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REVIEW LECTURE*

EXPERIMENTAL DISPLACEMENT OF INTRACELLULAR pH AND THE MECHANISM OF ITS SUBSEQUENT RECOVERY

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INTRODUCTION

Once upon a time those who cared believed that the intracellular pH (pH_i) of animal cells depended only on the external pH and the membrane potential. They thought H^+, OH^- and HCO_3^- ions were passively distributed across the cell membrane. We now know they were wrong: there is some mechanism in the cell membrane doing something equivalent to extruding acid. This keeps pH_i about the same as external pH.

The biochemical importance of pH_i is well understood. Although normal pH_i is near neutrality, and the H^+ ion concentration is less than 0·1 μM (a million times less than internal K^+) the H^+ ions are so reactive with proteins that pH_i needs to be closely regulated.

In this review lecture I will first describe ways of displacing pH_i, since it has proved easier to study the way pH_i recovers than the way (presumably the same) it is normally kept constant. Indeed, nearly all research on pH_i regulation has been on how pH_i recovers from acidification. Secondly, I will review the various mechanisms proposed for different cells, with examples of the experimental evidence.

For a detailed account of the whole field of intracellular pH, I recommend the exhaustive review by Roos & Boron (1981) together with the book edited by Nuccitelli & Deamer (1982).

METHODS OF INVESTIGATION

At present, four different types of method are used to measure pH_i: indicators, DMO (5,5-dimethyl-2,4-oxazolidinedione) distribution, nuclear magnetic resonance, and pH-sensitive micro-electrodes. Perhaps the oldest method is to use indicators: dyes that change colour or fluorescence with changes in pH. Such indicators can be used on single large cells or with suspensions or cultures of many small ones. Calibration may be difficult, but with new derivatives of fluorescein in particular, the method is gaining acceptance.

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A decade or so ago, the most widely used method was to measure the distribution of a weak acid or base. Radioactive DMO, a weak acid, gave the best results. The higher the pH_i, the more the DMO anion accumulates intracellularly. The main drawbacks of this method are its complexity, slowness, and destructiveness.

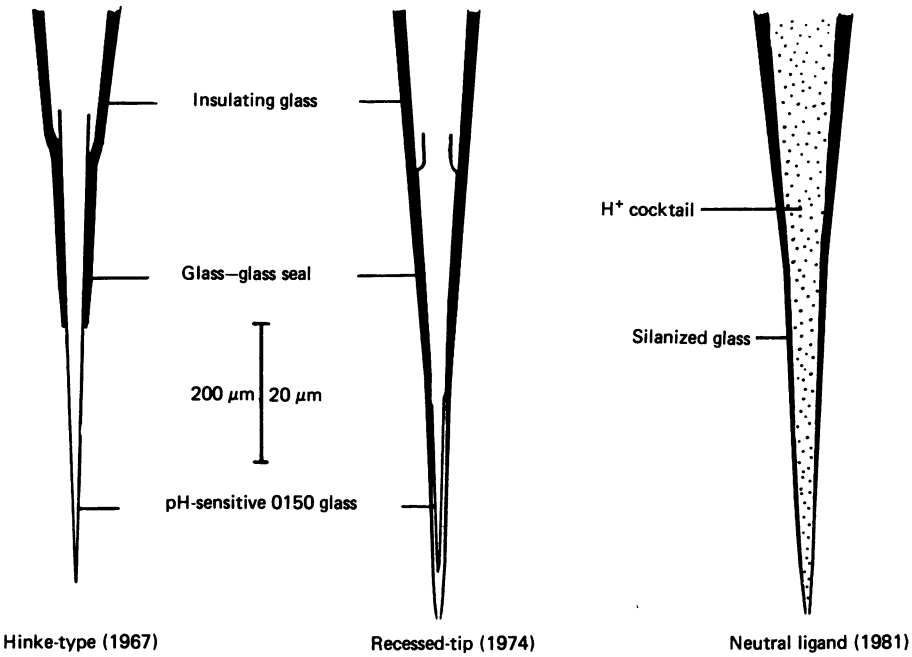

Fig. 1. Diagram of the sharp ends of three types of pH-sensitive micro-electrode. Scale 200 μm for the Hinke-type, 20 μm for the other two.

In contrast, nuclear magnetic resonance is non-destructive, non-invasive and novel. Unfortunately, it is expensive and requires relatively large tissue samples. It is often difficult to be sure in what part of the cell the pH is being measured, but the technique can measure much more than pH.

I think the best method, at least for studying pH_i regulation in single cells, is the pH-sensitive micro-electrode. Three different designs, as shown in Fig. 1, have been used recently. The Hinke-type was first described in 1967 (Hinke, 1967) and has been much used by Roos, Boron and their co-workers on barnacle muscle and squid giant axons: the exposed length of pH-sensitive glass (several hundred micrometres) is too long for other cells. The recessed-tip type was first described ten years ago (Thomas, 1974), and has been widely used to measure pH in medium-sized cells such as snail neurones. The pH-sensitive glass is inside the end of an insulating glass micropipette, so the micro-electrode is robust and very long-lasting. If sharp, however, it normally responds rather slowly, and it is tedious to make. The third type depends on a pH-sensitive cocktail developed in Zurich (Ammann, Lanter, Steiner, Schulthess, Shijo & Simon, 1981). This cocktail is an organic solution sensitive to pH which is held in the tip of a micropipette made hydrophobic by treatment with silanes. This

type of electrode is easy to make, responds rapidly, and can even be made into double-barrelled electrodes sharp enough to penetrate smooth muscle cells (Aickin, 1984). It tends to have a short and rather unreliable life, but at least if one electrode fails to work it is easy to make another, and no elaborate microforge is required as with the other designs.

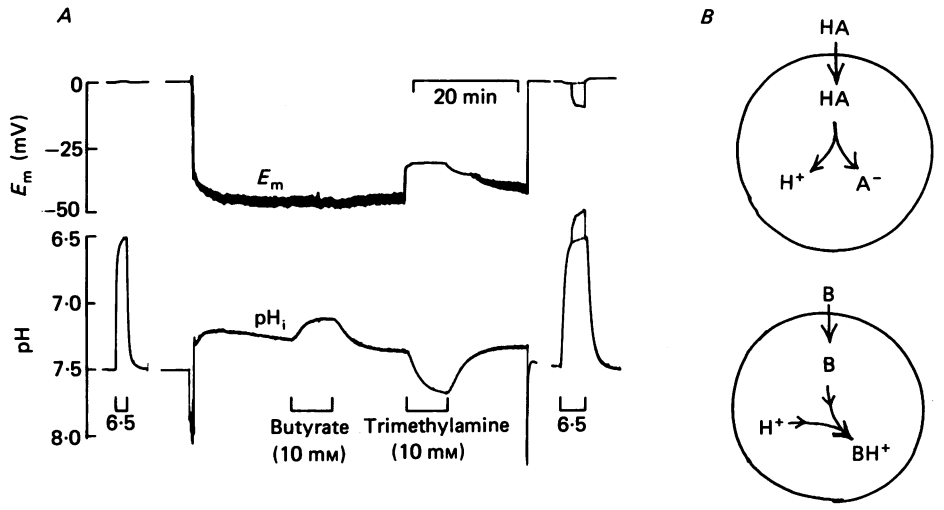

Fig. 2. Weak acid and base effects. *A*, experiment to show the effect of butyric acid and trimethylamine base on the E_m and pH_i of the snail neurone. All solutions were CO_2-free and buffered with HEPES to pH 7·5, except when the pH micro-electrode was calibrated with a pH 6·5 solution. (R. C. Thomas, recent unpublished experiment.) *B*, diagram showing weak acid (HA) producing H^+ ions inside a cell and a weak base B consuming them.

The electrical set-up required to measure pH_i with micro-electrodes is very simple: after all, the procedure is essentially the same as followed with a conventional pH meter. Normally, one has a very high impedance amplifier for the pH micro-electrode, a high impedance amplifier for a conventional micro-electrode (needed to serve as reference for the pH electrode as well as to measure the membrane potential of the cell), various micromanipulators, shielding, an experimental chamber with superfusion system and easily changed solutions, and the usual range of recording systems (for details see Thomas, 1978).

EXPERIMENTAL WORK

The rest of this review lecture is in two parts. First, I will describe various ways of changing pH_i without changing the pH of the external solution (pH_o). As examples, I will describe some recent results of mine on snail neurones, although most of what I will tell you has been known for over forty years. Secondly, I will survey the work on pH_i regulation in animal cells published over the last eight years.

Changing pH_i without changing pH_o

The easiest way to do this is to expose the cell to a weak acid or base, as shown in Fig. 2. Weak acids or bases such as butyric acid or trimethylamine are incompletely dissociated in aqueous solution, so even at pH 7·5 there will be some uncharged molecules of the acid or base present. As first shown by Jacobs (1920), cell membranes are generally much more permeable to such molecules than to charged ones of the same compound (see also Sharp & Thomas, 1981, Boron, 1983 and De Hemptinne, Marrannes & Vanheel, 1983).

In the experiment shown in Fig. 2*A*, I first checked the pH micro-electrode response to a pH 6·5 solution with both the pH and KCl-filled reference micro-electrodes poised above an exposed snail neurone in an experimental chamber. Next, I resolutely pushed the pH electrode into the chosen neurone. The pH record showed a deflexion of about 25 mV. Then I penetrated the same neurone with the KCl micro-electrode. Since the pH record in the Figure is the difference between the voltage recorded by the two micro-electrodes, both membrane-potential (E_m) and pH traces show the potential change as the KCl electrode penetrates the cell.

At first pH_i fell, probably due to injury, then began to recover. About 20 min after penetration, I changed the superfusing solution to one of the same composition (standard snail Ringer solution buffered with HEPES to pH 7·5) but with an additional 10 mM-Na butyrate. This solution has no effect on E_m, but caused pH_i to fall to about 7·1. When the butyrate was removed, pH_i returned to about 7·4. Next, I exposed the cell to 10 mM-trimethylamine HCl. This weak base caused pH_i to increase to about 7·7. Again, the effect was easily reversed. Finally, I pulled the electrodes out of the cell and checked the calibration.

The explanation of these pH_i responses is as follows (see also Fig. 2*B*). As well as the usual constituents of snail Ringer solution, the butyrate solution contains both butyrate ions and undissociated butyric acid. For any weak acid (HA) the relation between the concentrations of the anion and the acid are given by the Henderson–Hasselbalch equation:

$$pH = pK_a + \log \frac{[A^-]}{[HA]}.$$

The pK_a, or dissociation constant, is the pH at which a weak acid (or, by analogy, a weak base) is half-dissociated so that $[A^-] = [HA]$. The pK_a for butyric acid is 4·8. Entering this into the above equation, it is easy to calculate that at a pH of 7·5 and a butyrate concentration of 10 mM, the undissociated butyric acid concentration will be about 20 μM . This acid rapidly enters the neurone. Inside it dissociates to give H^+ and butyrate ions. This process continues until the undissociated butyric acid concentration is the same inside the cell as outside. It is then possible to calculate the internal butyrate concentration from the Henderson–Hasselbalch equation and the pH_i. In Fig. 2 the pH_i stabilized at 7·1, so the calculated internal butyrate is about 4 mM. Thus, during the few minutes that pH_i fell from about 7·3 to 7·1, enough butyric acid entered to make 4 mequiv/l cell water of both H^+ and butyrate ions. The smallness of the pH_i change is due to buffering; indeed the buffering power can be estimated by dividing the internal butyrate concentration by the pH change.

Since trimethylamine has a pK_a of 9·8, a 10 mM solution at pH 7·5 will contain about

50 μM free uncharged base. This enters the neurone easily, until the internal level equals the external. At (almost) steady state, the pH_i has increased to about 7·7. At this pH there will be about 6 mM-internal trimethylammonium ions, and there will have been a subtraction from the cell water of the same number of H^+ ions. This combination of internal H^+ ions with the entering uncharged base explains the pH_i increase.

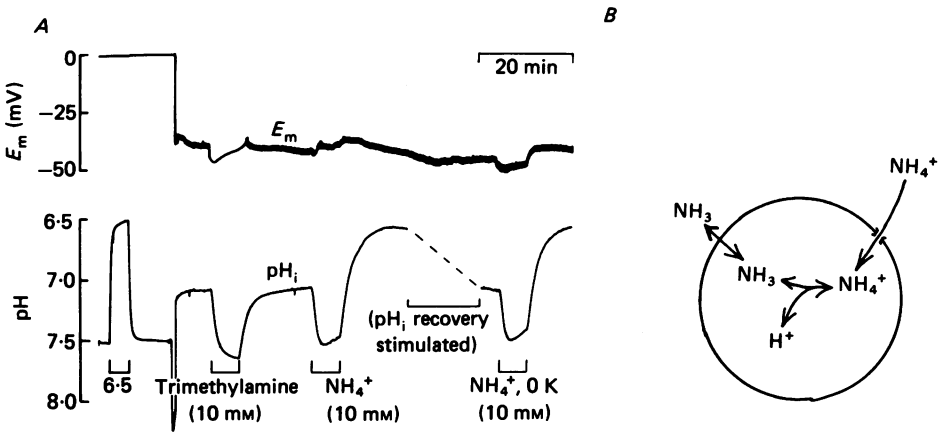

Fig. 3. *A*, experiment comparing the effects of trimethylamine and ammonium solutions on the E_m and pH_i of the snail neurone. Except for calibration, external pH was 7·5. Between the two NH_4Cl exposures, pH_i recovery was stimulated by adding bicarbonate to the Ringer solution. Otherwise all solutions were CO_2 free. The second time NH_4Cl was applied, it was in K-free Ringer solution. (R. C. Thomas, unpublished experiment.) *B*, diagram showing the two routes by which NH_3 and NH_4^+ can influence pH_i.

Although compounds such as trimethylamine are not often encountered, Zucker (1981) has reported that they can occur in some samples of K^+ channel blockers like tetraethylammonium (of which any fishy smell should make the user wary.) Quite small levels of impurity can have large effects on pH_i and cause much confusion.

Ammonia. Physiologically, probably the most important weak base is NH_3, which takes up H^+ ions to give NH_4^+ ions:

$$NH_3 + H^+ \rightleftharpoons NH_4^+.$$

The experiment shown in Fig. 3*A* compares the effect of 10 mM-trimethylamine HCl with that of 10 mM-NH_4Cl. At pH 7·5 the NH_4^+ solution will contain about 100 μM of uncharged NH_3, which permeates cell membranes very easily. As seen in Fig. 3 this causes a similar rapid pH_i increase as that produced by trimethylamine. With NH_4^+, however, the pH_i does not approach a steady high value, but begins to decrease again. This 'plateau-phase acidification' was first analysed in detail by Boron & de Weer (1976*a*), and is due to the cation NH_4^+ also entering the cell, driven by the membrane potential. NH_3 enters down only a concentration gradient, but NH_4^+ ions are attracted in by the membrane potential as well. The steady state for NH_3 is attained when internal NH_3 equals external due to diffusion equilibrium. According to the Law of Mass Action, the concentration of internal NH_4^+ is then about 10 mM

at the pH_i of 7·5 seen in Fig. 3. An equilibrium for NH_4^+ entry, given that E_m is about −40 mV, will only be attained when the internal NH_4^+ concentration reaches about 50 mM. The plateau-phase acidification occurs because the continuous entry of NH_4^+ drives the early conversion of NH_3 to NH_4^+ *backwards* (see Fig. 3*B*). As a consequence of this, the internal NH_3 concentration soon exceeds the external NH_3 level, so NH_3 starts to leave the cell. Thus as NH_4^+ ions continue to enter the cell some shed a H^+ ion and leave as NH_3. In other words NH_4^+ acts as a proton carrier.

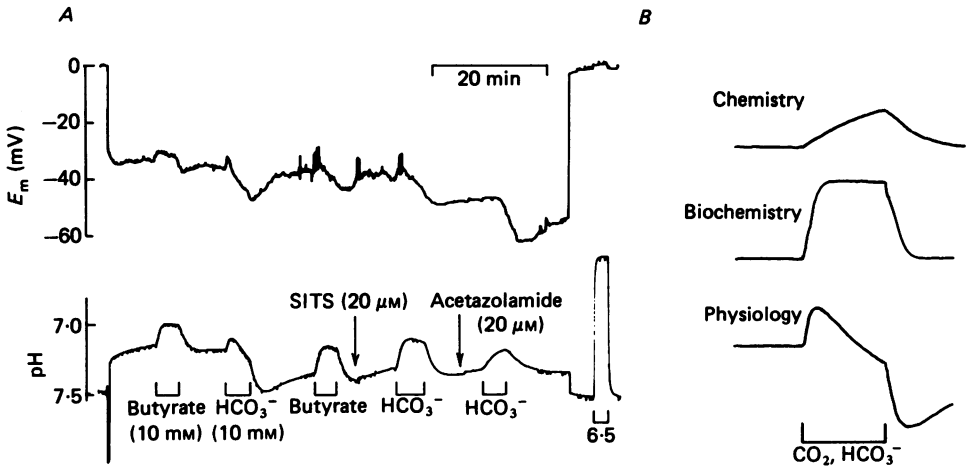

Fig. 4. *A*, experiment comparing the pH_i response of the snail neurone to butyrate and bicarbonate. External pH was 7·5 except for the pH electrode calibration. Bicarbonate solutions were equilibrated with 1·3 % CO_2 in air, the other solutions were CO_2 free. The arrows show where SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid) and acetazolamide were applied. (Recent unpublished experiment by R. C. Thomas.) *B*, diagram showing the three components of the pH_i response to CO_2.

By the time the external NH_4Cl is removed, the cell will have accumulated much more NH_4^+ than if it were impermeant and only NH_3 could enter. Almost all this NH_4^+ will leave as NH_3, since leaving as the ion would be against the electrical gradient. In leaving as NH_3 it sheds H^+ ions, causing the large fall in pH_i observed. The technique of acid-loading cells by NH_4^+ exposure was pioneered by Boron & de Weer (1976*b*) and has been widely used in investigations of pH_i regulation, as will be seen later.

The NH_4^+ ions probably enter largely through K^+ channels, although some may be carried in by the Na pump. In the experiment shown in Fig. 3, however, removal of external K during exposure to NH_4Cl did little to increase plateau-phase acidification. Much more spectacular effects of K removal were seen in mouse soleus muscle by Aickin & Thomas (1977) as described later.

Carbon dioxide. The physiologically important weak acid is, of course, carbonic acid, in equilibrium with CO_2. Its reaction in aqueous solution are probably the most important chemical reactions in acid–base physiology:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

The reaction of CO_2 with water is normally very slow, but is accelerated by the enzyme carbonic anhydrase. The dissociation of carbonic acid is effectively instantaneous.

While the effects of NH_3 on pH_i involve two processes, those of CO_2 on snail neurones involve three, as shown in Fig. 4.

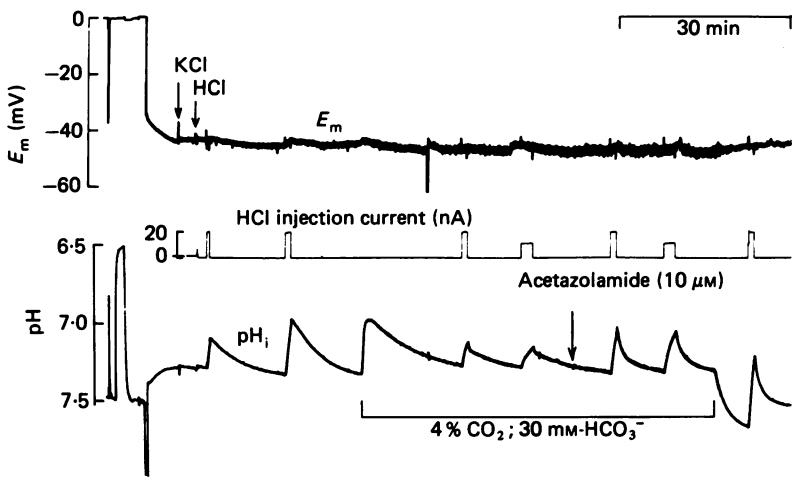

Fig. 5. Buffering power of a snail neurone and the effects of increasing the CO_2 from 0·5 to 4 % and of applying acetazolamide. External pH was 7·5 except for initial calibration. Before and after the period in 4 % CO_2 the preparation was superfused with Ringer solution equilibrated with 0·5 % CO_2. The arrows above the E_m record indicate when the two current-carrying electrodes were pushed into the cell. (Recent unpublished experiment by R. C. Thomas.)

In Fig. 4*A*, I have illustrated a recent experiment, done with a proton-cocktail micro-electrode, in which I compared the effects of 10 mM-butyrate with those of 10 mM-bicarbonate (with 1·3 % CO_2 to keep the pH at 7·5). As before, butyrate caused a fall in pH_i which reached a steady state after about 3 min, but when bicarbonate was applied there was only a brief acidification before pH_i began to increase, soon reaching a level higher than before bicarbonate application. This pH_i increase in the presence of bicarbonate must be due to bicarbonate stimulating pH_i recovery from acidification. When bicarbonate was applied after SITS (4-acetamido-4′isothiocyanato-stilbene-2,2′disulphonic acid, a blocker of pH_i regulation as described later) its effects became much like those of butyrate. When bicarbonate was applied after acetazolamide the pH_i changed only slowly.

Thus, as sketched in Fig. 4*B*, the pH_i response of the snail neurone to bicarbonate and CO_2 has three components: (1) the chemical reaction of CO_2 with water to produce acid, (2) the biochemical acceleration of this reaction by carbonic anhydrase, (3) the physiological stimulation of pH_i recovery by the bicarbonate. The large and rapid effects of CO_2 on pH_i have been known for a long time; indeed they were first directly recorded in squid axons by Caldwell (1958) over a quarter of a century ago. He used 100 % CO_2 at pH 4·7, and so saw no sign of pH_i recovery.

In the lecture CO_2 entry into a model cell was demonstrated using a small polyethylene bag containing a few millilitres of a solution of litmus in water. When dropped into a carbonated drink the blue litmus changed to red within a few minutes. (This was displayed on an overhead projector.)

HCl injection. In some cells it is possible to change pH_i directly without changing pH_o by injecting HCl, or by dialysing the cell interior with acid or alkaline solutions. A recent experiment in which I injected HCl to demonstrate the effects of CO_2 on buffering power is shown in Fig. 5. HCl was injected by passing a 10 or 20 nA current down a micro-electrode filled with HCl and up another filled with KCl. All except the

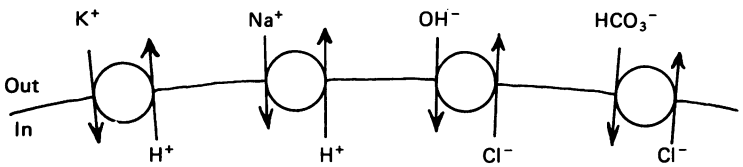

Fig. 6. Diagram of four possible electroneutral mechanisms for extruding acid.

first injection were made with the same charge. In 4% CO_2 the pH_i decreases were much smaller than when the cell was in 0·5%, showing that the buffering power had increased. Acetazolamide partially reversed the effect of the CO_2 increase. This suggests a possible role for carbonic anhydrase in maximizing intracellular buffering in CO_2, as originally reported by Thomas (1976*a*). The physiological importance of this mechanism is obscure.

A brief explanation of the more than doubling of the buffering power in 4% CO_2 is that at any pH_i there will be eight times more intracellular bicarbonate than in 0·5% CO_2. When CO_2 is constant, bicarbonate has a molar buffering power four times that of other buffers, and is effective over a wider pH range than when CO_2 varies.

Conclusions. For most cells the best way of decreasing pH_i is to load the cell with NH_4^+ ions, and then change to an NH_4^+-free solution. This causes a large acidification which can take place in any test solution desired. The best way of increasing pH_i is by loading the cell with bicarbonate in a solution equilibrated with a moderate level of CO_2 and buffered to normal pH with bicarbonate. When the CO_2 is removed, pH_i will increase as internal bicarbonate takes up H^+ ions and leaves as CO_2. Only in a few preparations can the pH_i be changed by direct injection or perfusion.

The ionic mechanisms of pH_i regulation

In this part I will concentrate on pH_i recovery from acidification. Animal cells are normally subjected to passive acidification, and often appear to have no special mechanism for accelerating a recovery from a pH_i increase.

By early 1976, reports had appeared that pH_i recovery from acidification seemed to be facilitated by bicarbonate, but otherwise little was known about the ionic mechanism. Various possible mechanisms for acid extrusion are shown in Fig. 6. Na–H exchange would require no metabolic energy, but the other three ion-exchange mechanisms would need some energy input. The two anion-exchanger systems would neutralize intracellular H^+ ions rather than extruding them.

Squid giant axon. The first publication completely devoted to the mechanism of pH_i regulation was a letter to *Nature* by Boron & de Weer; published early in 1976. Its title summarized the conclusions very well: 'Active proton transport stimulated by CO_2/HCO_3^-, blocked by cyanide.' Fig. 7 is from this paper, although I have inverted the pH_i records to make them clear to an international audience.

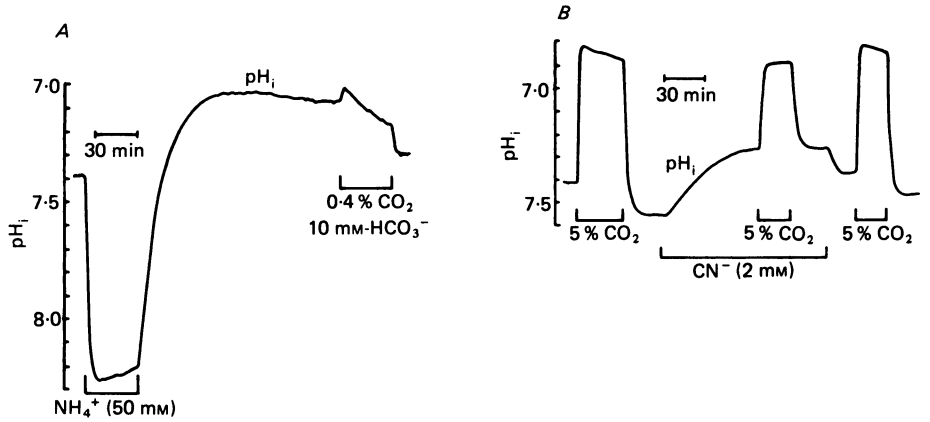

Fig. 7. Experiments on pH_i regulation by squid giant axons. *A*, the effect of 0·4 % CO_2, 10 mM-bicarbonate on pH_i recovery after acid-loading by the NH_4Cl method. *B*, the effect of cyanide on the pH_i recovery from the acidification caused by adding CO_2. (From Boron & de Weer 1976*b*, reproduced with permission of *Nature* and inverted.)

The record on the left shows that the pH_i did not recover from an NH_4^+-induced acidification until 10 mM-bicarbonate was added to the artificial sea water bathing the axon. The record on the right is from an experiment where CO_2 application was used to acidify pH_i. During the first and last applications of 5 % CO_2 (at pH 7·4) the pH_i began to recover during the CO_2 exposure, but when cyanide was present this pH_i recovery in CO_2 did not occur.

Later in 1976, Boron worked with Russell at Woods Hole on dialysed squid axons. Using this technique they could change the axoplasmic concentrations of Cl^-, ATP, and H^+ ions, and could follow radioactive Cl efflux as well as record pH_i changes.

Some of their results are shown in Fig. 8. Again I have inverted the pH_i record. Fig. 8*A* shows that only with both internal Cl and external bicarbonate does pH_i increase after it has been lowered by dialysis with an acid solution. SITS blocked the pH_i recovery. Fig. 8*B* illustrates the efflux of Cl^+ from an axon initially without, and later with, internal ATP. Only when ATP was present did bicarbonate stimulate Cl efflux. Thus, internal Cl was not only needed for pH_i recovery from acidification, but part of its efflux had the same requirements for ATP and bicarbonate. Russell & Boron (1976) therefore proposed that pH_i regulation in squid axons was by an ATP-driven Cl/HCO_3 exchange, as shown in Fig. 8*C*. Sadly, this scheme later proved incomplete.

Snail neurone. I started working on the pH_i of snail neurones about ten years ago. Not, I admit, from any realisation of the importance or virginity of the field, but only because I needed to demonstrate the usefulness of a new design of pH-sensitive

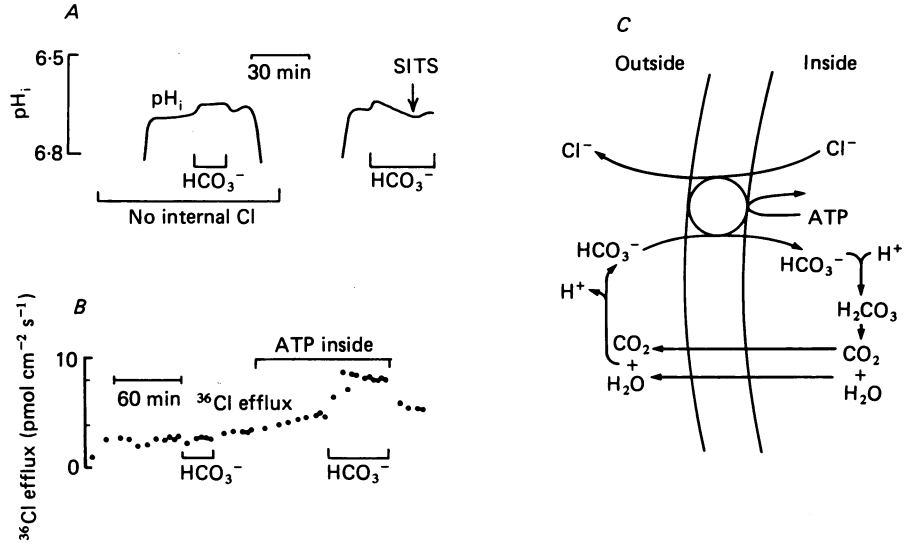

Fig. 8. Squid giant axon. *A*, experiment showing that pH_i recovery in dialysed squid axon only occurs in presence of internal Cl and external bicarbonate. *B*, radioactive Cl efflux from dialysed squid axon. Added bicarbonate (10 mM) stimulated extra efflux only when dialysis fluid also contained ATP. *C*, scheme for acid extrusion from squid axon. (From Russell & Boron, 1976, with permission of *Nature*.)

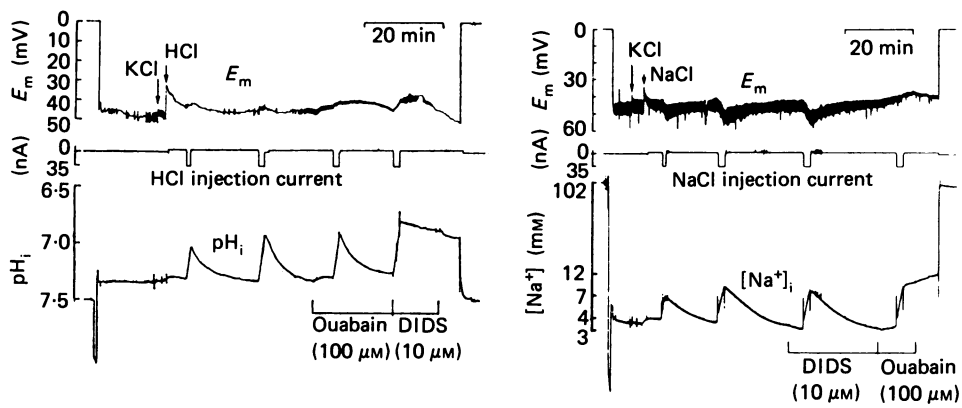

Fig. 9. Comparison of E_m and pH_i response of the snail neurone to HCl injection with the E_m and internal Na^+ response to NaCl injection. The transport inhibitors ouabain and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) were applied where indicated. All solutions were equilibrated with 2·5% CO_2. (From Thomas, 1976*b*, with permission of *Nature*.)

micro-electrode. At first I studied the effects of CO_2 and NH_3 and tried to understand intracellular buffering. Later, as the result of a conversation in Kiev with a Georgian biochemist named Kometiani, I set out to show that pH_i regulation did *not* involve the Na pump. Fig. 9 shows experiments from this project. Having both Na^+- and pH-sensitive micro-electrodes I was able to compare the pH_i response to HCl injections with the internal Na^+ ion concentration response to NaCl injection.

The pH_i recording shows that the responses to two control injections of HCl and to one in the presence of ouabain were much the same: ouabain did not block pH_i recovery, but DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) did. (Both SITS and DIDS specifically block anion exchange in red blood cells.) The second experiment shows that DIDS had no effect on the Na pump, and confirms that ouabain did. Thus, pH_i recovery from HCl injection and internal Na recovery from NaCl injection must have quite different mechanisms.

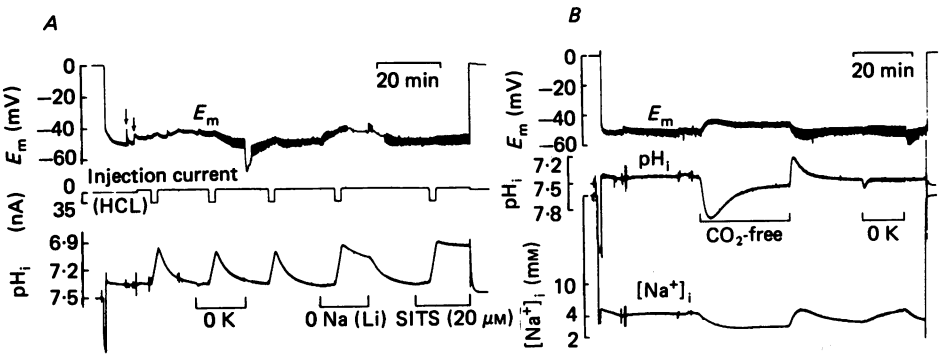

Fig. 10. The role of Na in pH_i recovery in snail neurones. *A*, the effect of removal of K and Na, and application of SITS, on pH_i recovery from HCl injections. All the solutions were equilibrated with 2·5% CO_2 (from Thomas, 1976*c*). *B*, the effect on the pH_i and internal Na^+ concentration ($[Na^+]_i$) of removing and replacing 2·5% CO_2, and external K. (Unpublished experiment by R. C. Thomas done in 1977, unlike the unpublished experiments of Figs. 2, 3, 4, 5 and 6, which were done in 1984.)

The blockage of pH_i recovery by DIDS and SITS almost led me to conclude that pH_i recovery was a simple anion-exchange process. Luckily, before the proofs of my letter to *Nature* (Thomas, 1976*b*) arrived, I tried the effects of removing external Na. To my surprise it greatly slowed PH_i recovery. So I deleted one sentence from the proofs and added a note mentioning this.

I described the effects of removing external Na at the centenary meeting of the Society. Fig. 10*A* shows the published result (Thomas, 1976*c*). Removing external K had no effect on the pH_i recovery from an HCl injection, but replacement of Na by Li caused perhaps 80% inhibition, and SITS appeared to block it completely.

It could be argued that external Na was merely required outside to stabilize the transport mechanism, and played no direct role in pH_i regulation. Experiments in which I recorded both internal Na and pH_i soon showed, however, that Na^+ ions appeared to enter as pH_i increased, presumably in exchange for H^+ ions leaving the cell. A previously unpublished experiment from 1977 is shown in Fig. 10*B*. The key part of the result is where I readmitted the CO_2 solution toward the end of the experiment. As CO_2 entered the cell the pH_i first fell, then rapidly recovered. As pH_i recovered there was a clear but transient increase in internal Na, exactly as expected if part of the acid extrusion was in exchange for external Na.

I have already mentioned that external bicarbonate stimulates pH_i recovery (Fig. 4). When allowance is made for the effects of CO_2 on the internal buffering

power, acid extrusion appears to be about fifteen times faster with 20 mM-bicarbonate than without (Thomas, 1977).

Is there a role for internal Cl? Fig. 11*A* shows that there is. (I think that this particular experiment is still my favourite.) As well as pH_i I recorded internal Cl with a Cl^--sensitive, liquid ion-exchanger micro-electrode. At the beginning the cell was

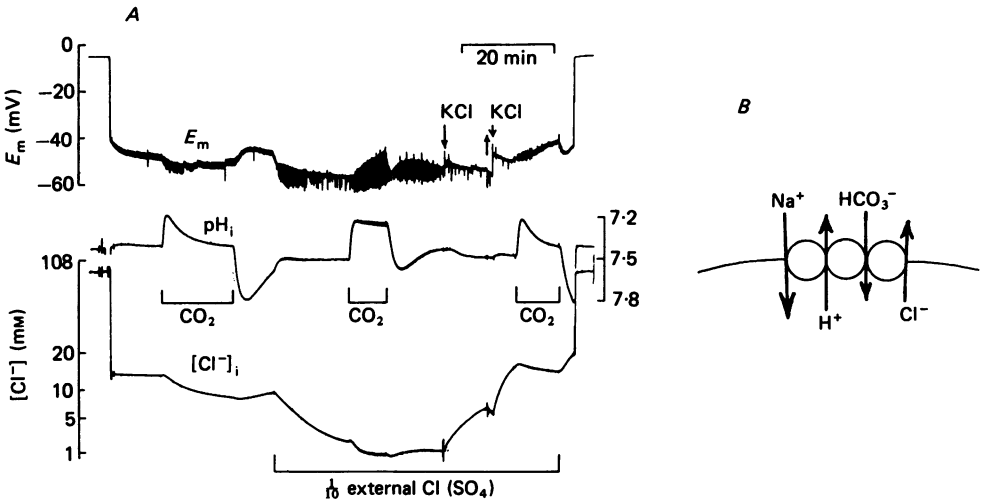

Fig. 11. *A*, the role of Cl in pH_i recovery in snail neurone. The experiment shows the effect of reducing external Cl on the pH_i and internal Cl^- ion concentration ($[Cl^-]_i$) response to adding and removing 2·3 % CO_2. The arrows above the E_m record indicate the points when an 18 MΩ KCl-filled micro-electrode was inserted, withdrawn (broken to 5 MΩ) and re-inserted. *B*, diagram showing proposed ionic mechanism of pH_i-regulating system in snail neurones. (*A* from, and *B* after Thomas, 1977.)

in a CO_2-free Ringer solution. After about 10 min this was replaced with one equilibrated with 2·3 % CO_2. As CO_2 entered, pH_i fell and then recovered. As pH_i recovered, internal Cl decreased, as expected if Cl^- ions were leaving the cell in exchange for entering bicarbonate ions.

I then removed CO_2, causing pH_i to increase, and allowed pH_i to recover for about 10 min before removing 90 % of the external Cl. This caused the internal Cl to decrease surprisingly rapidly to around 1 mM. I then applied CO_2 again, this time in low-Cl Ringer solution. As CO_2 entered the cell, pH_i again fell – but this time it did not recover. To show that this failure to recover was not due to the sulphate in the low-Cl Ringer solution, I later injected KCl from a broken micro-electrode. When internal Cl had reached its normal level I again applied CO_2 in low-Cl Ringer solution. This time pH_i recovered.

Thus, internal Cl decreases when pH_i recovers from a CO_2-induced acidification, and the recovery can be blocked by low internal Cl. Similarly, internal Na increases when pH_i recovers, and the recovery can be blocked by removal of external Na (more effectively when organic Na substitutes are used instead of Li). Finally, pH_i recovery is slow in the absence of bicarbonate. For these reasons I proposed the scheme for pH_i regulation shown in Fig. 11*B* (Thomas, 1977).

The ionic concentration gradients are all small, except for Na. I think the energy for pH_i regulation is provided by the Na gradient, and have failed in many attempts to demonstrate any requirement for metabolic energy. The ion movements are tightly coupled, and electroneutral: there is no effect of even a 50 mV increase in E_m. Presumably, the affinities of the various binding sites are affected by pH, since the

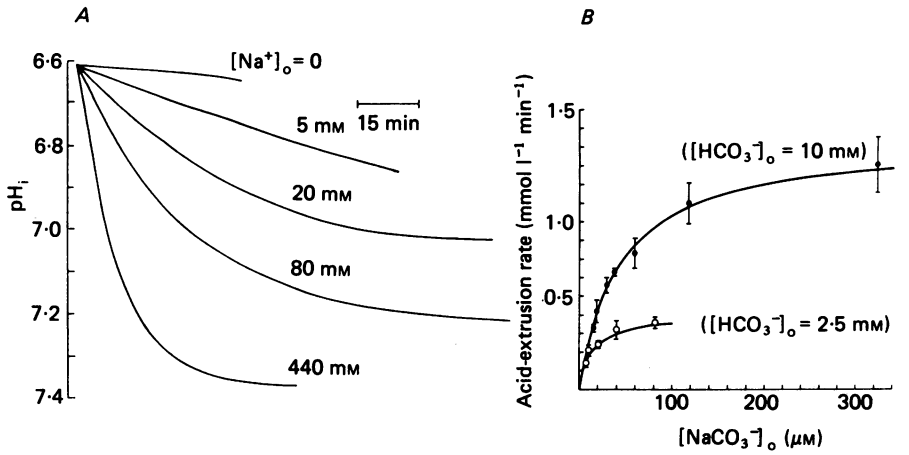

Fig. 12. pH_i recovery of barnacle muscle from acidification. *A*, recovery at five different levels of external Na after acid loading by exposure to 50 mM-NH_4Cl in choline barnacle saline. *B*, collected data from experiments at different external Na and bicarbonate levels showing acid extrusion rate as a function of the calculated concentration of the $NaCO_3$ ion pair. (From Boron, McCormick & Roos, 1981, with permission of the American Physiological Society.)

normal snail pH_i is more acid than it would be if all the energy in the Na and Cl gradients were devoted to moving H^+ and HCO_3^- ions. There is, incidentally, no evidence that H^+ ions participate at all – my results could well, but less symmetrically, be explained by a coupling of 2 HCO_3^-:1 Na^+:1 Cl^-.

Barnacle muscle and squid axon. Barnacle muscles are very large, and available all the year round even in places like Roos's laboratory in St. Louis. After various investigations of pH_i in this preparation (see Roos & Boron, 1981) its ionic mechanism was first described in detail some three years ago (Boron, McCormick & Roos, 1981). As shown in Fig. 12*A*, pH_i recovery from NH_4-induced acidification requires external Na. It also requires bicarbonate and Cl, like squid axons. Boron *et al.* (1981) also considered a mechanism of pH_i regulation suggested by Becker & Duhm (1978) in which a monovalent ion pair formed between one Na^+ ion and one CO_3^{2-} ion is exchanged for an internal Cl^- ion. As shown in Fig. 12*B*, Boron *et al.* were able to rule this model out. When acid extrusion rates were plotted against calculated $NaCO_3^-$ concentration, the points did not fall on a single curve as predicted. Thus, barnacle muscle pH_i regulation appears to have a similar mechanism to that suggested for snail neurones.

More recently, Boron & Russell (1983) have published a very comprehensive study of pH_i regulation in squid axons. They confirmed a brief mention of four years earlier that squid axons too require external Na. Then, exploiting to the full the use of radioactive tracers to follow ion fluxes, they measured the stoicheiometry of pH_i

regulation under a standard set of conditions. When the measured acid extrusion rate was 7·5 pmol cm^{-2} s^{-1} of H^+ ion equivalents, the net Na uptake was 3·4 and the net Cl efflux 3·9. These figures are very close to those expected for equal fluxes of Na^+, H^+, Cl^- and HCO_3^- ions, or for two HCO_3^- ions for each Na^+ and Cl^- ion.

While the ion-pair model for pH_i regulation was eliminated for barnacle muscle, Boron (1984) has briefly described some evidence that this model could explain his results with squid axons. The requirement of the squid axon for internal ATP has been confirmed, though its function is unclear.

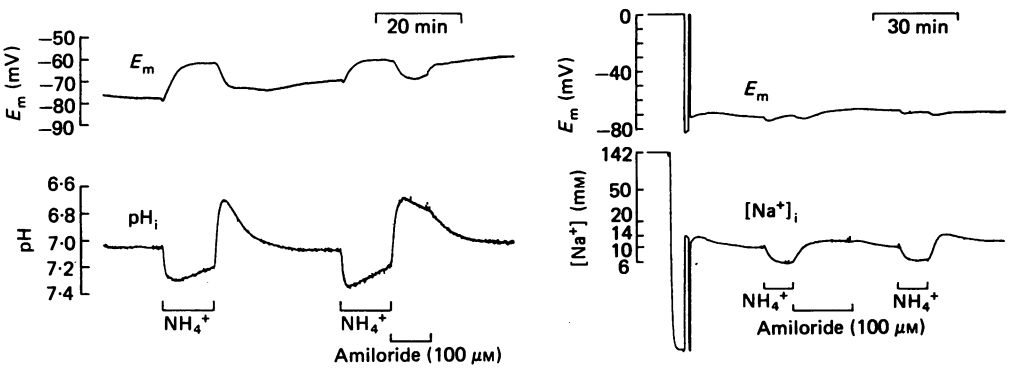

Fig. 13. Mouse soleus muscle. *A*, the effect of amiloride on pH_i recovery after exposure to 10 mM-ammonium sulphate. *B*, the effect of amiloride on the increase in internal Na ion concentration ($[Na^+]_i$) after NH_4 exposure. (From Aickin & Thomas, 1977.)

Thus, pH_i regulation by snail neurones, barnacle muscle and squid axons has the same ionic requirements, is inhibited by the same anion-exchange inhibitors, and may well have basically the same ionic mechanism. In snail neurones and barnacle muscle the pH_i regulator can be made to run backwards, but that is another story (see Russell, Boron & Brodwick, 1983; Evans & Thomas, 1984).

Mouse soleus muscle. This preparation was developed by Claire Aickin in Bristol for studying the ionic mechanism of pH_i regulation in mammalian striated muscle. It proved difficult to keep E_m high with both pH-sensitive and KCl-filled microelectrodes in the same fibre so external Ca was increased. It turned out that the mechanism of pH_i regulation was rather different from those described so far. Recovery from an NH_4-induced acidification was about three-quarters blocked by removing external Na or by the K-sparing diuretic amiloride, as shown in Fig. 13*A*. Amiloride has no effect on snail neurones, but was shown to block presumed Na–H exchange in sea-urchin eggs by Johnson, Epel & Paul (1976).

Amiloride also reduced the rise in internal Na seen on removal of NH_4, as shown in Fig. 13*B*, and low Na solutions slowed pH_i recovery. Thus, a major part of the pH_i recovery in mouse muscle appears due to Na–H exchange.

Removal of CO_2 or application of SITS caused some inhibition of pH_i recovery, suggesting that perhaps 20% of the acid extrusion was due to Cl–HCO_3 exchange. We could find no evidence that external K was required, in spite of earlier reports

that K depletion upset acid–base balance in whole animals. A long exposure to K-free Ringer solution caused only a small acidification, and had no effect on the recovery from an NH_4-induced acidification, as seen in Fig. 14. One interesting effect of the K-free Ringer solution and the low E_m is that the muscle membrane became highly permeable to NH_4^+ ions, as shown by the rapid acidification *during* NH_4^+ exposure. The particularly rapid acidification in K-free Ringer solution, and the increase in E_m during NH_4^+ exposure shows that NH_4^+ can substitute for K^+ in the Na pump. The E_m increase presumably occurs because the pump is electrogenic.

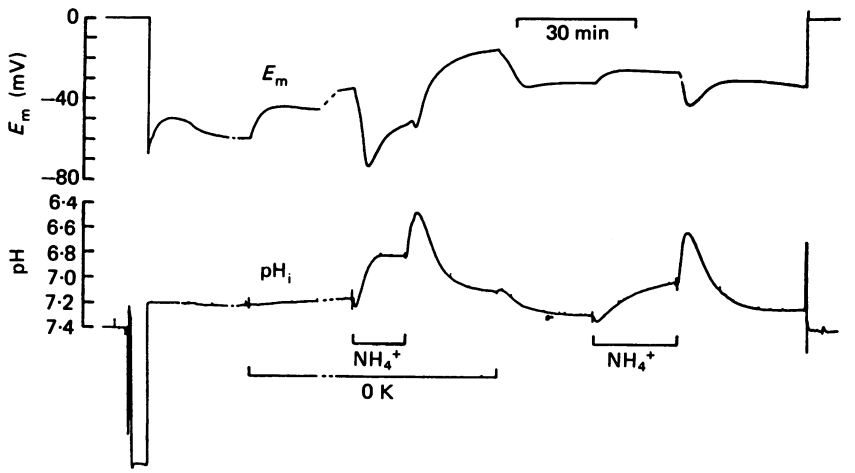

Fig. 14. Mouse soleus muscle. The effect of the removal of external K on E_m and pH_i and the recovery of pH_i following exposure to 10 mM-ammonium sulphate. The two blanks in the records were for 10 and 40 min. (From Aickin & Thomas, 1977.)

Crayfish neurones. Not all invertebrates have similar pH_i regulating systems. Moody (1981) discovered that crayfish neurones regulated their pH_i surprisingly rapidly even in the absence of bicarbonate. Two of his results are shown in Fig. 15. In the experiment shown in Fig. 15*A* the cell was acid-loaded by the ammonium technique. Each time external Na was removed, pH_i recovery stopped. In Fig. 15*B*, HCl was injected ionophoretically three times. After the first injection pH_i recovery was accelerated by 25 mM-bicarbonate. Then SITS was applied. After the second injection pH_i recovered as before, but there was no acceleration when bicarbonate was added.

Thus, pH_i regulation had an absolute requirement for external Na. SITS blocked the stimulation seen with bicarbonate, but had no effect on pH_i recovery in its absence. Moody concluded that there were two separate mechanisms for pH_i recovery from acidification in crayfish neurones. One was a Na-dependent Cl–HCO_3 exchange, as in snail neurones and barnacle muscle, the other was probably simple Na–H exchange. Rather surprisingly, recent evidence suggests that a similar pair of mechanisms may be involved in pH_i regulation by frog skeletal muscle (Abercrombie, Putnam & Roos, 1983).

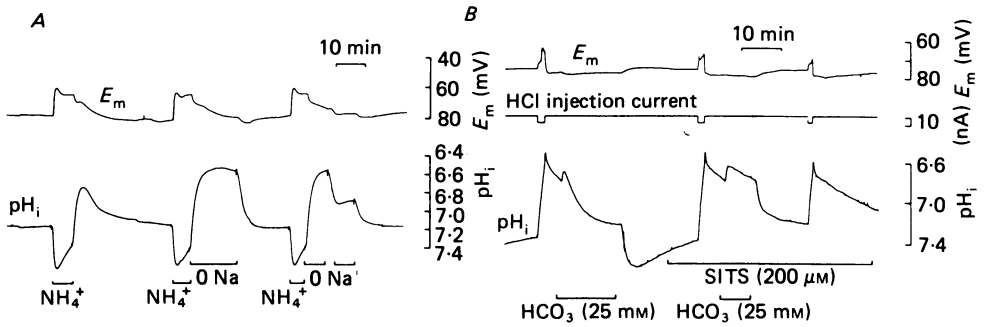

Fig. 15. pH_i regulation in the crayfish neurone, *A*, the effect of removing external Na on E_m and pH_i recovery from ammonium-induced acidification. Bicarbonate concentration: 5 mM. *B*, the effect of bicarbonate and SITS on pH_i recovery from HCl injection. (From Moody, 1981.)

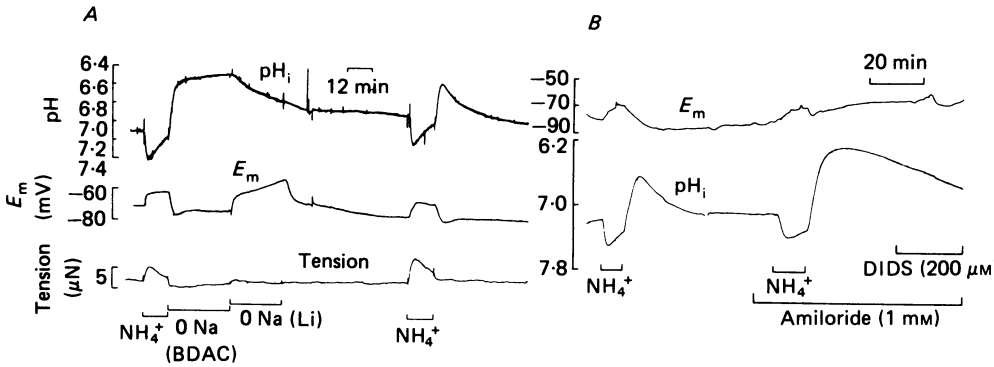

Fig. 16. Sheep heart Purkinje fibre pH_i recovery from ammonium-induced acidification. *A*, the effect of Na-free solutions on pH_i recovery. After the first exposure to ammonium, NaCl was replaced first by BDAC (bis (2-hydroxyethyl) dimethylammonium chloride) and then by LiCl. (D. Ellis & K. T. MacLeod, unpublished observations.) *B*, the effect of amiloride and DIDS on pH_i recovery in bicarbonate-buffered solution. (R. D. Vaughan-Jones, unpublished observations.)

Sheep heart Purkinje fibres. Intracellular pH regulation in this preparation was first investigated in detail by Deitmer & Ellis (1980) working in Bristol, but their results were somewhat inconclusive. D. Ellis & K. T. MacLeod (unpublished observations) have since shown very clearly that pH_i recovery is blocked by an organic Na substitute but not by Li. One of their results is shown in Fig. 16*A*. Working on the same preparation, R. D. Vaughan-Jones (also unpublished) has shown that high levels of amiloride almost completely block pH_i recovery, but DIDS has no effect, as shown in Fig. 16*B*. Thus pH_i recovery from acidification appears to depend largely on Na–H exchange, although Vaughan-Jones (1981) suggests a role for Cl–HCO_3 exchange in restoring pH_i after alkalinization. In this case HCO_3^- ions would be carried out and Cl^- ions in.

Salamander proximal tubule. With skill and dedication, Boron has since 1978 been

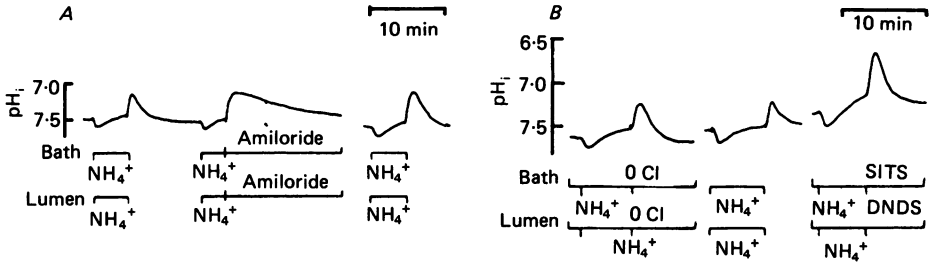

Fig. 17. pH_i recovery in the proximal tubule of Salamander kidney. An isolated tubule section was perfused and superfused with different solutions while the pH_i was recorded with a micro-electrode. Intracellular acidification was induced by NH_4 exposure. *A*, shows the effect of amiloride (2 mM) on the pH_i recovery, *B*, shows the effects of Cl-free solutions and treatment with SITS and a SITS analogue, DNDS (4,4′-dinitrostilbene-2,2′-disulphonate). (From Boron & Boulpaep, inverted and reproduced from the *Journal of General Physiology* (1983), vol. 81, pp. 29–52, by copyright permission of The Rockefeller University Press.)

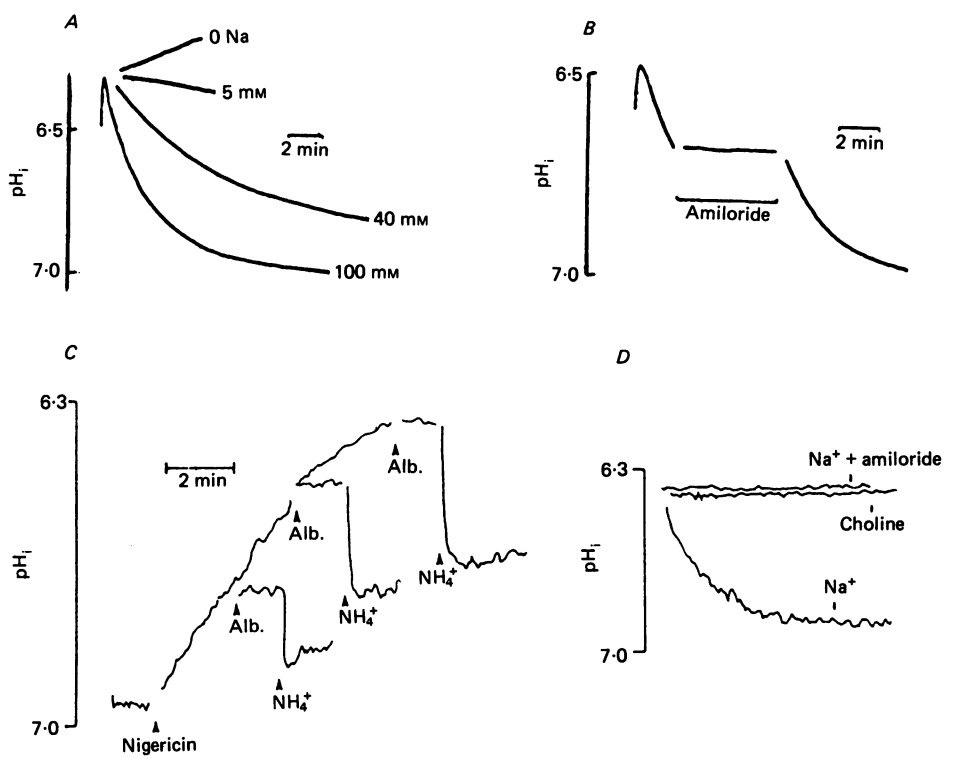

Fig. 18. Experiments with fluorescein derivatives to measure pH_i. *A* and *B*, human fibroblasts and the effect of varying external Na and applying amiloride (1 mM) on pH_i recovery. All media were bicarbonate-free, acidification was induced by NH_4 treatment. (From Moolenaar *et al.* (1983), with permission of *Nature*, inverted.) *C* and *D*, rat thymic lymphocytes. Acidification was started by exposure to nigericin and stopped by adding albumin (alb.). *C* shows the use of NH_4 to measure intracellular buffering power; *D* shows the effects of Na-free solutions and amiloride on pH_i recovery. All solutions bicarbonate free. (From Grinstein *et al*, reproduced from the *Journal of General Physiology* (1984), vol. 83, pp. 341–369, by copyright permission of The Rockefeller University Press and inverted.)

working on the above preparation in Boulpaep's and later his own laboratory at Yale. As shown in Fig. 17, the cells of the proximal tubule regulate their pH_i very well, by a mechanism inhibited by amiloride but not by SITS. So Boron & Boulpaep (1983) concluded that pH_i regulation was primarily by Na–H exchange. The mechanism for acid transport *across* the tubule is as yet unknown, but luckily does not concern me here.

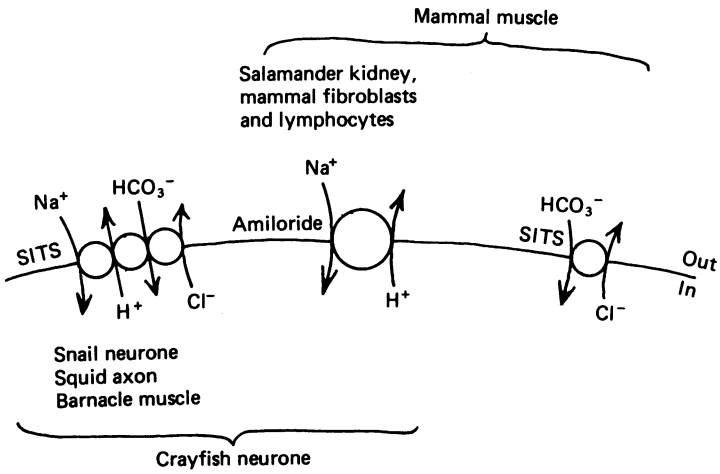

Fig. 19. The three mechanisms by which pH_i is regulated, and their contribution in different animal cells. As yet no one has proposed that all three mechanisms are involved in a single preparation.

Fibroblasts and lymphocytes. Finally, I would like to mention two recent investigations in which pH_i was measured using fluorescein derivatives. As shown in Fig. 18*A*, the pH_i of human foreskin fibroblasts needs external Na to recover from an NH_4 -induced acidification. Recovery was also blocked by amiloride, suggesting that it involves Na–H exchange as in mammalian muscle (Moolenaar, Tsien, van der Saag & de Laat, 1983, see also Moolenaar, Tertoolen & de Laat, 1984 and Frelin, Vigne & Lazdunski, 1983).

Three workers in Canada, Grinstein, Cohen & Rothstein (1984), used a similar method to measure pH_i in rat thymic lymphocytes, rather than the previous preparation, but acid-loaded their cells by treatment with nigericin. They also found that pH_i recovery required external Na and was blocked by amiloride, as shown in Fig. 18*B*.

CONCLUSION

There seem to be three separate mechanisms for pH_i regulation in the animal cells studied so far, as shown in Fig. 19. They are Na–H–Cl–HCO_3 exchange (or more strictly Na-dependent Cl–HCO_3 exchange), Cl–HCO_3 exchange, and Na–H exchange. Their involvement in the different types of cell is indicated in Fig. 19. The mechanisms involving Na are probably driven by the Na gradient, and thus ultimately depend on the Na pump, but the source of energy for Cl–HCO_3 exchange has not been established.

I would like to thank my colleagues in Bristol for their advice and help in preparing this lecture, the M.R.C. for support and the Committee of the Physiological Society for their invitation.

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