brown *et al.* 1978; Moser, 1985), corresponding to an intracellular  $K^+$  concentration  $[K^+]_i$  of about  $200 \text{ mmol l}^{-1}$ . However, there are advantages in using this preparation, as there is indirect evidence from intracellular pH (pHi) measurements that entry of  $NH_4^+$  is large in the SR neurone compared with other cells (Boron and de Weer, 1976; Moody, 1981; Moser, 1985, 1987; Galler and Moser, 1986).

This study is mainly methodological. Its purpose is threefold: (1) to assess the limitations of the  $NH_4^+$ -selective microelectrode ( $NH_4ISM$ ) during intracellular as well as extracellular use, (2) to demonstrate and to discuss problems specific to the  $NH_4ISM$  and (3) to provide some practical hints on its use. We show that  $NH_4ISM$ s can be successfully used intracellularly even at a background  $K^+$  concentration of about  $200 \text{ mmol l}^{-1}$ .

Preliminary results were presented as an abstract (Moser *et al.* 1989) at a satellite symposium of the XXXIst International Physiological Meeting in Helsinki.

### Materials and methods

A double-barrelled electrode, to measure membrane potential ( $E_m$ ) and intracellular pH (pHi), and two single-barrelled ammonium electrodes, for extra- and intracellular use, were prepared and connected to the headstages of voltmeters. When all the electrodes had been successfully calibrated (see below), the slowly adapting stretch receptor (SR) of *Astacus astacus* L. was dissected and mounted in the experimental chamber (Brown *et al.* 1978; Moser, 1985). A flow-through system (driven by a peristaltic pump, Pharmacia, model P3) continuously exchanged the saline in the experimental chamber about 12 times per minute.

### Chemicals and solutions

Chemicals of highest purity were obtained from Merck (Darmstadt, BRD), Sigma (Deisenhofen, BRD) and Fluka (Buchs, Switzerland). The ammonium cocktail was a gift from Professor W. Simon (ETH, Zürich). The pH of all solutions was measured with an Orion 8162 (Ross-type) glass electrode. Two sets of calibrating solutions were used. (1) NAS (normal *Astacus* saline), containing a low  $[K^+]$  and corresponding to the composition of *A. astacus* blood, was modified from that devised by van Harreveld (1936). It had the following composition ( $\text{mmol l}^{-1}$ ): 207 NaCl; 5.4 KCl; 2.4  $MgCl_2$ ; 13.5  $CaCl_2$ ; 10 Hepes adjusted to pH 7.4 with NaOH. In  $NH_4^+$ -NAS or  $K^+$ -NAS,  $NH_4^+$  or  $K^+$  was substituted for  $20 \text{ mmol l}^{-1} Na^+$ . Small amounts of  $NH_4Cl$  or other substances (up to  $1 \text{ mmol l}^{-1}$ ) were added without compensation. (2) SIS (simulated intracellular saline) consisting of a Hepes-buffered saline ( $10 \text{ mmol l}^{-1}$ , pH 7.4) with  $194 \text{ mmol l}^{-1} KCl$

and  $21 \text{ mmol l}^{-1}$  NaCl as background ions. To calibrate the  $\text{NH}_4\text{ISM}$ s, measured amounts of  $\text{NH}_4\text{Cl}$  were added to SIS at pH 7.4. The intracellular pH electrode was also calibrated in SIS, using  $10 \text{ mmol l}^{-1}$  Hepes at pH 8.0 and 7.4 and  $10 \text{ mmol l}^{-1}$  Pipes at pH 6.4. The SIS solutions were made alkaline by adding measured amounts of KOH (the amount of  $\text{K}^+$  added being included in the total  $\text{K}^+$  concentration) and then adjusted to the proper pH by adding HCl. The composition of the calibrating solutions is shown in Fig. 1. Additional calibrating solutions are described in Table 1 (see below).

### *Electrodes*

Glass for both single- (GC 150) and double-barrelled (TGC 150) electrodes was obtained from Clark (Reading, UK) and pulled vertically (model 700C, Kopf Instruments, USA). Single-barrelled  $\text{NH}_4\text{ISM}$ s were made after the method of Bührer *et al.* (1988). The pulled pipettes were kept overnight in an oven at  $160\text{--}180^\circ\text{C}$  and then a small quantity of silane was added to the glass beaker covering them. The pipettes were baked in the silane vapour for an additional 30 min and allowed to cool. The cold pipettes were back-filled under pressure with filling solution, consisting of  $10 \text{ mmol l}^{-1}$   $\text{NH}_4\text{Cl}$ , before the  $\text{NH}_4^+$  cocktail was sucked in by applying negative pressure, to give cocktail columns of between 100 and  $300 \mu\text{m}$ . After the electrodes had been conditioned (see Results) in a  $20 \text{ mmol l}^{-1}$   $\text{NH}_4\text{Cl}$  solution to stabilize them, they were ready for calibration. Double-barrelled electrodes were made and silane-coated following the instructions given by Grafe *et al.* (1985). For pH<sub>i</sub> measurements we preferred the tri-*n*-dodecylamine cocktail (Fluka), which proved to be much less susceptible to interference from a variety of substances, such as various ion-transport blockers. The filling solution of the  $E_m$  electrode, which also served as an internal reference, was a mixture of  $0.6 \text{ mol l}^{-1}$   $\text{K}_2\text{SO}_4$  (85 %) and  $1.5 \text{ mol l}^{-1}$  KCl (15 %). The resistance of the  $E_m$  barrel was  $12\text{--}30 \text{ M}\Omega$ . The calibration of this electrode is described in the Results section.

### *Electrical arrangement, recording and display*

The signals from the  $\text{NH}_4\text{ISM}$  were recorded either with an electrometer (WPI, model FD 223) or with a varactor amplifier (Analog Devices 311J) according to the design of Thomas (1978). The signals from the  $E_m$  electrode and from an additional bath electrode were measured with an electrometer (WPI 750) against a grounded calomel electrode (Amagruss, Helmstadt-Bargen, Germany) connected to the bath through a  $3 \text{ mol l}^{-1}$  KCl/agar bridge. All signals were fed into an oscilloscope (Tektronix, model 5113), a personal computer for further calculations and pen-recorders (BBC-Goerz: Servogor 220; SE 130), from which the records were photocopied for illustration.

## **Results**

### *Electrode conditioning*

Conditioning time should be kept to a minimum or problems will arise when fine

electrodes ( $0.5\text{--}0.8\ \mu\text{m}$  tip diameter) penetrate the membrane. The conditioning saline appears to affect the mechanical stability of the electrode tip. We found that conditioning times below 2 h sufficed and we could further improve the number of successful penetrations by using normal-walled GC 150 glass (instead of thin-walled GC 150T). However, if 'blunt' electrode tips were used, the prepared  $\text{NH}_4\text{ISM}$ s could be stored in the conditioning solution for at least 1 week.

*Calibration of ammonium-selective electrodes for intracellular application*

Calibration of  $\text{NH}_4\text{ISM}$ s is rather time-consuming, taking at least 40 min (without conditioning). We combined the calibrating salines in such a way as to calibrate both  $\text{NH}_4^+$ -sensitive and pH electrodes simultaneously. Fig. 1 shows the second part of such a calibration curve, the first part (not shown) can be thought of

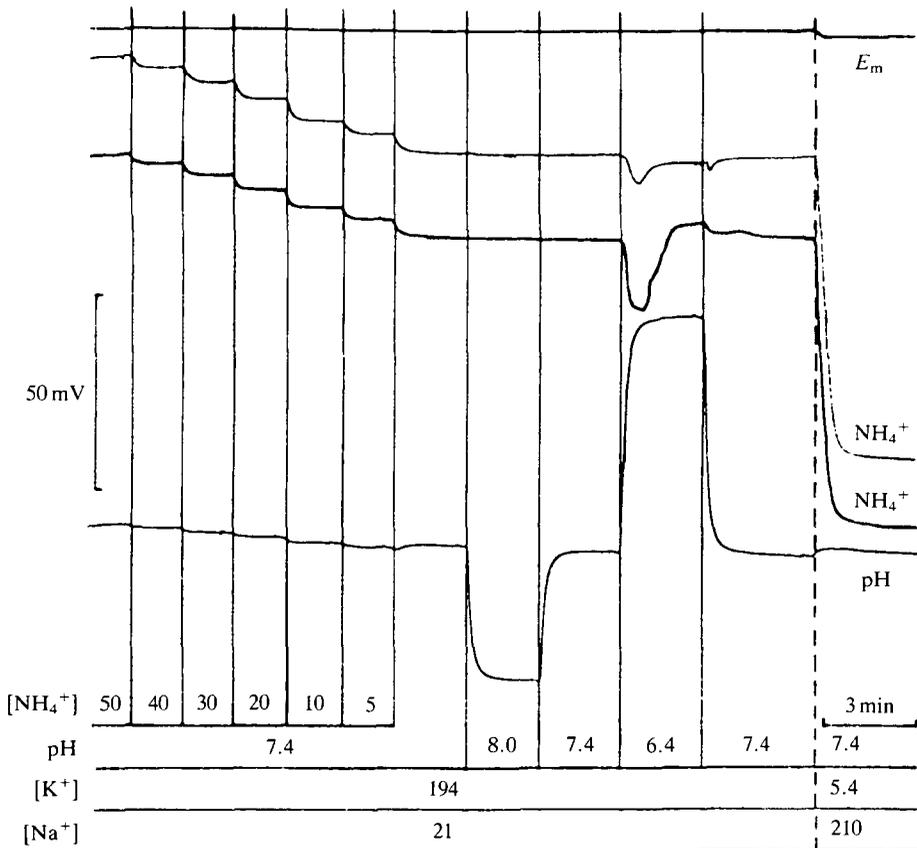


Fig. 1. Calibration of microelectrodes: pen recordings showing the effects of calibrating solutions on a membrane potential ( $E_m$ ) electrode (upper trace), two  $\text{NH}_4^+$ -sensitive microelectrodes ( $\text{NH}_4\text{ISM}$ s; middle traces) and one pH electrode (lower trace). The potential is measured in single mode between the microelectrodes and a calomel half-cell connected to the bath by an agar bridge in a flow-through chamber. Ion concentrations are given in  $\text{mmol l}^{-1}$ .

as a mirror image on the left side of the picture. In the part shown, the electrodes are usually less 'jumpy' and drift is less pronounced and, therefore, we took these values as precalibration curves. Some other features should also be noted. In the case of the pH electrodes, we regularly found (1) that in the presence of  $\text{NH}_4\text{Cl}$  the response shifted in the acid direction and (2) that the calibrations in SIS gave values that were shifted towards the alkaline direction by about 0.05 pH units compared with similar calibrations in NAS. In the case of  $\text{NH}_4\text{ISM}$ s, a change from SIS to NAS was accompanied by a potential change of about 72 mV, mainly due to the change in  $[\text{K}^+]$  (from 194 to  $5.4 \text{ mmol l}^{-1}$ ). Potassium was the dominant interfering ion, the interference from other ions being small by comparison.

Most  $\text{NH}_4\text{ISM}$ s showed a short, transient sensitivity to acidic pH. As two electrodes were calibrated simultaneously we could choose the one that was less sensitive to pH for intracellular measurements. In Fig. 1 this is the  $\text{NH}_4\text{ISM}$  shown in the upper trace. The temporal resolution of  $\text{NH}_4\text{ISM}$ s is about the same as that of the pH electrode. It seems to be limited by the mixing time in the experimental chamber.

Both pre- and postcalibration curves for  $\text{NH}_4\text{ISM}$ s are shown in Fig. 2. Despite a background concentration of  $194 \text{ mmol l}^{-1} \text{ K}^+$ ,  $\text{NH}_4^+$  could be measured at concentrations as low as  $5 \text{ mmol l}^{-1}$ . When electrodes were exposed to SIS and  $\text{NH}_4^+$  concentrations were increased the potentials of the  $\text{NH}_4\text{ISM}$ s also increased. Precalibrations of 130  $\text{NH}_4\text{ISM}$ s showed that a change from SIS to SIS containing  $5 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$  resulted in a potential change of approximately 3–4 mV, whereas the potential change resulting from a transfer from SIS to SIS containing  $50 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$  was between 20 and 25 mV. A comparison between

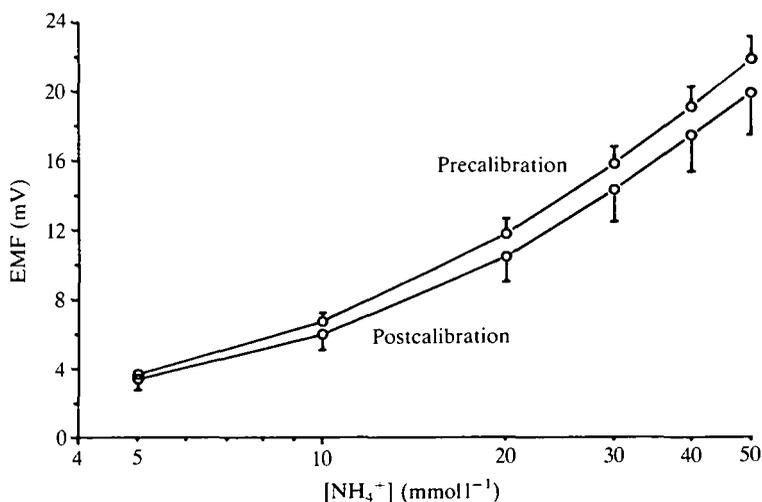


Fig. 2. A graph showing mean pre- and postcalibration values for 29  $\text{NH}_4\text{ISM}$ s in simulated intracellular solution (SIS1). The microelectrodes had punctured the membrane of the sensory neurone.

pre- and postcalibration curves obtained before and after intracellular measurements lasting 4–11 h revealed two important points. Pre- and postcalibration curves were never identical; the electrode response decreased by about 10–15 % after intracellular measurements. The potential difference when SIS was replaced with NAS could decrease by 50 % in postcalibration measurements with electrodes that had been used intracellularly; this did not occur in electrodes that remained outside the cell. It seems possible that this phenomenon explains most of the drift seen when the electrode was withdrawn from the cell. This point will be discussed below as it is important in evaluating the experiments.

To check the influence of several variables on the response of the  $\text{NH}_4\text{ISMs}$  additional calibrations were performed. As  $a_{\text{K}^+}$  may vary between 160 and 90  $\text{mmol l}^{-1}$  from one SR neurone to another (Brown *et al.* 1978; Moser, 1985) we calibrated  $\text{NH}_4\text{ISMs}$  in SIS1 or SIS2, simulating a high- $\text{K}^+$  or a low- $\text{K}^+$  cell. The results are shown in Fig. 3A, where two families of curves can be distinguished. The total voltage response was smaller in SIS1 (filled symbols) than in SIS2 (open

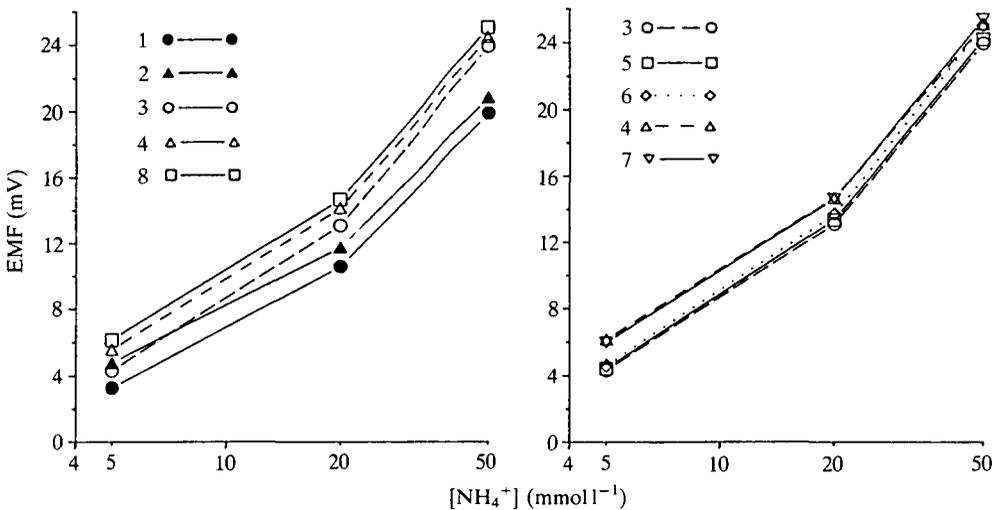


Fig. 3. Calibration curves for  $\text{NH}_4\text{ISMs}$  in several intracellular salines. The effects of background  $[\text{K}^+]$  and  $[\text{Na}^+]$ , the ratio  $[\text{K}^+]/[\text{Na}^+]$ , ionic strength and/or osmotic pressure are tested. The numbers given refer also to Table 1. Saline 1 was SIS1, 2 was SIS1 compensated for  $\text{Na}^+$  (see Table 1), 3 was SIS2, 4 was SIS2 compensated for  $\text{Na}^+$ , 5 was SIS2 with an additional 44  $\text{mmol l}^{-1}$  NaCl, 6 was SIS2 with an additional 44  $\text{mmol l}^{-1}$  glucose, 7 was SIS2 with an additional 44  $\text{mmol l}^{-1}$  NaCl and compensated for  $\text{Na}^+$ , and 8 was SIS2 with an additional 44  $\text{mmol l}^{-1}$  NaCl and compensated for  $\text{Na}^+$ . The symbols represent mean values for the number of electrodes tested (numbers of electrodes are given in Table 1). Standard deviation was omitted as it was mostly within the symbols or close to them. (A) The effects of variable (curves 1 and 3) or constant (curves 2, 4 and 8) ionic strength and/or osmolarity in high- $\text{K}^+$  saline (filled symbols) or low- $\text{K}^+$  saline (open symbols). (B) The effects of low- $\text{K}^+$  salines (curves 3–7) in the presence of 44  $\text{mmol l}^{-1}$  NaCl (curve 5) or glucose (curve 6) and compensated for  $\text{Na}^+$  (curve 7).

symbols), since larger amounts of the main interfering ion ( $K^+$ ) were present in SIS1. As SIS1 was always used for calibrations when working with the SR neurone, the results overestimate the ammonium concentration. Overestimation could be about 30 % at  $5 \text{ mmol l}^{-1} \text{ NH}_4^+$  and about 15 % at  $50 \text{ mmol l}^{-1} \text{ NH}_4^+$ . Table 1 shows that ionic strength was changed from 181 to  $275 \text{ mmol l}^{-1}$ , resulting in a change in the activity coefficient for monovalent ions of about 0.03, calculated according to Ammann (1986, page 76). As the effects are rather small even when amplified under the condition of low  $[K^+]$  in SIS2, we need not go into detailed descriptions of all the variables investigated. In summary, we conclude that  $\text{NH}_4\text{ISM}$ s are sensitive to a number of different variables, especially at low  $\text{NH}_4^+$  concentrations.

Compared with control calibrations we find that osmotic pressure is of some importance for measurements with  $\text{NH}_4\text{ISM}$ s at low  $[\text{NH}_4^+]$  (about 7 % at the  $5 \text{ mmol l}^{-1} \text{ NH}_4^+$  level), but the most important variable is background  $[K^+]$ . Cell volume changes causing alterations of intracellular  $[K^+]$  are critical as they constitute one of the main sources of erroneous measurements for  $[\text{NH}_4^+]$ , while alterations in intracellular  $[\text{Na}^+]$  (by up to  $44 \text{ mmol l}^{-1}$ ) can be ignored.

#### *Penetration of the ammonium-sensitive microelectrode into a cell*

There are two reasons for puncturing a cell with the  $E_m$  electrode before inserting an  $\text{NH}_4\text{ISM}$ . (1) The intracellular  $K^+$  interference produced a potential shift of about +70 mV. The magnitude of this potential was about the same as the membrane potential ( $E_m$ ) and, since the potential of the intracellular  $\text{NH}_4\text{ISM}$  is the sum of  $E_{\text{NH}_4, K}$  and  $E_m$ , this value will be close to 0. It was, therefore, difficult to detect the entry of the  $\text{NH}_4\text{ISM}$ , since a potential change of only a few millivolts could be expected. (2) When the  $\text{NH}_4\text{ISM}$  touched the cell surface, a gradually increasing positive potential shift of up to 30 mV developed as the tip pressed against the membrane. This can be seen in Fig. 4. Comparable effects have never been found with other ISMs measuring intracellular pH and  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentrations.

Fig. 4 shows voltage recordings from an  $E_m$  electrode and an  $\text{NH}_4\text{ISM}$  before and after cell penetration and from an extracellular  $\text{NH}_4\text{ISM}$ . At point A the  $E_m$  electrode crossed the membrane, reaching a value of about -65 mV. As  $[\text{NH}_4^+]_i$  is measured differentially, penetration of the  $E_m$  electrode caused its reading to move in the 'opposite' direction. To show this more clearly a small portion of the recording of the  $[\text{NH}_4^+]_i$  electrode has been replaced by a dashed line. At B the  $\text{NH}_4\text{ISM}$  touched the cell and, as it was pressed against the membrane, a positive potential shift of +20 to +30 mV occurred. Several gentle attempts to push the  $\text{NH}_4\text{ISM}$  into the cell failed. A stable potential value seems to be reached at point C. Exposure to  $K^+$ -NAS ( $25.5 \text{ mmol l}^{-1}$ ) at point D caused the cell to depolarize by about 25 mV, a value similar to that recorded by the extracellular  $\text{NH}_4\text{ISM}$ . The small change in potential indicates that the supposed 'intracellular'  $\text{NH}_4\text{ISM}$  was not yet within the cell. At E the  $\text{NH}_4\text{ISM}$  was pushed inside the cell, as shown by the second depolarization induced by  $K^+$ -NAS at F. Isopotentiality resulted,

Table 1. Effects of simulated intracellular salines (SISs) on the voltage response (EMF) with respect to  $\text{NH}_4\text{Cl}$ -free salines, measured in a number (N) of  $\text{NH}_4^+$ -selective microelectrodes

Curve in Fig. 3	Original saline	Background $[\text{K}^+]/[\text{Na}^+]$	Ionic strength ( $\text{mmol l}^{-1}$ )	Osmolarity ( $\text{mosmol l}^{-1}$ )	Mean EMF (mV) at 5;20;50 $\text{mmol l}^{-1}$ $\text{NH}_4^+$	N
1	SIS1	194/21	225-275	450-550	3.25;10.57;19.86	24
2	SIS1 <sup>1</sup>	194/21-66	275	550	4.75;11.73;20.80	9
3	SIS2	150/21	181-231	362-462	4.30;13.08;23.90	21
4	SIS2 <sup>1</sup>	150/21-66	231	462	5.55;14.13;24.43	9
5	SIS2 <sup>a</sup>	150/65	225-275	450-550	4.40;13.34;24.14	9
6	SIS2 <sup>b</sup>	150/21	181-231	406-506	4.58;13.69;24.98	9
7	SIS2 <sup>1b</sup>	150/21-66	231	506	5.95;14.62;25.34	9
8	SIS2 <sup>1a</sup>	150/65-110	275	550	6.13;14.66;25.01	9

Original salines (in  $\text{mmol l}^{-1}$ ): SIS1, 194 KCl; 21 NaCl; 10 Hepes (pH7.4) plus 0, 5, 20 or 50  $\text{mmol l}^{-1}$   $\text{NH}_4\text{Cl}$ . SIS2, 150 KCl; 21 NaCl; 10 Hepes (pH7.4) plus 0, 5, 20 or 50  $\text{mmol l}^{-1}$   $\text{NH}_4\text{Cl}$ .

<sup>a,b</sup> Additional 44  $\text{mmol l}^{-1}$  NaCl (a) or 44  $\text{mmol l}^{-1}$  glucose (b) as background.

<sup>1</sup> Ionic strength and osmolarity were kept constant in  $\text{NH}_4^+$ -containing salines, as  $\text{NH}_4\text{Cl} + \text{NaCl} = 50 \text{ mmol l}^{-1}$ . In all other solutions these quantities were varied according to the amount of  $\text{NH}_4\text{Cl}$  added.

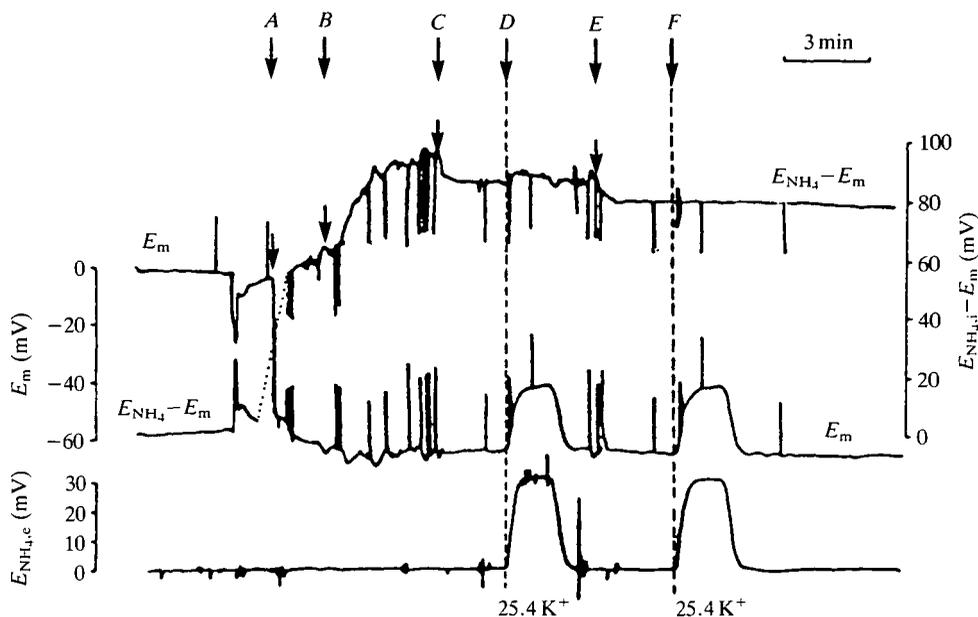


Fig. 4. Pen recordings showing the voltage response from electrodes measuring extracellular  $[NH_4^+]$  (bottom trace,  $E_{NH_4,e}$ ), membrane potential ( $E_m$ ) and intracellular  $[NH_4^+]$  before and after cell penetration (top traces). The calibration for the  $E_m$  electrode is on the left, that for the intracellular  $NH_4ISM$  ( $E_{NH_4,i}$ ) on the right.  $[NH_4^+]_e$  was measured in single mode,  $[NH_4^+]_i$  was measured differentially. Note that the traces for  $E_m$  and  $[NH_4^+]_i$  cross over between points A and B. For a detailed description of points A–F see text.

which strongly suggests that both electrodes were in the same cell. During the experiments we included several depolarizations induced by  $K^+$ -NAS to ensure that the  $NH_4ISM$  remained within the cell.

#### *Intracellular and extracellular measurements of $[NH_4^+]$*

In  $NH_4^+$ -NAS an equilibrium between  $NH_4^+$ ,  $NH_3$  and  $H^+$  will be established, the dissociation constant (pK) being 9.25. Hence, in  $NH_4^+$ -NAS (at a pH of 7.4) about 98% of the ammonium will be present as  $NH_4^+$  and about 2% as  $NH_3$ . The lipophilic, electroneutral  $NH_3$  and the hydrophilic, charged  $NH_4^+$  exert specific effects on the membrane, and they may pass through different pathways at different velocities. Depending on which enters the cell earlier, pHi will change in a particular direction.  $NH_3$  entry causes a decrease in intracellular proton concentration, whereas  $NH_4^+$  entry elicits an increase in intracellular proton concentration. Boron and de Weer (1976) attribute the initial alkalization to the rapid entry of  $NH_3$ , and the subsequent slow acidification (degradation of alkalization, Moser *et al.* 1988) to the entry of  $NH_4^+$ . Upon return to NAS, a period of fast acidification starts, due to a rapid loss of  $NH_3$  converted from intracellular  $NH_4^+$  that had previously entered the cell. An example of simul-

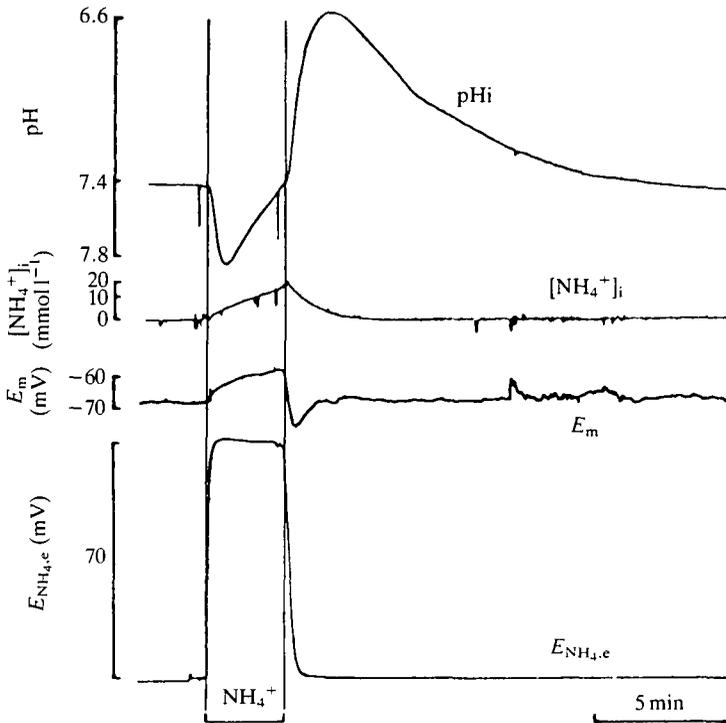


Fig. 5. Pen recordings of simultaneous measurements of pHi (top trace), intracellular ammonium concentration ( $[\text{NH}_4^+]_i$ ; second trace), membrane potential ( $E_m$ ; third trace) and extracellular ammonium concentration ( $E_{\text{NH}_4,e}$ ; bottom trace).  $E_{\text{NH}_4,e}$  was measured in single mode,  $[\text{NH}_4^+]_i$  was measured differentially. The slowly adapting sensory neurone was exposed to  $20 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$  saline for 3 min.

taneous measurements of intracellular pH,  $[\text{NH}_4^+]_i$ ,  $E_m$  and  $[\text{NH}_4^+]_e$  is given in Fig. 5. The cell was punctured with a double-barrelled and a single-barrelled microelectrode and kept in NAS for about 15 min. When all variables had stabilized, the cell was ready to be acid-loaded by exposure to  $\text{NH}_4^+$ -NAS.

Measurements in more than 30 cells are qualitatively and quantitatively similar to the example given in Fig. 5.  $\text{NH}_3$  entered the cell rapidly by diffusion, as shown by the fast alkalization. To account for this alkalosis one must assume an initial increase in  $[\text{NH}_4^+]_i$  of several millimoles per litre. But such an initial, rapid change, which should be well above the detection limit of the  $\text{NH}_4\text{ISM}$ , was not found. Instead,  $[\text{NH}_4^+]_i$  increased slowly in the SR neurone, and this seems to reflect an entry of  $\text{NH}_4^+$  rather than a conversion of intracellular  $\text{NH}_3$  (see above and Discussion).  $\text{NH}_4^+$  may be transported either through channels or by carriers (e.g. a  $\text{Na}^+/\text{K}^+$  pump; Moser, 1987). During a 3 min exposure to  $\text{NH}_4^+$ -NAS, intracellular  $[\text{NH}_4^+]_i$  increased to  $19.6 \pm 4.94 \text{ mmol l}^{-1}$  (mean  $\pm$  s.d.;  $N=142$ ). At the same time, the depolarization increased, but pHi, intracellular  $[\text{NH}_4^+]_i$  and  $E_m$  did not reach steady-state values within 12 min of exposure. During washout in NAS these three variables changed rapidly: pHi exhibited fast acidification

'and the process of acid extrusion,  $E_m$  repolarized and/or hyperpolarized with additional after-effects, and  $[\text{NH}_4^+]_i$  fell exponentially and reached a minimum value close to the initial one. At the point of maximum acidification, nearly all the intracellular  $\text{NH}_4^+$  had left the cell as  $\text{NH}_3$ , which followed its outwardly directed chemical gradient. ( $E_m$  and the electrical gradient tend to keep cations inside the cell; from the experiments shown in this study, we cannot exclude any participation of processes transporting  $\text{NH}_4^+$  out of the cell.) In a few measurements we even found that the potential of the  $\text{NH}_4\text{ISM}$  transiently undershot its original value, a phenomenon that will be discussed below.

#### Extracellular application of the $\text{NH}_4\text{ISM}$

20  $\text{mmol l}^{-1}$   $\text{NH}_4\text{Cl}$  caused the external  $\text{NH}_4\text{ISM}$  to change its potential by about 72 mV (Fig. 5). This indicates that the detection limit for  $\text{NH}_4^+$  is lower in an extracellular solution such as NAS. We carried out several calibrations to determine the detection limit and the effect of adding of 0.1 or 1  $\text{mmol l}^{-1}$  KCl to NAS. The results are shown in Fig. 6.  $\text{NH}_4^+$  concentrations as low as 0.02  $\text{mmol l}^{-1}$  were obtained by diluting  $\text{NH}_4^+$ -NAS with NAS. A change from NAS to NAS containing 0.02  $\text{mmol l}^{-1}$   $\text{NH}_4^+$  caused a potential shift of about 2 mV. The potential difference between 0.02 and 0.2  $\text{mmol l}^{-1}$   $\text{NH}_4^+$  is about 3.5 mV, between 0.2 and 2  $\text{mmol l}^{-1}$   $\text{NH}_4^+$  it is about 21 mV and between 2 and 20  $\text{mmol l}^{-1}$   $\text{NH}_4^+$  it is about 46 mV. Compared with SIS1, the detection limit for

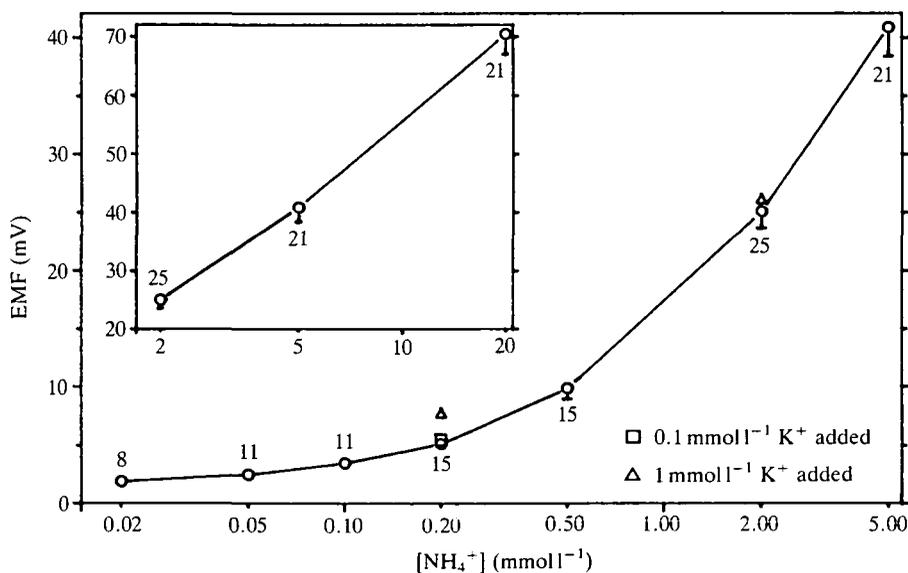


Fig. 6. Calibration curve of  $\text{NH}_4\text{ISMs}$  in normal *Astacus* saline (NAS) in the absence (○) or presence of additional 0.1  $\text{mmol l}^{-1}$  KCl (◻) or 1  $\text{mmol l}^{-1}$  KCl (◻). The inset is the continuation of the calibration curve in which the numbers near the symbols indicate  $N$ , from which the mean value and standard deviation (s.d.) were calculated.

$\text{NH}_4^+$  was at least 10 times (about  $100 \mu\text{mol l}^{-1}$ ) better in NAS (at a background  $\text{K}^+$  concentration of  $5.4 \text{ mmol l}^{-1}$ ). At specified  $\text{NH}_4^+$  levels known concentrations of KCl were added (Fig. 6). At  $0.2 \text{ mmol l}^{-1}$   $\text{NH}_4^+$ , addition of  $0.1 \text{ mmol l}^{-1}$  KCl produced a negligible potential change. At the same  $\text{NH}_4^+$  concentration addition of  $1 \text{ mmol l}^{-1}$  KCl caused a positive potential change which could give rise to an error of about 50 % for  $[\text{NH}_4^+]$ . In contrast to this, addition of  $1 \text{ mmol l}^{-1}$  KCl when  $[\text{NH}_4^+]$  was  $2 \text{ mmol l}^{-1}$  produced an error of only 15 %. This result again indicates the importance of the main interfering ion ( $\text{K}^+$ ) for the detection limit of the  $\text{NH}_4\text{ISM}$ . For exact measurements with the  $\text{NH}_4\text{ISM}$ , a knowledge of background  $[\text{K}^+]$  and its possible alterations during experimentation seems essential.

### Discussion

#### *Concept of evaluation; absolute versus apparent values of $[\text{NH}_4^+]$*

$\text{NH}_4\text{ISM}$ s show a remarkable drift within 4–11 h, mainly in the positive direction ( $22.1 \pm 15.9 \text{ mV}$ ; mean  $\pm$  s.d.;  $N=26$ ) when the baseline (in NAS) is considered before and after intracellular measurement. Because of this drift, the NAS value cannot be used as a reference for calibration of intracellular  $[\text{NH}_4^+]$ . The source of this drift could be either changes in the electrode, caused by its penetration through the plasma membrane, or ionic alterations in the preparation during the experiment.

As mentioned above and partially shown in Figs 1 and 2, postcalibration curves differ from precalibration curves in the magnitude of their potential changes (about 10 %) and in their response to changes from NAS to SIS (about 50 %). Each factor by itself, and both together, can cause electrode drift. Intracellular ionic alterations can be seen if we follow the intracellular baseline (IB) of the  $\text{NH}_4\text{ISM}$ . Values of IB can be obtained from the potential of the differential signal of the intracellular  $\text{NH}_4\text{ISM}$  before an  $\text{NH}_4^+$  exposure or after recovery of the cell from an  $\text{NH}_4^+$  exposure. An hour-by-hour comparison of the mean value of the IB of 31 cells shows that within 7 h of experimentation the mean values differed by a maximum of 1 mV, whereas the standard deviation increased from  $\pm 3.4 \text{ mV}$  (after 2h) to  $\pm 5.6 \text{ mV}$  (after 7h). From the relatively stable IB we conclude (1) that minor changes occur in  $[\text{NH}_4^+]_i$  and  $[\text{K}^+]_i$  when the cells are allowed to recover from an exposure; (2) that the amount of  $\text{NH}_4^+$  produced or taken up by various metabolic pathways (Kvamme, 1987) is either precisely regulated, shown by its tendency towards a constant value during recovery, or it is below the detection limit of the  $\text{NH}_4\text{ISM}$ ; (3) that the main changes in the electrode response occur during impalement or when the electrode is withdrawn. We propose to take the IB as a reference for the evaluation of the  $[\text{NH}_4^+]$  measurements. In doing so, we cannot give absolute values of  $[\text{NH}_4^+]_i$  (which would be difficult in any case owing to interference from mostly unknown amounts of intracellular  $\text{K}^+$ ), but only apparent values. The apparent value must be corrected by the basic background level of  $\text{NH}_4^+$  to obtain absolute values. The presentation of apparent values

instead of absolute values is, in principle, similar to measurements with chloride-sensitive microelectrodes (e.g. Deisz and Lux, 1982).

We have not been able to measure background  $[\text{NH}_4^+]_i$  for several reasons: (1) the amount of intracellular  $\text{K}^+$  is unknown, (2) there is a positive potential shift when attempts are made to penetrate the cell, (3) there is a severe drift of the response of the  $\text{NH}_4\text{ISM}$  with respect to the reference level in NAS, (4)  $\text{NH}_4^+$  level could be below the limit of detection of the  $\text{NH}_4\text{ISM}$ , and (5) it is questionable whether all metabolic pathways in which  $\text{NH}_3/\text{NH}_4^+$  is involved can be blocked without killing the preparation. Thus, an  $\text{NH}_3/\text{NH}_4^+$ -free cell cannot be obtained (in contrast to a  $\text{Cl}^-$ -free cell). Points 2–4 also mean that intracellular  $[\text{K}^+]$  can be neither estimated nor calculated from the IB.

For evaluation, the postcalibration curve was used and matched to the actual value of the IB. Therefore, the apparent values given must be considered to be maximum values (Fig. 2). In contrast, during extracellular application of the  $\text{NH}_4\text{ISM}$  the measurement of absolute values of  $[\text{NH}_4^+]$  seems to be possible, especially when  $[\text{K}^+]$  is known during calibration and when it is ascertained that  $[\text{K}^+]$  does not change.

#### *Detection limits of the $\text{NH}_4^+$ -sensitive electrode*

The detection limit of the  $\text{NH}_4^+$ -sensitive electrode is affected by the concentration of  $\text{K}^+$  present in the solutions (Figs 2 and 6). From calibration curves in SIS1 salines with high ( $194\text{ mmol l}^{-1}$ ) background  $\text{K}^+$  levels (Fig. 3), we deduce that  $\text{NH}_4^+$  concentrations below  $5\text{ mmol l}^{-1}$  are estimates rather than measurements. Between 5 and  $20\text{ mmol l}^{-1}$   $\text{NH}_4^+$  a potential change of 1 mV corresponds to a concentration change of approximately 20%. In other words, the differential potential measurements must be accurate to 1 mV to give estimates for  $[\text{NH}_4^+]_i$  within a 20% error limit. Between 20 and  $50\text{ mmol l}^{-1}$   $\text{NH}_4^+$  a 1 mV potential shift represents a 10% change in  $\text{NH}_4^+$  concentration.

In addition to these limitations and the relatively poor resolution when  $\text{NH}_4\text{ISM}$ s are applied intracellularly, there are additional sources of error which may mimic the presence of, or changes in,  $[\text{NH}_4^+]_i$ . These include changes in  $[\text{K}^+]$ , ionic strength, osmolarity, volume and pH. Their influence is discussed in the text and is apparent from Fig. 3. It is almost impossible to exclude changes in all these factors during an experiment. To improve the detection limit of the  $\text{NH}_4\text{ISM}$ , simultaneous measurement of intracellular  $[\text{K}^+]$  is essential and the measured  $[\text{K}^+]$  should be used for calibrations.

Extracellular measurements with  $\text{NH}_4\text{ISM}$ s are not only easier to perform but also much more accurate, because in  $\text{NH}_4^+$ -NAS the slope of the electrode response is steeper and the detection limit is at least 25 times lower than that of intracellular measurements (Figs 5 and 6). Between 0.2 and  $0.5\text{ mmol l}^{-1}$   $\text{NH}_4^+$ -NAS a potential change of 1 mV corresponds to a change in  $\text{NH}_4^+$  concentration of 20%. For comparison, this is the same value as that found for intracellular calibration in  $\text{NH}_4^+$ -SIS between 5 and  $20\text{ mmol l}^{-1}$   $\text{NH}_4^+$ . Again, the detection

limit can be improved when the background  $[K^+]$  is less than  $5.4 \text{ mmol l}^{-1}$  or when  $[K^+]$  and ionic strength do not change during the experiment.

#### *Undershoot of the internal baseline*

In a number of experiments we found that, after removal of  $\text{NH}_4\text{Cl}$ , the voltage of the  $\text{NH}_4^+$ -sensitive electrode did not return to its original value of the internal baseline but showed a transient undershoot lasting for a few minutes. Drift of the  $\text{NH}_4\text{ISM}$  can be excluded because of the transient nature of the undershoot. The effects of intracellular acidification (Fig. 1) can be excluded since the time courses are too different. The increase in intracellular  $[\text{Na}^+]$  due to pH<sub>i</sub> regulation is too small (Fig. 3) to have an effect and, more importantly, it is in the opposite direction to the undershoot. For potassium the situation is as follows: a rather wide range of intracellular  $K^+$  activities ( $90\text{--}165 \text{ mmol l}^{-1}$ ) has been found in this preparation (Brown *et al.* 1978; Moser, 1985). An assumed change of  $10 \text{ mmol l}^{-1} K^+$  in a background of  $90 \text{ mmol l}^{-1}$  will be sensed by the  $\text{NH}_4^+$ -sensitive electrode as larger than the same change in a background of  $160 \text{ mmol l}^{-1}$ , as verified by calibrations. Changes in  $[K^+]_i$  can be caused to some extent by substitution of  $\text{NH}_4^+$  for  $K^+$  (Moser, 1987) or by volume changes (e.g. Ballanyi and Grafe, 1988). Before drawing any conclusions about this factor it is essential to measure  $[K^+]_i$ .

The undershoot phenomenon could also mean that the intracellular baseline includes a substantial amount of  $\text{NH}_4^+$ . To exclude or to verify this factor, a systematic analysis of the undershoot phenomenon should be carried out. However, this was not the subject of the present work.

#### *Positive potential shift*

Touching the plasma membrane with an  $\text{NH}_4\text{ISM}$  (Fig. 4) causes a positive potential to develop. This is not a general phenomenon for all ISMs but seems to be specific for  $\text{NH}_4\text{ISM}$ s. The potential could arise either from  $\text{NH}_4^+$  or from  $K^+$ . We assume that the potential is caused by  $K^+$  accumulated close to the external surface of the membrane. This effect may be linked to the destruction of small glial cells which surround the sensory neurone.

#### *The model of Boron and de Weer*

When comparing directly measured intracellular  $[\text{NH}_4^+]_i$  with that (indirectly) predicted from pH<sub>i</sub> measurements by the Boron and de Weer (1976) model, we find clear quantitative differences. Although the quantitative aspect will be examined more closely in a separate paper, Fig. 5 shows only a slow increase in  $[\text{NH}_4^+]_i$  during the period of initial alkalization. According to the Boron and de Weer model, a rapid increase in  $[\text{NH}_4^+]_i$  of  $10\text{--}20 \text{ mmol l}^{-1}$  was to be expected (depending on pH<sub>i</sub>). In agreement with the model, we see a slow increase in  $[\text{NH}_4^+]_i$  during the period of slow acidification and a rapid decrease in  $[\text{NH}_4^+]_i$  during the period of fast acidification.

It is too early to decide what kind of additional assumptions have to be included in the model. For example, one can argue that the differences between our results

and those of the model might be due to some kind of intracellular  $\text{NH}_4^+$  buffering and/or involvement of  $\text{NH}_4^+$  in glutamine and glutamate metabolism, but this cannot yet be confirmed. This study provides evidence that the quantitative difference in determination of  $[\text{NH}_4^+]_i$  cannot be attributed to the temporal and/or quantitative limitations of  $\text{NH}_4\text{ISM}$ s.

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