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INTRACELLULAR pH OF SNAIL NEURONES MEASURED WITH A NEW pH-SENSITIVE GLASS MICRO-ELECTRODE

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SUMMARY

1. The construction and properties of a new design of pH-sensitive micro-electrode are described. The electrodes are very durable, and have a recessed configuration so that only the extreme tip, which can be as small as 1 μm in diameter, needs to enter the cell.

2. The average intracellular pH in thirty-two snail neurones was 7·4. This was not in accord with a passive distribution of H^+ ions across the cell membrane.

3. Changing membrane potential or external pH had only slow effects on internal pH.

4. Removing external K had no effect, and removing external Na had only slow and variable effects on intracellular pH.

5. Anoxia, azide and DNP all caused a slow fall in internal pH.

6. External CO_2 caused large and rapid decreases in internal pH, which external bicarbonate appeared to offset slowly. Injected bicarbonate increased internal pH.

7. The size of the pH changes caused by CO_2 suggested a minimum intracellular buffering power of 25 m-equiv H^+/unit pH per l., equivalent to that of 150 mM Tris maleate, pH 7·4.

8. External ammonia caused a large and rapid increase in internal pH, while the injection of ammonium ions had the opposite effect.

INTRODUCTION

The experiments described in this paper were done to test a new design of pH-sensitive microelectrode and to investigate the intracellular pH of snail neurones.

The two techniques most widely and successfully used in the last 20 years to measure intracellular pH involve either pH-sensitive micro-electrodes inserted directly in cells, or studies of the distribution of weak acids from which the internal pH can be calculated (Waddell & Bates,

1969). The directness of the micro-electrode method makes it in some ways the ideal technique, but difficulties in constructing suitably small electrodes have so far limited the method to use in very large cells.

Glass electrodes were first used to measure intracellular pH by Caldwell (1954, 1958), but his electrodes were too large to be used in cells smaller than crustacean muscle fibres or cephalopod giant axons. Kostyuk & Sorokina (1960) developed pH sensitive micro-electrodes small enough to be used in frog muscle cells, but they were difficult to make and had poor durability. Carter, Rector, Campion & Seldin (1967) used glaze-insulated pH glass micro-electrodes to measure rat muscle pH, but both their results and their electrodes have been recently criticized (Roos, 1971; Paillard, 1972). Finally, a new design of antimony micro-electrode has recently been used for intracellular pH measurement (Bicher & Ohki, 1972) but it has a rather short life and a non-linear pH sensitivity. All these micro-electrodes have the disadvantage that at least several microns of the electrode tip must be inserted into the cell to obtain a valid reading.

The new design of pH-sensitive microelectrode described in this paper has the advantage that only the extreme tip needs to be inserted into the cell to be studied. This tip can be as small as 1 μm in external diameter. The design is based on that of the recessed-tip Na^+-sensitive microelectrode described earlier (Thomas, 1972) and has the pH-sensitive glass inside the tip of a borosilicate glass micropipette. The electrodes are perhaps rather difficult to make, but once prepared can be used for many weeks, and can give continuous measurements of intracellular pH from one cell for many hours.

The experiments on snail neurones were designed to determine the normal value of the intracellular pH *in vitro*, and to investigate the effects on it of changes in membrane potential, external pH and the concentration of other ions. The effects on intracellular pH of various metabolic inhibitors, external CO_2 and bicarbonate ions, external ammonia and ammonium ions, and injected bicarbonate and ammonium ions were also investigated.

METHODS

Most of the methods were the same as those described earlier (Thomas, 1972) except that pH-sensitive rather than Na^+-sensitive micro-electrodes were used. The experiments were done on large neurones of the snail, *Helix aspersa*. About half of the experiments were done on the large Dinhi cell at the rear of the right pallial ganglion, with the rest being done on other cells in the same ganglion and in the visceral ganglion.

General. The brain was removed from dormant snails, and mounted, dorsal surface up, on a P.T.F.E. block. The outer connective tissue over part of the suboesophageal ganglion was then removed, the block placed in the experimental chamber and covered with snail Ringer. Then the thin inner connective tissue covering the right pallial and visceral ganglia was torn with a tungsten hook. The ganglia were superfused

with snail Ringer throughout the experiments, at a rate of about 1·5 ml., or about five chamber volumes, per minute. The experimental solutions were kept in bottles, mounted above the recording apparatus, which were connected by plastic tubing to a twelve-way P.T.F.E. tap. The tap allowed solution changes to be made without interrupting the flow. It was mounted close to the experimental chamber, and connected to it by a short length of narrow bore tubing to minimize the dead space. All tubing used for the solutions was polyethylene inside nylon, to reduce gas interchange between the solutions and air.

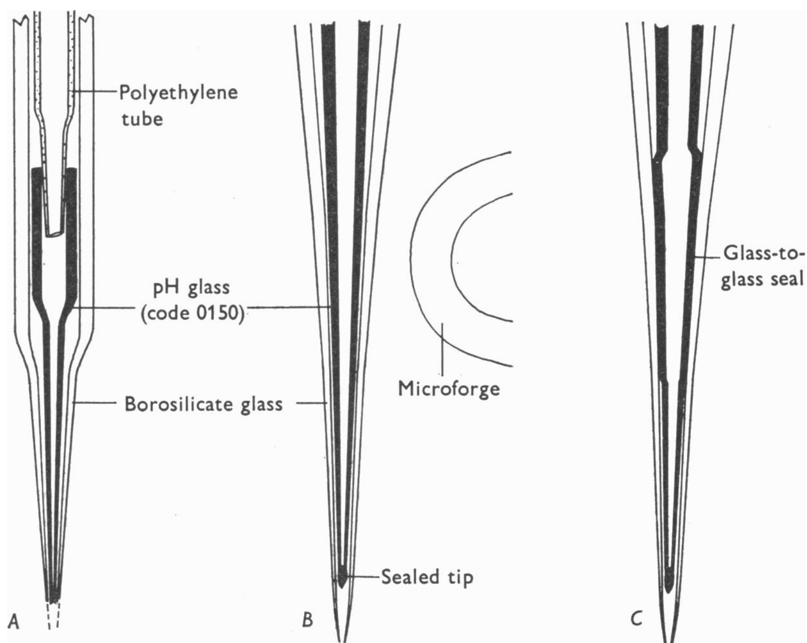
Solutions. The normal snail Ringer used had a pH of 8·0 and the following composition: KCl 4 mM, NaCl 80 mM, $CaCl_2$ 7 mM, $MgCl_2$ 5 mM, Tris maleate buffer 20 mM. (the buffer concentration was higher than used previously to make the pH micro-electrode response time reasonably fast during calibration, see Results section). Solutions of the same composition but different pH were made using buffer solutions with the same amount of Tris base added, but different amounts of maleic acid. For the O_2-free Ringer, N_2 was bubbled, via a wash bottle, through about 200 ml. Ringer for several hours before use. For the CO_2-bicarbonate solutions, CO_2 in O_2 was bubbled, via a wash bottle, through unbuffered Ringer for several hours; the pH was then adjusted by adding predetermined quantities of $NaHCO_3$, and the CO_2 bubbling continued until the end of the experiment. Additional NaCl was added to the unbuffered Ringer as necessary to maintain the osmotic pressure. The pH of all solutions was carefully checked, and adjusted as necessary, before and after each series of experiments. All solution pH measurements were made using a digital pH meter, which was carefully calibrated daily with an electrometrically standardized pH 7·00 phosphate buffer solution (BDH Chemicals Ltd).

Conventional micro-electrodes. Micro-electrodes for recording the membrane potential and for current injection were made from lengths of 2 mm diameter borosilicate glass tubing which had a filament of the same glass glued inside. Recording micro-electrodes were made some hours before use and then filled with distilled water from a syringe. After a few minutes most of the distilled water was sucked out and replaced with 2·5 M-KCl, and the electrodes were kept with their tips immersed in distilled water until use. Electrodes used had resistances between 15 and 30 MΩ, and had tip potentials of less than 3 mV. Current-passing electrodes were made and filled directly with the final filling solution shortly before use.

pH micro-electrodes. Except for changes in technique made necessary by the low melting point of the pH sensitive glass, these were made in a similar way to that described for recessed-tip Na^+-sensitive micro-electrodes (Thomas, 1972). The pH sensitive glass, code 0150, was obtained from Corning Glass Works, Scientific Instruments Division, Medfield, Massachusetts, as 1 mm diameter tubing. It was cut up into 3 cm lengths and pulled into micropipettes on a vertical electrode puller. The internal diameter of the heater coil was 3·3 mm, and it consisted of eleven turns of 22 gauge Kanthal A wire. One of each pair of micropipettes was carefully measured at 100 and 200 μm from the tip, preferred external diameters being close to 6 and 10 μm respectively.

Next a batch of borosilicate glass micropipettes was made, using the same heater coil on the electrode puller. The borosilicate tubing was the same as used for the conventional micro-electrodes, and it had external and internal diameters of about 2 and 1·2 mm respectively. A two-stage process was used, the tubing being first heated and elongated by about 1 cm, then recentred in the heater coil, and pulled again with a relatively low heat. The internal dimensions of the final micropipettes were measured at 100 and 200 μm from the tip. Adjustments were made to the low, second, heat so that a range of differing internal dimensions were obtained to match, as far as possible, the outer dimensions of the pH glass micropipettes.

Then the tips of each pH micropipette were sealed in a microforge. This apparatus consisted of a 25 μm diameter Pt:Rh wire loop, heated electrically, which was mounted on a micromanipulator and held at the focus of a horizontally mounted compound microscope. Each pH micropipette was held vertically in a second micromanipulator and brought down briefly on to the heated wire so that its tip was melted to form a small seal of solid glass.



Text-fig. 1. Diagram showing construction of recessed-tip pH-sensitive micro-electrode. *A* and *B* show the micro-electrode just before, and *C* after, the formation of the final glass-to-glass seal. The length of electrode shown in *A* is about 1·5 cm, in *B* and *C* about 300 μm.

A selected borosilicate glass micropipette was then mounted vertically in the microforge, held by the second micromanipulator. A sealed pH micropipette was chosen with external dimensions near the tip similar to the corresponding internal dimensions of the borosilicate micropipette. It was connected to a fine polyethylene tube held by a third micromanipulator and carefully introduced into the top of the borosilicate micropipette. The third micromanipulator was then used to lower the pH micropipette into position inside the borosilicate micropipette as shown in Text-fig. 1*A* and 1*B*. The other end of the fine polyethylene tube was already connected to a 2·5 ml. syringe full of air. The cold microforge wire loop was placed close to the micropipettes about 150 μm from their tips, as shown in Text-fig. 1*B*. Then the current to the wire loop was turned on, and, simultaneously, air pressure was applied, via the polyethylene tube, to the inside of the pH micropipette. The softening point of the pH glass is over 150° C lower than that of the borosilicate glass, so that as the heat was increased the pH glass was softened and blown outwards to seal to the inside of the borosilicate glass, as illustrated in Text-fig. 1*C*. The heating current was then

switched off. The required air pressure was about 50 lb/sq.in., which was obtained by squeezing the syringe between fingers and thumb. Finally, the polyethylene tube was withdrawn, usually carrying with it the surplus pH glass from above the seal. A microphotograph of a finished electrode is reproduced as Pl. 1. The finished micro-electrode dimensions that were aimed at were (*a*) an exposed length of pH glass of about 100 μm from the bottom of the glass to glass seal to the sealed tip and (*b*) the minimum possible internal volume between the inside of the borosilicate glass and the outside of the pH glass.

The electrode stem was filled, using a syringe, with a solution containing approximately 100 mM-NaCl and 100 mM citrate buffer, pH 6, and the air in the shank was removed with the aid of a cat's whisker. Then a chlorided silver wire was permanently mounted in the stem of the electrode, using a silicone rubber sealant. Completed electrodes were stored with their tips immersed in distilled water or chromic acid cleaning solution.

Electrical arrangements. The membrane potential micro-electrode was connected to a unity gain current amplifier, the output of which was led to an oscilloscope, pen-recorder and, via a low pass filter, to the low impedance input of a vibrating capacitator electrometer. The pH micro-electrode was connected to the high impedance input of the electrometer, the output of which was recorded on another channel of the pen-recorder. A photon-coupled floating current clamp was used to pass current across the cell membrane or between two intracellular micro-electrodes for salt injections.

RESULTS

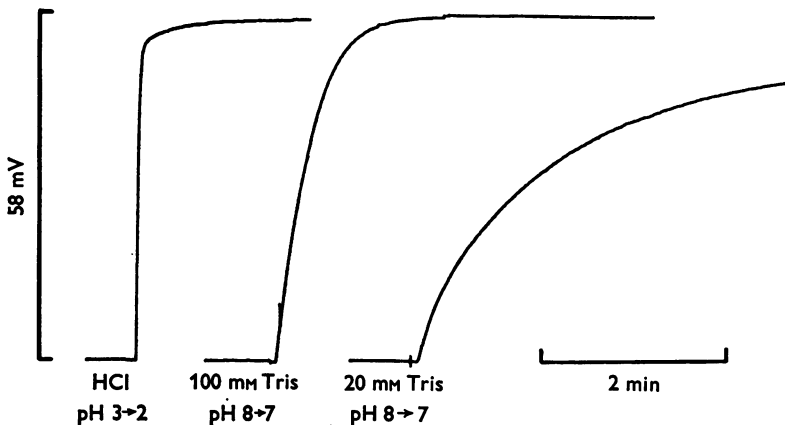
Properties of the recessed-tip pH-sensitive microelectrodes

In contrast to the Na^+-sensitive microelectrodes described earlier, which gave their best responses immediately after filling, the pH-sensitive micro-electrodes gave at first only a small and noisy response to pH changes. Two or 3 weeks of soaking in water or chromic acid were needed before they would give their maximum response with least noise.

One or two days after filling, a typical pH micro-electrode was unstable, had a resistance of about 4×10^{11} Ω, and gave a response of about 40 mV (instead of the theoretical 58 mV) to a unit pH change. After soaking for 3 weeks the resistance had fallen to 7×10^{10} Ω, the response had increased to 55 mV/pH unit, and the noise level was less than 1 mV. The soaked pH micro-electrodes gave good responses to pH changes for many experiments and for at least several weeks after the initial soaking; no micro-electrode remained unbroken long enough to exhibit spontaneous loss of function.

As expected from the properties of the code 0150 pH-sensitive glass, the electrodes gave linear responses to pH changes over at least the range pH 2–9 and were not affected by changes in the concentration of other ions. The *speed* of response depended on the tip diameter and the volume of the recess, and was also greatly affected by both the pH and the buffer concentration. Text-fig. 2 illustrates the response of a pH micro-electrode to approximately unity pH changes. In acid conditions the response was relatively fast, with a similar time course to that obtained with Na^+-sensitive micro-electrode

having the same dimensions. But in the pH range 8–7, where the hydrogen ion concentration is less than micromolar, the response was very much slower, and appeared to depend principally on the buffer concentration. Thus with 100 mM Tris maleate the response was several times faster than the response in 20 mM buffer, and the response in 20 mM buffer was in turn much faster than the response in 5 mM buffer (not shown).



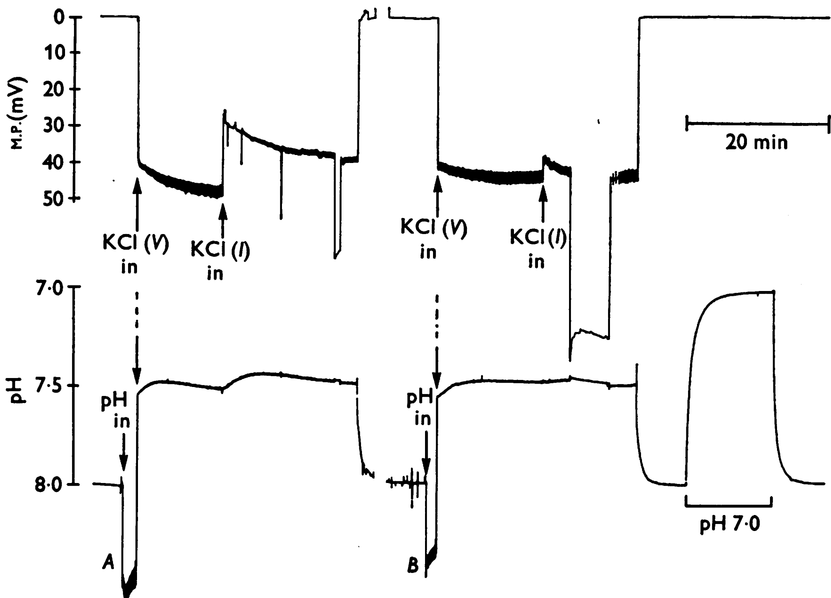
Text-fig. 2. Pen-recordings of the responses of a pH-sensitive micro-electrode to unit pH changes. The electrode tip was in a bath through which the test solutions flowed continuously. In the first record the test solution was changed from 10 mM-HCl to 100 mM-HCl, in the second record it was changed from 100 mM-Tris maleate pH 8 to 100 mM-Tris maleate pH 7, and in the third record it was changed from 20 mM-Tris maleate pH 8 to 20 mM-Tris maleate pH 7.

With different pH micro-electrodes the size of the response to pH changes varied slightly, ranging from 53 to 56 mV/pH unit, but with a given electrode the response remained the same over many days, and was the same size at the end of a long experiment as at the beginning. The speed of the response, however, was often slower at the end of an experiment, presumably because the electrode tip tended to become partially blocked. Soaking for a few hours in chromic acid restored the original speed of response.

Normal intracellular pH and the effects of membrane potential

Text-fig. 3 illustrates pen-recordings from experiments on two neurones in the same preparation. Three micro-electrodes were used; one to record pH, a second to record the membrane potential, and a third to pass current for changing the membrane potential. The electrodes were first equilibrated outside the selected neurone for some time. Then, in this and all other

experiments, the pH-sensitive micro-electrode was inserted first since it had the largest tip. As it crossed the cell membrane, there was a downward deflexion of the pH record, and spontaneous action potentials (seen as a thickening of the trace), were recorded, greatly reduced in size by the long time constant of the recording system. After a few minutes the second micro-electrode, a conventional, KCl filled micro-electrode for measuring the membrane potential, was inserted, as shown by the downward deflexion



Text-fig. 3. Pen-recordings of an experiment on two different neurones to determine the normal intracellular pH and the effect of changing membrane potential. The voltage recorded by the KCl-filled membrane potential electrode is shown at the top, and the voltage recorded by the pH-sensitive micro-electrode is shown at the bottom. In each experiment, the pH electrode was inserted first, then the membrane potential electrode (KCl (*V*)), and finally a third micro-electrode (KCl (*I*)) for passing current across the cell membrane. At the end of each experiment the membrane potential and pH electrodes were withdrawn simultaneously. After the second experiment the pH electrode was calibrated by recording its response to pH 7·0 Ringer. In the latter part of each experiment a current of 5 nA was passed across the cell membrane as shown by the deflexion on the membrane potential record.

on the upper trace. Since the membrane potential was automatically subtracted from the potential recorded by the pH electrode, there was a simultaneous upward jump in the pH trace. From this point in the experiment the pH trace gives a direct reading of intracellular pH, and the

spontaneous action potentials appear only on the membrane potential record.

The pH electrode measured both intracellular pH and, with a faster response time, membrane potential. Thus with only the pH electrode inside the cell, the large deflexion seen in this experiment, and in those shown in Text-figs. 4, 7 and 10, is caused largely by a difference between the membrane potential and the equilibrium potential for hydrogen ions across the cell membrane (the fact that the pH electrode response is a few millivolts less than theoretical would contribute only a few millivolts to this difference). Thus only one penetration of the cell is required to show that the membrane potential is not the same as the hydrogen ion equilibrium potential.

In the experiment shown in Text-fig. 3*A*, the insertion of the third micro-electrode some 10 min after the second damaged the cell membrane sufficiently to cause a depolarization of nearly 20 mV. The cell did not recover rapidly from this injury, so the experiment was ended without more than a brief hyperpolarization of the cell. The membrane potential and pH micro-electrodes were withdrawn simultaneously from the cell, and both traces allowed to return to their original levels. (On some occasions the KCl electrode potential did not return to a value close to that recorded before the cell was penetrated, usually due to top blockage. If this potential change was more than a few millivolts, the result was discarded.)

The same experiment was attempted in a second cell in the same preparation, as shown in Text-fig. 3*B*. In this case the insertion of the third micro-electrode caused only slight damage. About 4 min later a current of 5 nA was passed across the cell membrane, increasing the membrane potential by over 40 mV. As the pH record shows, this hyperpolarization had very little effect on the intracellular pH. The virtual absence of artifacts in the pH record confirms that both pH and membrane potential micro-electrodes were recording the same change of membrane potential from the same cell, and that the electrical subtraction was working well.

Both pH and membrane potential electrodes were then withdrawn from the cell, but left with their tips in the bath. The pH electrode calibration was then checked by perfusing the bath for a period with a pH 7·0 Ringer solution instead of the normal pH 8·0 Ringer. As shown in Text-fig. 3 the KCl micro-electrode used for membrane potential recording did not normally respond to pH changes. Occasionally the KCl micro-electrode developed a high resistance and became sensitive to pH changes; experiments in which this happened were discarded.

Since injury to the cell clearly caused a fall in intracellular pH, determinations of the 'normal' value were made by taking the value of internal pH at least 20 min after penetration of the cell, and only on cells that were

not badly damaged. For thirty-two cells that met these criteria, the mean intracellular pH was $7 \cdot 41 \pm 0 \cdot 015$ (S.E. of mean) and the mean membrane potential was $44 \cdot 2 \pm 0 \cdot 76$ mV. There was no correlation between these two values in the thirty-two cells. The individual pH values were converted to intracellular hydrogen ion concentrations, taking pH to be, to a first approximation, the negative logarithm of the hydrogen ion concentration. The mean intracellular hydrogen ion concentration was then $40 \cdot 1$ nM $\pm 1 \cdot 3$ corresponding to an intracellular pH of 7·40.

The smallness of the variation in the internal pH suggests that it is closely controlled, and that the measurement error is quite small. The mean intracellular pH is 0·22 units higher than the value corresponding to a passive distribution of hydrogen ions across the membrane, suggesting that some mechanism may exist to expel hydrogen ions from the cell interior.

These results are in reasonable agreement with the measurements made on *Helix pomatia* neurones by Sorokina (1965, see also Kostyuk, Sorokina & Kholodova, 1969). She used a snail Ringer with a pH of 7·8 and reported an average intracellular pH of 7·26, and an average membrane potential of 54 mV. These values also did not correspond to those expected if hydrogen ions were passively distributed across the cell membrane.

The effect of changing extracellular pH

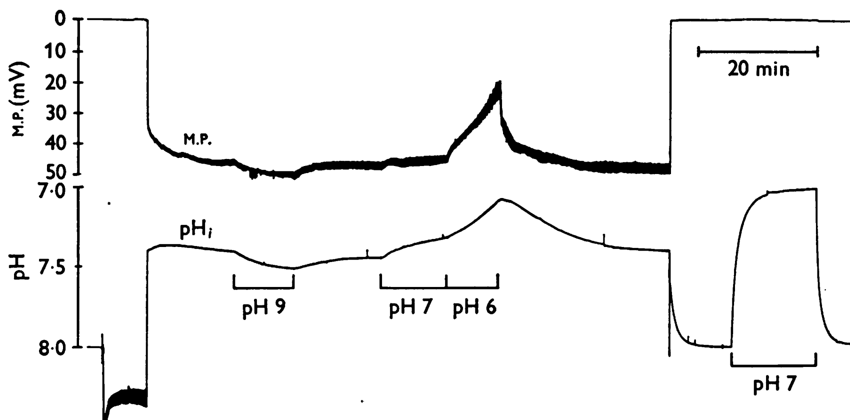
If hydrogen ions were in equilibrium across the cell membrane, then changing external pH should cause a corresponding change in internal pH. This was tested in experiments such as that shown in Text-fig. 4. The preparation was, as usual, perfused for some time before the experiment with snail Ringer of pH 8·0. About 15 min after the second micro-electrode had been placed in the cell, the preparation was exposed in turn to solutions of pH 9·0, 8·0, 7·0 and 6·0.

With the pH 9·0 and 7·0 solutions the pH changed by about 0·1 units over a 10 min period, the rate of change being fastest immediately after the pH change. The results do not suggest that the internal pH was changing over a short period by an amount equivalent to the external pH change, or that hydrogen ions are in equilibrium across the cell membrane. With the pH 6·0 solution, both the membrane potential and internal pH decreased at rates which increased during the period of exposure, suggesting that the membrane permeability was significantly changed. The change in internal pH, however, was still relatively slow.

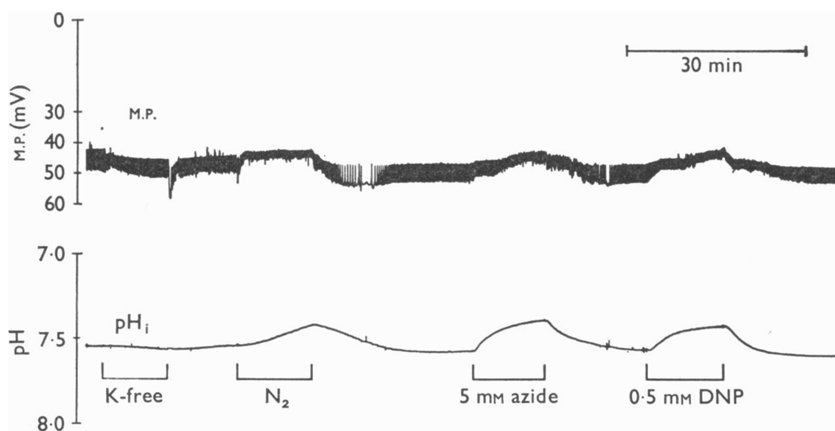
The effect of changing external K or Na

Removing external K inhibits the sodium pump and thus causes a rise in internal Na. But, as shown in Text-fig. 5, this treatment had only a very small effect on intracellular pH. In seven experiments the largest pH change

seen was an increase of less than 0·05 pH units after 10 min of exposure to K-free Ringer. Increasing external K fourfold, to 16 mM, was a little more effective, causing a fall in pH of about 0·1 unit in 10 min; but this was possibly a secondary effect of the stimulation caused by the high K.



Text-fig. 4. Pen recording of an experiment to show the effects on the membrane potential and internal pH of changing the external pH.



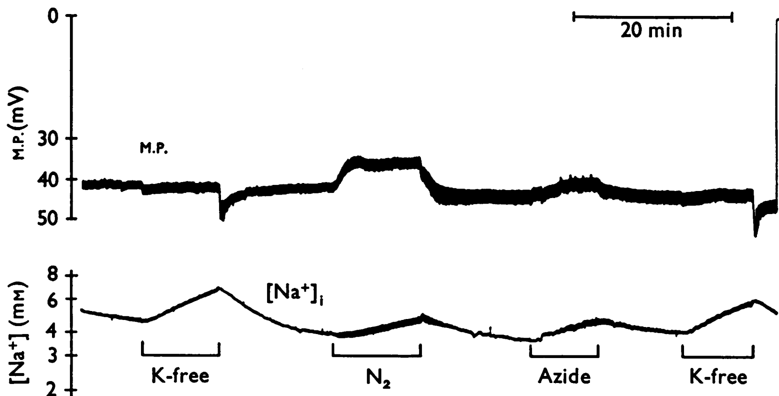
Text-fig. 5. Pen recording of part of an experiment to show the effects on the membrane potential (M.P.) and internal pH of exposing the preparation of K-free Ringer, anoxic Ringer, sodium azide and DNP.

Removing external Na did cause slow changes in internal pH, but the effects varied with the cell tested and with the ion used to replace Na. Replacement of Na by Li had no effect or caused a small decrease in intracellular pH. Replacement by Tris increased the intracellular pH in two

cases, and decreased it in one. Replacement by choline decreased the intracellular pH in the two experiments in which it was tested. All the responses occurred only after a delay of several minutes, which suggest that the effects may be secondary to a lowering of internal Na, but the results varied so much that they are not easy to explain.

The effect of metabolic inhibitors

If there is a metabolically driven hydrogen ion pump in the cell membrane, then metabolic inhibitors may reduce the energy available to drive it. If this effect is sufficient to inhibit the proposed pump then metabolic inhibitors should decrease the difference between the membrane potential and the hydrogen ion equilibrium potential, or E_H. Text-fig. 5 shows the effects of anoxia, 5 mM Na azide and 0·5 mM 2,4 dinitrophenol (DNP) on intracellular pH. All three treatments reduced the internal pH by about 0·2 units in 10 min. The external pH was kept at 8·0, so that this internal acidification does correspond to a decrease in the difference between the membrane potential and E_H. Unfortunately, this experiment was only performed at pH 8·0 and for rather a short time, so that this decrease may have been fortuitous. Both azide and DNP might also be acting as hydrogen ion carriers across the cell membrane, thus increasing hydrogen ion influx, rather than reducing extrusion.



Text-fig. 6. Pen recording showing the effects on membrane potential and $[Na^+]_i$ of K-free Ringer, anoxic Ringer, and Na azide.

To see how effective these treatments were in reducing the metabolic energy available to the Na pump, this type of experiment was repeated with a Na^+-sensitive micro-electrode rather than a pH-sensitive one. As described earlier (Thomas, 1972), the Na-sensitive electrode allows some assessment of the Na pump activity if one assumes that the Na influx remains constant. Text-fig. 6 shows part of such an experiment. K-free

Ringer clearly caused the expected rise in internal Na which results from Na pump inhibition. Anoxia and azide, however, caused only a much slower rise in internal Na, suggesting that they are less effective than K removal at inhibiting the Na pump. This suggests that anoxia and azide cause only a small reduction in supplies of metabolic energy for the Na pump. If so, it is unlikely that these metabolic inhibitors would completely inhibit a hydrogen ion pump, so that their effects on internal pH are perhaps more likely to be due to a change from aerobic to anaerobic metabolism by the cell.

The effects of external carbon dioxide and bicarbonate

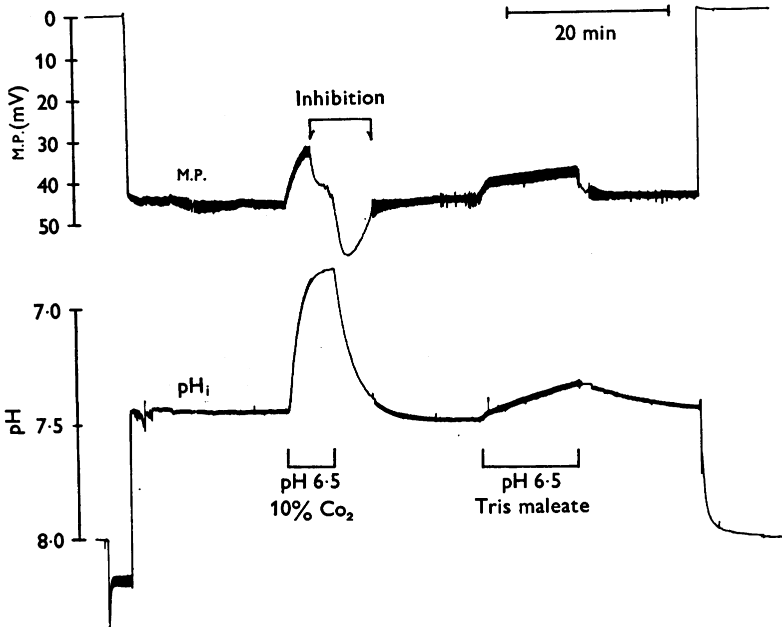
It is well established that cell membranes are highly permeable to CO_2. Thus when a cell is exposed to a solution containing dissolved CO_2 it rapidly enters the cell. Inside the cell the CO_2 combines with water to produce carbonic acid, which dissociates to produce hydrogen and bicarbonate ions. The cell interior thus becomes more acid. This effect of CO_2 on intracellular pH has been known for many years, and exposure to CO_2 was the only treatment Caldwell (1958) found which rapidly changed the intracellular pH of crustacean and cephalopod axons.

Because of the low solubility of calcium and magnesium carbonates, CO_2 could only be applied to the snail preparation in solutions more acid than the normal pH 8 Ringer. The effects of CO_2 were therefore always compared with the effects of Tris-buffered Ringer of the same pH. Text-fig. 7 shows an experiment where the effects of Ringer solution with and without CO_2 at pH 6·5 were compared. About 20 min after the beginning of the experiment the normal, pH 8 Ringer was changed to one which had been equilibrated with a 10 % CO_2, 90 % O_2 gas mixture, and had been adjusted to pH 6·5 by adding 4 mM-$NaHCO_3$. The internal pH rapidly fell, changing by about 0·6 pH units, in less than 5 min. On return to normal Ringer, the internal pH rapidly recovered.

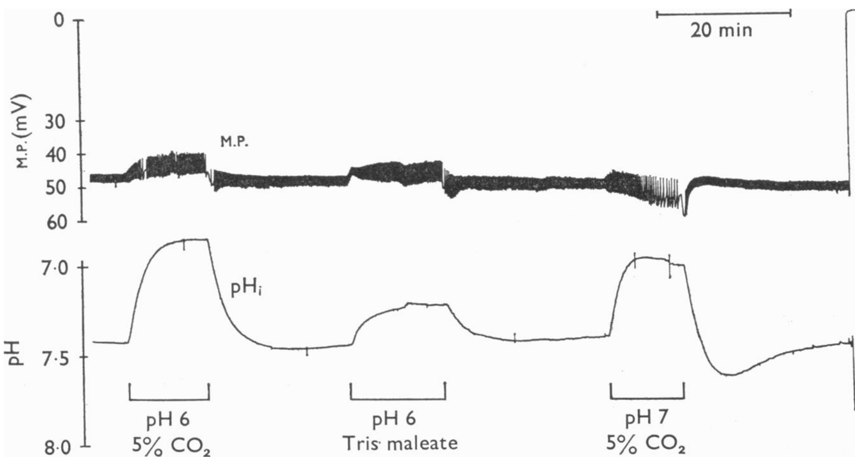
During the CO_2 application the cell began to receive synaptic inhibition, as seen by the deflexion on the membrane potential trace. Presumably the depolarization caused by the CO_2 treatment excited other cells in the preparation, one of which had an inhibitory input to the cell being recorded and had been previously silent.

As a control, Tris-buffered, pH 6·5 was applied to the cell when it had recovered from the CO_2 treatment. As expected, it had only a small, slow effect on the internal pH.

Text-fig. 8 illustrates an experiment in which the same concentration of CO_2 was applied at two different pH's. The cell was first exposed to a Ringer equilibrated with 5 % CO_2 and adjusted to pH 6·0 by adding $NaHCO_3$ to a final concentration of 1·3 mM, then to pH 6·0 Tris maleate Ringer, and



Text-fig. 7. Pen recording of an experiment to show the effects on membrane potential and internal pH of external CO_2 and pH changes. The cell was first exposed to a Ringer of pH 6·5 equilibrated with 10 % CO_2, 90 % O_2, and secondly to a Ringer of pH 6·5, buffered with Tris maleate, as indicated below the pH record. For a period during and after the CO_2 exposure, the cell was subject to synaptic inhibition, as shown above the membrane potential record.



Text-fig. 8. Pen recording of an experiment comparing the effects of 5 % CO_2 at pH 6 and 7 with the effects of Tris maleate buffered Ringer at pH 6. The $NaHCO_3$ concentration of the two CO_2 solutions was 1·3 and 13 mM respectively.

finally to a solution equilibrated with 5 % CO_2 and adjusted to pH 7·0 by adding $NaHCO_3$ to a concentration of 13 mM. Thus the CO_2 in the first and third test solutions was the same, but the bicarbonate concentration was ten times greater in the third. It is clear that the initial rapid decrease in intracellular pH is very similar in these two exposures to CO_2. With the higher level of bicarbonate, however, the internal pH began to return towards the normal value after a few minutes. Then, when the solution was changed back to normal, the internal pH increased to a value beyond the normal level, and then slowly returned to the control level.

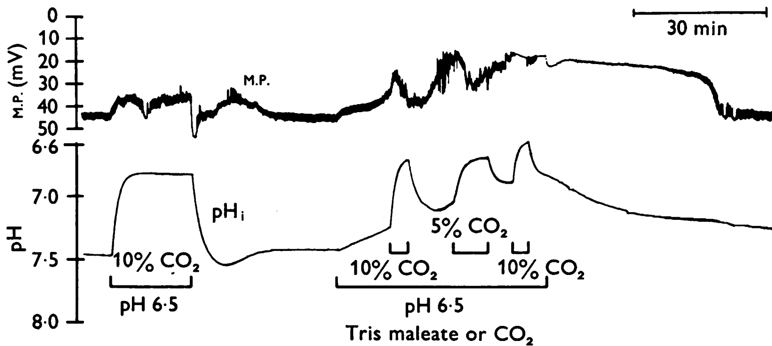
To show the effects of applying external CO_2 at constant external pH, experiments such as that illustrated in Text-fig. 9 were done. First the cell was exposed to a 10 % CO_2 solution at pH 6·5, but for a longer time than in the experiment shown in Text-fig. 7. The internal pH fell by about 0·6 units, and remained constant until the end of the exposure. On return to normal Ringer the internal pH increased and briefly overshot the normal level, as in the last part of Text-fig. 8. After some time in normal Ringer, three different solutions, all at pH 6·5, were tested. Before and after the CO_2 solutions, the solution was buffered with Tris maleate. 10 % CO_2 was applied next, then 5 % and finally 10 % again. The long period of pH 6·5 greatly decreased the cell's membrane potential, and recovery was very slow. The decrease in pH caused by the 10 % CO_2 solutions became less as the internal pH before CO_2 application decreased. Thus the first application of 10 % CO_2 at the beginning of the experiment caused a change of 0·62 pH units, and the third a change of 0·28 units. If these pH changes are converted to changes in hydrogen ion concentration, they become concentration increases of 114, 123 and 122 nM respectively. These are, of course, rough estimates only, but they do suggest that the buffering capacity of the snail neuron does not vary greatly over the internal pH range 7·5 to 6·5.

The buffering power of a solution was first defined by Van Slyke (1922) as the number of equivalents of strong acid or base required to change the pH of a litre of the solution by one pH unit, or d Base/d pH. This unit is still used in chemistry and biochemistry, but in physiology a unit one thousand times smaller is more usual. The smaller unit was first used by Van Slyke, Hastings, Heidelberger & Neill (1922) and was later named a 'slyke' by Woodbury (1965). It can be defined as the number of milliequivalents of acid or base per unit pH per litre. An alternative measure of the physiological buffering power is given by the CO_2 buffering capacity, i.e. the change in the logarithm of the P_{CO_2} per unit pH change, or d log P_{CO_2}/d pH. Since the effective acidity of CO_2 varies with the pH, the CO_2 buffering capacity is not linearly related to the Van Slyke buffering power.

In a total of ten experiments with 5 % CO_2, the intracellular pH change occurring after 5 min of exposure was $0·47 \pm 0·01$ units, and in five similar experiments with 10 % CO_2 the change was $0·58 \pm 0·02$. Thus the CO_2 buffering capacity was 2·7 over the range 5–10 % CO_2. To estimate the Van Slyke buffering power of snail neurones, 10 % CO_2 was bubbled through a series of solutions containing different concentrations of pH 7·4 Tris-maleate. The concentration of the solution whose pH changed by

0·58 units was 150 mM. The buffering power of this solution was determined by titration with HCl. Over the pH range 7·4–6·8 it was 0·025 equiv H^+/pH per l. or 25 slykes. (In measuring the internal pH changes with external CO_2, no allowance was made for the effects of the external pH change. Thus the internal pH change with CO_2 was probably over-estimated, so that the calculated buffering power should be taken as a minimum value.)

The overshoot of the internal pH seen in Text-figs. 8 and 9 on return to normal Ringer after exposure to CO_2 was apparently correlated with high levels of external bicarbonate. Presumably bicarbonate ions enter the cell passively during the period of exposure, to add to those being formed there as the CO_2 is hydrated. To investigate the effects of intracellular bicarbonate when there is no CO_2 outside the cell, potassium bicarbonate was injected into cells maintained in normal Ringer. Text-fig. 10 illustrates such an experiment. The pH and membrane potential electrodes were inserted as usual. Then a third micro-electrode, filled with KCl, was inserted, causing very little damage. Finally, with some difficulty, a fourth electrode, filled with $KHCO_3$, was pushed into the cell. Once the fourth electrode was in the cell a 4 nA current was passed between the KCl and $KHCO_3$ electrodes, with the latter positive, to reduce leakage of the bicarbonate anions. This current appeared ineffective, so it was later increased to 9 nA, as indicated.



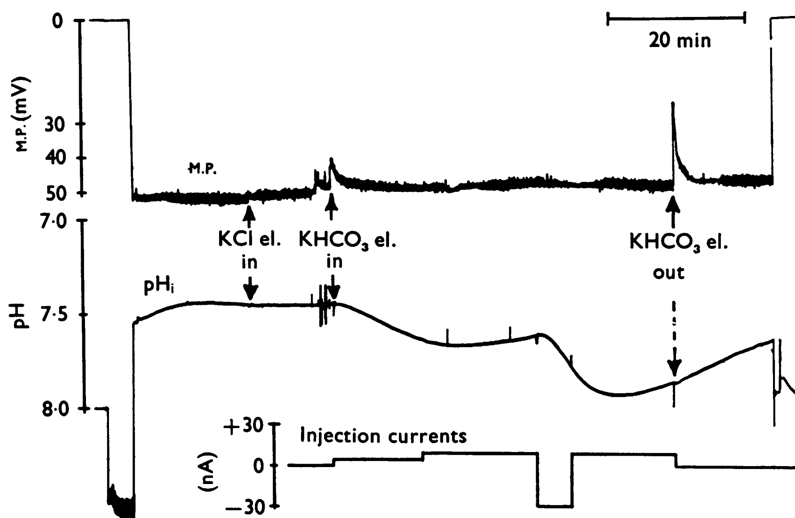
Text-fig. 9. Pen recording of an experiment showing the effects of the membrane potential and internal pH of Ringer solutions of pH 6·5 containing 5 % CO_2 and 4 mM-$NaHCO_3$, or 10 % CO_2 and 8 mM-$NaHCO_3$, or 20 mM-Tris maleate.

This larger backing current appeared to have reduced leakage enough to allow the intracellular pH to return toward normal, so the current was reversed and increased to 29 nA for 4 min. This interbarrel injection should have increased internal bicarbonate by at least 10 mM. The response of the internal pH to this injection was a slow, but large, increase. Some of the injected bicarbonate probably left the cell passively as bicarbonate; but the pH changes show that some accepted H^+ to make carbonic acid, which

in turn was converted to water and CO_2, a reaction which would be greatly favoured by the rapid loss of the CO_2 to the cell exterior.

The effect of external ammonium ions and ammonia

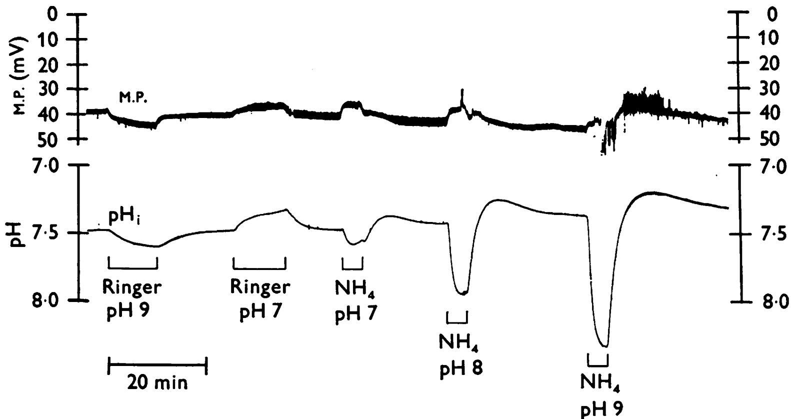
Like CO_2, ammonia has long been known to penetrate cell membranes relatively easily (Jacobs, 1940). Like CO_2, it reacts with water, but instead of yielding hydrogen ions it in effect takes them up to produce ammonium ions. To investigate the effects of NH_3 and ammonium ions on snail neurones cells were exposed to $(NH_4)_2SO_4$ solutions at different pH. The $(NH_4)_2SO_4$ was added as a solid to the Ringer (buffered with 20 mM Tris maleate) just before use, so that the dissolved NH_3 in equilibrium with the NH_4^+ ions would not be lost to the atmosphere.



Text-fig. 10. Pen-recordings of the membrane potential, internal pH and injection current in an experiment to show the effects of the intracellular injection of $KHCO_3$. Arrows indicate the insertion of the KCl and $KHCO_3$ injection electrodes. The direction of the current indicated refers to the potential applied to the $KHCO_3$ electrode.

Text-fig. 11 shows an experiment in which 5 mM $(NH_4)_2SO_4$ was applied for 4 min periods at three different pH's. At pH 7, where the NH_3 concentration would be about 0·04 mM, the ammonium solution caused a relatively brief increase in internal pH (although the same solution without the ammonium caused a change in the opposite direction) with a small overshoot on return to the normal Ringer. At pH 8, where the NH_3 concentration would be about 0·4 mM, there was a rapid pH increase of about 0·5 units, with again an overshoot on return to normal. Finally, at pH 9, and

an NH_3 concentration of about 3 mM, there was an equally rapid pH increase of almost 1 unit, with again an overshoot on return to normal. It is clear that the increasing size of the response as the pH increases is correlated with the increasing NH_3 concentration. The higher the external concentration the greater will be the NH_3 influx, and the greater the subsequent H^+ ion uptake during the conversion of NH_3 to NH_4^+ (the pK of this reaction is about 9·3).



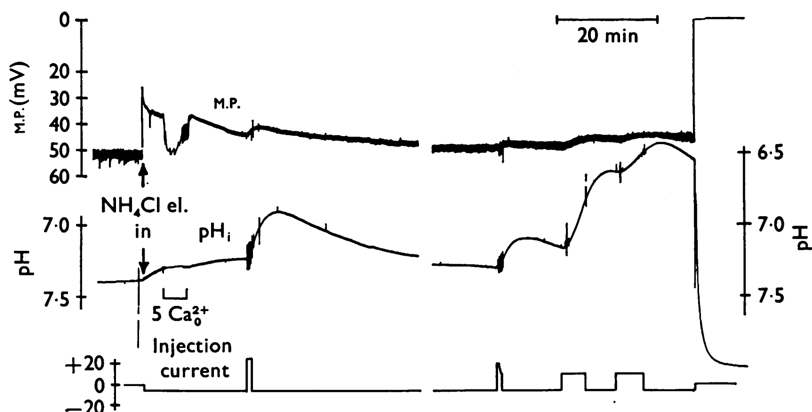
Text-fig. 11. Pen recording of an experiment to show the effects on the membrane potential and intracellular pH of external $(NH_4)_2SO_4$, 5 mM, applied at pH 7, and 9. Control applications of the pH 7 and 9 Ringer were made at the beginning of the experiment.

The rebound or overshoot of the internal pH seen on return to normal Ringer is presumably due to the accumulation of NH_4^+ ions inside the cell. With the removal of the external NH_4^+ and NH_3, the internal NH_4^+ would give up H^+ ions and tend to leave the cell as NH_3. In Text-fig. 11, the increase in the size of the rebound seen with increasing pH of the test solutions suggests that a large fraction of the internal NH_4 comes from the NH_3 entry rather than from NH_4 entry, since the external NH_4 if anything decreased with increasing pH. This agrees with results obtained on frog muscle by Fenn, Haege, Sheriden & Flick (1944) who found that the rate of accumulation of ammonium was greater at high pH than at low.

The effect of injecting ammonium chloride

To confirm that intracellular NH_4^+ ions would tend to acidify the cell interior as they were converted to NH_3, NH_4^+ ions were injected into snail neurones by interbarrel iontophoresis. Text-fig. 12 is a recording from an experiment in which NH_4^+ and Cl^- ions were injected by passing a current between a KCl and a NH_4Cl-filled micro-electrode. Between injections a

backing current was passed, as shown, to minimize NH_4^+ leakage. The result shows clearly that NH_4^+ injection causes a large, if slow, decrease in intracellular pH. (The noise occurring on the pH trace during the injections was due to intermittent partial blockage of the injection electrode.)



Text-fig. 12. Pen recordings of the membrane potential, internal pH and injection current from an experiment to show the effects of the intracellular injection of NH_4Cl. Shortly after the NH_4Cl injection electrode was inserted the cell was exposed, as indicated, to a Ringer solution containing five times the normal Ca. The sign given on the current scale refers to the NH_4Cl electrode. (Current scale in nA.)

DISCUSSION

The results show that hydrogen ions are not in equilibrium across the snail nerve cell membrane *in vitro*, and that intracellular pH is little affected by changes in membrane potential, extracellular pH or other extracellular ions. Metabolic inhibitors cause moderate falls in intracellular pH. The only agents causing large or rapid changes of the internal pH are NH_3 and CO_2.

The finding that there are fewer hydrogen ions inside the cell than would occur if they were simply passively distributed across the cell membrane agrees with most previous findings using pH-sensitive intracellular electrodes (Caldwell, 1958; Kostyuk & Sorokina, 1960; Sorokina, 1965). It also adds additional weight to the conclusion by Paillard (1972) that the conflicting results obtained by Carter *et al.* (1967) were due to badly insulated electrodes.

The new pH-sensitive micro-electrode's slow response in poorly buffered solutions could be turned to advantage and made use of in a technique for determining both pH and membrane potential with a single electrode. If a recessed tip pH-sensitive microelectrode is inserted into a cell and left long

enough it will give an accurate measure of the sum of the membrane potential and pH gradient across the membrane. If the cell is then superfused by a Ringer with a low buffering power when the pH electrode is withdrawn from the cell it will at first still register the intracellular pH, but now without the membrane potential. This procedure was not tried in the present series of experiments, but some idea of how it would work can be obtained from the pH records at the beginning and end of several of the results illustrated, especially the one shown in Text-fig. 4.

Some consideration should be given to possible sources of error in the present experiments. Errors could arise from incorrect determination of either the membrane potential or the intracellular pH. The KCl-filled micro-electrodes used to measure the membrane potential were carefully checked for low tip potential and almost certainly were as accurate as such electrodes can be. The pH electrodes measured the same changes in membrane potential as the KCl electrodes, and had the same pH response at the end as at the beginning of an experiment. It is possible that the intracellular environment contained some compound that caused an offset voltage on the pH electrode while it was inside the cell, but it would have to be freely diffusing to enter and leave the space inside the electrode tip quickly enough not to cause a visible distortion in the pH record. It would also have to have a very similar effect on different pH electrodes to account for the small variation in intracellular pH found in different cells with different electrodes (the results described in this paper were obtained using three different pH-sensitive micro-electrodes). There is no reason to suspect that such a compound exists. Finally, it is possible that the pH electrode does not measure the same absolute membrane potential as the KCl electrode. The most likely reason for this would be the existence inside, but not outside, the cell of a junction potential between the inside of the pH electrode tip and the solution outside. But since the volume of this space is small compared to the cell volume, and the electrode tip is open, any difference between the solution inside the electrode tip and inside the cell would not last long enough to give a consistent error.

The effects of external CO_2 and bicarbonate on intracellular pH were rather complex. Since carbonic acid is a weak acid, the pH change expected from the entry of CO_2 into a cell will depend not only on the buffering power but also on the final pH of the cell interior. Soon after the beginning of the exposure to CO_2 the intracellular CO_2 can be assumed to be effectively equal to the extracellular CO_2. Inside the cell the CO_2 will be hydrated to form carbonic acid, which will dissociate to form equal numbers of H^+ and HCO_3^- ions. The number of HCO_3^- ions (and thus the number of H^+ ions) produced will depend on the final H^+ concentration: the more H^+ there is from other sources the less will be the pH change caused by the CO_2. Thus,

given a constant buffering power, the pH change caused by CO_2 will decrease as the intracellular pH decreases, as seen in Text-fig. 9.

Any intracellular HCO_3^- ions that do not arise from the dissociation of the carbonic acid produced by the entering CO_2 will also tend to reduce the pH change caused by CO_2 entry. But it is hard to see how HCO_3^- entry from outside the cell, or any other passive ion movements, could cause the intracellular pH to begin to increase again after the initial decrease with CO_2, as seen in the third part of Text-fig. 8. After five minutes of exposure to CO_2 at pH 7 the intracellular pH is also close to pH 7, and presumably the intracellular HCO_3^- will be close to the extracellular level of 13 mM. Since the bicarbonate ion is negative, purely passive distribution across the cell membrane would give an intracellular level of about 2 mM, so that HCO_3^- ions would be expected to leave passively, rather than enter. Similarly, passive movements of H^+ or OH^- cannot explain the partial reversal of the pH change: perhaps it is caused by active transport of hydrogen ions at a rate large enough to overcome the acidifying effects of passive loss of HCO_3^- ions.

With the application of external NH_4^+ and NH_3 there is no need to invoke active transport to explain the secondary changes in intracellular pH. The passive entry of NH_4^+ ions is electrically 'downhill', and an accumulation of NH_4^+ internally would tend to offset the increase in pH caused by NH_3 entry, and cause an overshoot of the base line on return to normal Ringer.

Otherwise the responses of the snail neurones to external NH_3 and CO_2 are perhaps surprisingly similar, although the pH changes are in opposite directions. Comparing the CO_2 application shown in Text-fig. 8 with the NH_3 applications shown in Text-fig. 11, the speed of the pH change is much the same, suggesting that the membrane permeability to CO_2 and NH_3 are of the same order, if the reaction of CO_2 with water is not rate-limiting.

The intracellular buffering power of invertebrate neurones has not been previously measured, but the intracellular buffering power of mammalian brain has been estimated using two different techniques. From the distribution of [^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO) between brain, plasma and cerebrospinal fluid in the cat, Roos (1965) estimated the CO_2 buffering capacity of the brain as 2·38 over the range P_{CO_2} 20–90 mmHg. From the bicarbonate distribution, Kjällquist, Nardine & Siesjo (1969) have estimated the rat brain CO_2 buffering capacity as 2·3 at a P_{CO_2} of 40 mmHg. Considering the difference in preparation and technique, the value for snail neurones of 2·7 for the range P_{CO_2} about 40–80 mmHg is close to these values.

The Van Slyke (1922) buffering power for snail brain of 0·025 equiv H^+/pH per l. also agrees reasonably well with the value of 0·037 equiv H^+/pH per l. estimated for cat brain by Roos (1965). It is, however, higher

than the value of 0·0107 given for rat skeletal muscle by Paillard (1972), which is the only previous estimate of buffering power made using a pH-sensitive micro-electrode.

In comparing the responses of the cell to injected bicarbonate and ammonium ions there are two points that are difficult to understand. First, assuming that the buffering power of the cell is relatively constant over the pH range 7–8, why is the size of the pH response to NH_4^+ injection so similar to that obtained with the injection of much larger quantities of HCO_3^-? Theoretically, if only CO_2 or NH_3 left the cell, all the injected $NaHCO_3$ should effectively be converted to NaOH, and all the injected NH_4Cl to HCl. Without careful measurements it is difficult to rule out differences in cell size, but perhaps a more likely reason for the difference is that relatively more of the injected HCO_3^- than NH_4^+ is lost from the cell as ions, without accepting or producing H^+ ions. Secondly the slowness of the internal pH response to injected NH_4^+ is rather puzzling. The reaction of CO_2 with water is well known to be slow, but the reaction of NH_3 with water is about a million times faster (Eigen, 1964). Perhaps the membrane permeability to NH_3 becomes the limiting factor in this case.

Using a rough estimate of the cell volume, the size of the observed pH change with NH_4^+ injection can be compared to that expected if all the injection current through the NH_4Cl electrode was carried by NH_4^+ ions, and all the NH_4^+ ions were converted to H^+ ions. For the first injection of Text-fig. 12, the internal H^+ ion concentration should have risen by between 10 and 20 mM. If the cell's buffering capacity is taken as equivalent to 150 mM Tris maleate, this addition of H^+ ions would cause a pH change of 0·3–0·6 units. The observed pH change was about 0·3 units, suggesting that a sizeable fraction of the injected NH_4^+ ions must indeed have left the cell as NH_3, leaving behind the H^+ ions.

The present results strongly suggest that the recessed-tip design of pH-sensitive micro-electrode gives an accurate and direct reading of intracellular pH, and shows that such an electrode can give a continuous recording from one cell for several hours. The principal advantage of the new electrode over previous designs is that only its extreme tip, the external diameter of which can be less than one micron, needs to penetrate the cell. The main disadvantage is perhaps its very slow response in poorly buffered solutions.

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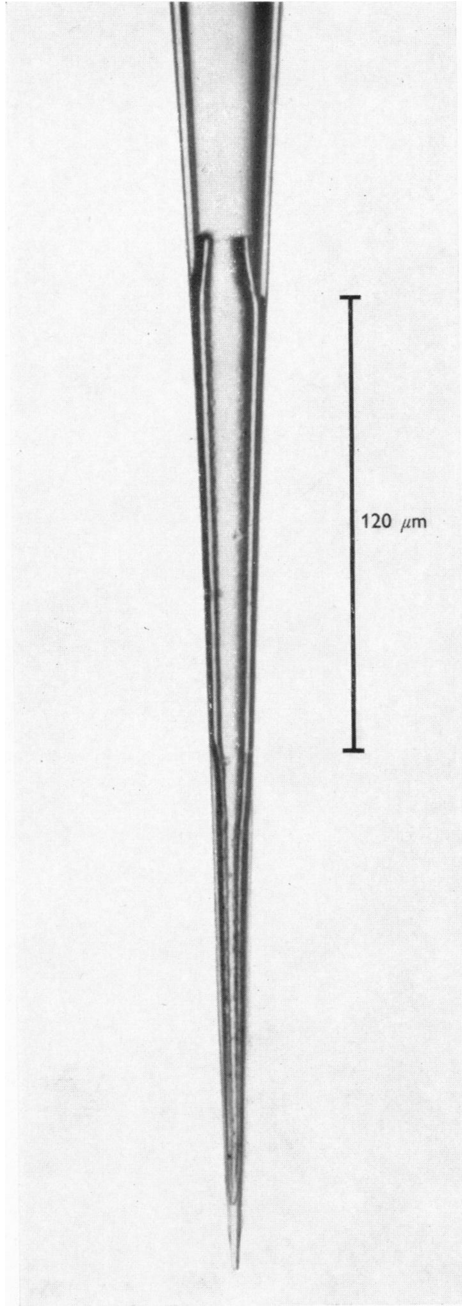
EXPLANATION OF PLATE

Photomicrograph of the terminal 0·3 mm of a finished pH-sensitive micro-electrode of the recessed-tip design. The scale indicates the position of the glass–glass seal. See also Text-fig. 1.



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