Control of Intracellular pH

Predominant Role of Oxidative Metabolism, Not Proton Transport, in the Eukaryotic Microorganism Neurospora

DALE SANDERS and CLIFFORD L. SLAYMAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT Recessed-tip microelectrodes were used to measure internal pH (pHi) in the fungus Neurospora, and to examine the response of pHi to several kinds of stress: changes of extracellular pH (pHo), inhibition of the principal proton pump in the plasma membrane, and inhibition of respiration. Under control conditions, at pHo = 5.8, pHi in Neurospora is 7.19 ± 0.04. Changes of pHo between 3.9 and 9.3 affect pHi linearly but with a slope of only ~0.1 unit pHi per unit pHo, stable pHi being reached within 3 min of changed pHo. Despite a postulated high passive permeability of the Neurospora membrane to protons (Slayman, 1970), neither active nor passive H+ transport appears critical to pHi because (a) specific inhibition of the proton pump by orthovanadate has little effect on pHi, and (b) cytoplasmic acidification produced by respiratory blockade is unaffected by the size or direction of proton gradient. To convert measured changes in pHi into net proton fluxes, intracellular buffering capacity (βi) was measured by the weak acid/weak base technique. At pHi = 7.2, βi was (−35 mmol H+ (liter cell water)]−1 (pH unit)−1, but βi increased substantially in both the acid and alkaline directions, which suggests that amino acid side chains are the principal source of buffer.

INTRODUCTION

There is now substantial evidence from a variety of cell types that intracellular pH (pHi) is maintained within relatively narrow limits (animals: Roos and Boron, 1981; plants: Smith, 1980; fungi: Navon et al., 1979; bacteria: Slonczewski et al., 1981). This conforms with the general expectation, based on the pH optima of cytoplasmic enzymes, that large deviations of pH from neutrality would be severely inhibitory to metabolism. It is also clear that this constancy of internal pH represents a steady state, not true equilibrium; in all circumstances where metabolism is active, there is a steady flux of protons into and out of the cytoplasmic proton pool.

Previous work on nerve and muscle has established a predominant role for
membrane transport in control of $pH_i$. In response to an acid load on the cytoplasm, a variety of excitable cells uses electroneutral exchanges of $Cl^-$ for $HCO_3^-$ and of $Na^+$ for $H^+$ (either in separate or combined transport systems) to return cytoplasmic $pH$ to more alkaline levels (Roos and Boron, 1981). Energy for these exchanges comes primarily from the gradient of $Na^+$, which means ultimately from work done by the sodium pump.

The situation in microorganisms must be quite different, however, because the primary pump in the plasma membrane is an outwardly directed, electrogenic $H^+$ pump (Slayman, 1974). It therefore seemed reasonable to expect that regulation of cytoplasmic $pH$ in these cells would be accomplished by the proton pump, and cogent theoretical arguments have been made that this is indeed the case (Raven and Smith, 1974, 1976a). However, the magnitude of influence that the pump has on cytoplasmic $pH$ will be limited by at least two circumstances. First, the pump consumes a large fraction (25%; Slayman et al., 1973) of the total phosphate bond energy, which is itself tightly controlled in respiring cells (Atkinson, 1977). This will set a limit on the degree to which the pump can be stimulated in the presence of a proton load. Second, metabolism can liberate or consume substantial quantities of protons, depending on the predominant pathways being used at any particular time (Raven and Smith, 1976a, b). Early evidence from bacteria (Gale, 1946) had, in fact, suggested that metabolism can act as a controller of intracellular $pH$, and more recent studies using $^{31}P$ nuclear magnetic resonance (NMR) have identified a very considerable sensitivity of $pH_i$ to imposed metabolic conditions (Ugurbil et al., 1978; Navon et al., 1979; den Hollander et al., 1981).

However, limitations intrinsic to the NMR technique have retarded attempts to assess the relative contributions of metabolism and the proton pump to $pH_i$ control (for example, when one process, but not the other is inhibited), and we have adopted a more direct approach using intracellular $pH$ microelectrodes on the relatively large cells of the mycelial fungus Neurospora. Our two main findings have been (a) that, despite a large inward transmembrane gradient for protons, the dominant source of cytoplasmic protons is intracellularly produced acidity; and (b) that metabolism alone is capable of controlling $pH_i$, even when the proton pump is inhibited.

**MATERIALS AND METHODS**

**Growth of Cells**

For internal $pH$ measurements, wild-type *Neurospora crassa*, strain RL21a, was grown on scratched cellophane underlaid with minimal medium plus 2% sucrose (Slayman, 1965). After ~30 h growth, a portion of the hyphal mat and cellophane was removed and soaked in the standard buffer solution (see below) for 1-2 h before the commencement of experiments, at room temperature (usually 23°C). This pretreatment allowed the cells to come to a new steady state in which they were actively respiring, but no longer growing. Hyphae that had adhered firmly to the cellophane and reached 12-14 $\mu$m diameter were used. Measurements of respiration were made at 25°C on cells that had been grown 12-15 h in shaking cultures, as described by Slayman and Tatum (1964) and Lambowitz and Slayman (1971).
All cells were grown in Vogel's (1956) minimal medium, pH 5.8. Measurements of respiratory rate, and in some cases (Fig. 6) of internal pH, were performed in phosphate-free dilute (0.3X) minimal medium (P-free DMM), to which were added 1% glucose, 2.3 mM CaCl₂, and 20 mM dimethylglutaric acid (DMG) titrated to pH 5.8 with NaOH. The standard buffer medium (designated "standard DMG") for most experiments was 20 mM DMG, 1 mM CaCl₂, and 1% glucose, brought to pH 5.8 with KOH (final K⁺ concentration 25 mM). DMG titrated with KOH was also used for experiments below pH 5.8; HEPES (30 mM, pH 7.5-8.4, usually 8.2) or glycylglycine (30 mM, pH 9.0; 18 mM, pH 9.2-9.5) was used at higher pH's. In all cases, the solutions were titrated to the desired pH with freshly prepared KOH, and the final K⁺ concentration was brought to 25 mM with KCl. Additions to these solutions (inhibitors, weak acids, or weak bases) were made from concentrated stock solutions brought to the appropriate pH with NaOH or HCl. Orthovanadate was used from a freshly boiled 200-mM stock solution of the Na⁺ salt, pH 9.5, and subsequently neutralized with HCl.

**Measurement of Internal pH: the Electrodes**

Recessed-tip electrodes of the Thomas (1974, 1978) design were fabricated for each experiment. Efforts were made to reduce the outside tip diameter and the dead volume of the electrodes below sizes conventionally described (Thomas, 1978), in order to minimize penetration damage and increase response speed. However, test studies showed that with very small dead spaces (resistances on the order of 5 × 10¹² Ω), the time constant for response to a step change of pH was essentially identical to the time constant for response to a current pulse; i.e., the sensing time constant (τPH) and the electrical time constant (τα) were the same. For larger electrodes this relationship did not hold, and τα was generally faster than τPH, which presumably means that H⁺ diffusion into the dead volume was rate limiting. Optimization of the various factors in construction and use of the pH electrodes led to the following dimensions: tip diameter (outside), 0.5 μm; recessed distance between casing alumino-silicate glass and the pH glass, 2-6 μm; overall length of exposed pH glass, 25-35 μm; sensitivity, >55 mV/unit pH; electrical resistance, 10¹² Ω; τPH, 5-20 s (usually close to τα).

**Experimental Protocol and Correction of the pH Trace during Rapid Changes of Membrane Potential**

All measurements were made with a high-impedance differential amplifier (Model F-223; WP Instruments, Inc., Hamden, CT), which compared the signal from the pH electrode with a second, intracellular, microcapillary reference electrode reporting membrane potential alone. Fig. 1 shows a sample recording that demonstrates the general experimental protocol and some of the problems associated with the use of recessed-tip microelectrodes. Initially, the reference microelectrode was inserted into the hypha, and after a stable voltage was achieved, the pH electrode was positioned in the same cell. The sudden depolarization accompanying entry of the pH electrode is typical, but recovery was taken as an indication that the cell was not damaged by entry of the (comparatively coarse) pH electrode. Addition of CN⁻ results in rapid depolarization by ~100 mV. This event produces a transient artifact in the pH trace (apparent alkalization) because of the markedly different electrical time constants of the two electrodes. A segmental linear method for correcting the artifact is described in the Appendix, and the results of the correction are shown in Fig. 1 (dashed line). (Note that since Fig. 1 is traced from an original pen recording, the pH trace is
Figure 1. Insertion of recessed-tip pH electrode and potential-measuring electrode into *Neurospora*, and the effect of rapid depolarization on measured internal pH. Upper trace: voltage difference between signals from the pH electrode and a conventional, KCl-filled microcapillary electrode. Lower trace: output from the microcapillary electrode alone. Before insertion of either electrode, the upper trace displays external pH = 5.70. Upon insertion, the microcapillary electrode registered membrane potential of $-157$ mV; note accompanying equal and opposite deflection in the difference trace. 4.7 min later, the pH electrode was inserted, which resulted in a transient depolarization of $47$ mV. On attainment of a stable reading for internal pH (7.32), the preparation was pulsed with 1 mM NaCN for 1 min. The apparent alkalization that paralleled depolarization was caused by the markedly different time constants of the two electrodes. Similarly, the (slower) repolarization produced a (smaller) apparent acidification upon washout of NaCN. Dashed trace shows the “true” internal pH, after correction of pH trace for $\tau_{el}$ (see Appendix). $\tau_{el}$ in this case was 8.7 s. Further discussion of the effects of CN$^-$ on pH is given in the Results.

Inverted. Throughout the rest of this paper, pH data have been oriented with increasing pH values upward, after correction for the membrane potential artifact.

Normally, experiments were terminated after 15–20 min by “sealing off” of the pH electrode by a cytoplasmic cap (Umrath, 1932; Walker, 1955; Slayman, 1965), although cells were capable of surviving for ≥2 h with the pH- and potential-recording electrodes inside.
Correction of pH Traces for Slow Response to pH

In some experiments, pH was monitored simultaneously inside and outside the cells, using separate pH electrodes. In these cases, we wanted to know the exact behavior of pH in both phases, so correction for the response time of each pH electrode was necessary. Again, reconstruction of the actual time course of pH change from the observed traces was performed by segmental linear analysis, using the \( \tau_{pH} \) for each electrode (see Appendix).

Measurement of Cytoplasmic Buffering Capacity

Weak acids (or bases) can be used in conjunction with pH electrodes to estimate the buffering capacity \( (\beta_i) \) of cytoplasm, as has been demonstrated by numerous previous investigators (see Roos and Boron, 1981). When the internal pH has attained a new steady level in the presence of a weak acid, it is assumed that the undissociated acid is at equilibrium across the cell membrane. The internal concentration of the acid anion can then be calculated from the Henderson-Hasselbalch equation (Waddell and Bates, 1969), provided the internal pH and \( pK_a \) are also known. Under normal conditions, the stable amount of acid anion closely approximates the quantity of protons absorbed by the cytoplasmic buffer (only a very small fraction of the total protons released from the acid remains free in the cytoplasm) so that \( \beta_i \) can be written as

\[
\beta_i = (-) \frac{dA^-}{dpH_i} \tag{1a}
\]

where \( dA^- \) is the change in internal concentration of the weak acid anion, and \( dpH_i \) is the change in internal pH. For a weak base, analogous arguments hold and the corresponding equation is

\[
\beta_i = (+) \frac{dB^+}{dpH_i} \tag{1b}
\]

where \( dB^+ \) is the change in internal concentration of the weak base cation.\(^1\)

The \( pK_a \)'s of butyric acid (the weak acid in the present study) and procaine (the weak base) were measured by titration either in 25 mM KCl plus 1 mM CaCl\(_2\) (similar to the external medium in these experiments) or in 200 mM KCl (a simulation of cytoplasm). For butyric acid, the measured \( pK_a \) in both cases was 4.81, in agreement with the published value (Weast, 1971). For procaine, values of 9.03 and 9.13, respectively, were obtained, these values being used for the determination of \( \beta_i \).

Experimental Checks on Assumptions Made by the Weak Acid/Base Method of Determining \( \beta_i \)

Possible Metabolism of the Weak Acid

The method assumes that any changes in internal pH result simply from dissociation of free acid inside the cell. Since microorganisms are known for their ability to use simple organic acids as carbon sources, it seemed important to determine whether butyrate might be metabolized and thereby alter internal pH. To answer this question, cells were allowed to take up

\(^1\) Although the introduction of high concentrations of weak acid or base into the cytoplasm in itself raises the total cytoplasmic buffering capacity, the method measures only the intrinsic cytoplasmic buffering, since the sole participant in proton exchange with the acid or base is the preexisting cytoplasmic buffer.
1-14C-butyric acid for 30 min (15 mM butyrate, pH 5.8). Evolution of 14CO2 was monitored, and at intervals, aliquots of cells were removed, extracted with ethanol, and analyzed by thin-layer chromatography. No release of 14CO2 was detectable in 30 min, and >95% of total 14C counts co-chromatographed with pure butyric acid, independently of the incubation time.

**Comparative permeabilities of the charged and noncharged forms.** Clearly, if the membrane is significantly permeable to the charged form of the weak acid or weak base, the condition that the noncharged form alone is at concentration equilibrium across the membrane will be violated. However, the following preliminary experiments demonstrated, both for butyric acid and for procaine, that the charged species have low permeability coefficients. (a) To test whether influx was proportional to the free acid concentration or the acid anion concentration, the initial uptake of 5 mM 14C-labeled butyric acid was measured at two different external pH values. Raising pH from 5.80 to 8.02 resulted in a 300-fold reduction of influx, compared with a 150-fold reduction of free acid, and a 10% increase of acid anion. The discrepancy is in the wrong direction to be accounted for by significant anion permeability. (b) In electrical experiments, butyrate (applied at high pH) and procaine (applied at low pH) had no effects on membrane potential. High passive permeability to either charged species should have been signaled by hyperpolarization (butyrate) or depolarization (procaine).

These experiments demonstrate that interference of the buffering capacity measurement by entry of the charged forms is probably minimal. However, errors could arise from significant exit of the charged forms, particularly in the case of an acid for which the driving force on the anion will be strongly outward in the steady state. If the ionized form of the acid leaks from the cell, a biphasic change in pH would be expected—the initial phase corresponding to free acid equilibration, and the second to further acid uptake as the anion leaks out. For butyric acid, no such biphasic changes were observed. In addition, initial rate measurements showed that influx of 14C butyric acid (measured at 5 mM total acid) is at least fourfold greater than efflux of H+ through the pump; the effect of pump activity on the estimate of βI would therefore be largely nullified by continued free acid entry. On application of procaine, pH often did show a transient overshoot, which might indicate either regulation of pH or the slower entry of the charged form of the base (cf. Boron, 1977). Either could produce an error in the estimate of βI. However, the transient response was nearly abolished in the presence of CN− and the estimates of βI made with procaine in the presence of CN− did not deviate significantly from those over the same pH range in the absence of CN−.

**Statistics**

Throughout the manuscript, averaged results are stated as the mean ± 1 SEM.

**RESULTS**

**Normal Intracellular pH of Neurospora**

In standard DMG, pH 5.8, pH in 24 cells was measured as 7.19 ± 0.04 on initial impalement. In standard HEPES, pH 8.2, pH on initial impalement was 7.41 ± 0.04 (n = 12). Rather wide variability was observed between hyphae: at pHo = 5.8, recorded pH ranged from 6.85 to 7.62. The origin of this variability is not known, but similar variability has also been observed in measurements of membrane potential (range −160 to −240 mV under stan-
standard conditions); it may arise, at least in part, from differences in the physiological ages of hyphae sampled in a petri plate culture. It is important to note, however, that the steady pH$_i$ did not correlate either with the membrane potential or with the magnitude or direction of pH response to experimental tests.

**Effect of pH$_o$ on pH$_i$**

A large amount of circumstantial evidence in the literature has led to the belief that major electrical conductance of plasma membranes in *Neurospora* and other eukaryotic microorganisms is that for protons (Slayman, 1977; Harold, 1977; Walker, 1980). However, determinations of intracellular pH over a wide range of extracellular values show that pH$_i$ is only slightly sensitive even to large changes of pH$_o$. This fact is demonstrated for alkaline and acid shifts of pH$_o$ in Figs. 2 and 3, respectively.

For these experiments, a chamber of small volume (0.8 ml) was perfused

![Figure 2](image-url)
Figure 3. The effect of lowered external pH on internal pH and membrane potential. Bathing medium was changed from pH 5.7 to pH 3.9 and back to pH 5.7. As in Fig. 2, pH$_e$ was monitored with a pH microelectrode at the cell surface. Medium at pH 5.7 was standard DMG, and the lower pH medium was also buffered with 20 mM DMG, as described in Materials and Methods. The middle trace shows the response of pH$_i$, and the bottom trace shows that of the membrane potential. Again, both pH traces were corrected for lag in electrode responses. Note the fivefold magnification of the ordinate for pH$_i$ compared with that for external pH. The pseudo-first-order time constants for the changes in external pH and internal pH (fitted by nonlinear least squares) were, respectively, 0.49 and 1.79 min. The dashed line superimposed on the trace of membrane potential is drawn as the sum of two exponentials whose time constants were identical with those for the changes in external and internal pH; the fitting equation is

$$V_m = 82 + 75 \exp(-t/0.49) + 103 \exp(-t/1.79),$$

where $V_m$ is membrane potential (mV) and $t$ is time (min).

rapidly (12 ml/min), and an extracellular pH microelectrode was positioned 2 $\mu$m from the hypha being studied. Readings were obtained simultaneously from this electrode and the intracellular one. Fig. 2 shows that increasing pH$_e$ from the normal value (near 5.8) to 9.25 caused pH$_i$ to rise, from 7.50 to 7.84 in this case. It should be noted, also, that the time course of change of pH$_i$ followed closely that of pH$_e$; there was neither an initial transient nor any
longer-term (10–20 min) tendency of pHi to return to the control value with sustained high pHo. This simple behavior of pHi in response to changing pHo contrasts with the complex changes of membrane potential (bottom trace, Fig. 2): a slight transient depolarization, which was followed by a much larger hyperpolarization peaking at the time of maximal slope in the pH curves \([dpH/d(time)]\), and then a return to the control value. Slayman (1965) has reported that membrane potential rises monotonically with increasing extracellular pH, but those original data extend only to pH 9.0; at still more alkaline values, the voltage response to pHo is less stable.

For acid changes of pHo (from the control value near pH 5.8), pHi also moved in the acid direction, but with a substantial delay. As can be seen in the example of Fig. 3 (middle trace), pHi did not begin to fall until external acidification was almost complete; the time constant for internal acidification was \(\sim 1.8\) min. On return of pHo to the control value, pHi also returned, but once again with a delay. As in the case of external alkalization, changes of pHi were monotonic, with no indication of either rapid transients or long-term correction.

The response of membrane potential followed the general pattern observable in most microorganisms and plants (Slayman, 1970): depolarization accompanied the decrease in pH. However, an important feature of the data in Fig. 3 is that not all of the depolarization occurred concurrently with the change in external pH; about half the overall change in membrane potential was observed after the external pH reached the new steady level. The dashed line on the voltage trace in Fig. 3 shows that the depolarization can be fitted well as the sum of two declining exponentials whose time constants are identical with those for the change in external and internal pH (0.5 and 1.8 min, respectively). Repolarization on returning to the original pHo was also biphasic in the same fashion. Thus, the dependence of membrane potential on pHo in the acid range consists of at least two components—one directly related to pHo, and the other probably mediated by an intracellular event, perhaps the small change in pHi.

Absence of Detectable Feedback Control of pHi

Fig. 4 is a summary plot for the results from two types of experiments. Closed symbols represent pHi recorded after long-term (1–2 h) preincubation at the specified pH; open symbols represent data from shorter-term experiments of the type in Figs. 2 and 3, in which the steady pHi was recorded after times >3 min. In the short-term experiments, pHo was always returned to the reference value of 5.8 between each treatment. The two sets of data are readily described by a single straight line function (slope = 0.08 unit pHi/unit pHo), which indicates that there is little or no long-term tendency for pHi to deviate from the value obtained only a few minutes after the change in pHo. Thus, pHi either is effectively controlled in short term or is insulated from effects of pHo. This behavior of Neurospora contrasts with that of Escherichia coli after acid jumps in pHo; there, a transient fall in pHi occurs in the first 30 s, and is followed by spontaneous recovery over the ensuing 3–4 min, so that pHi is
restored to a value close to the original one (R. M. Macnab, personal communication). The resultant steady state dependence of pH$_i$ on pH$_o$ in *E. coli*, however, is very similar to that in *Neurospora*: $-0.1$ unit pH$_i$/unit pH$_o$.

**Effects of Proton Pump Inhibitors on pH$_i$**

The internal H$^+$ concentration ([H$^+$]$_i$) rests at a level significantly lower than if H$^+$ were at equilibrium across the plasma membrane ($E_H = +75$ mV in standard DMG, membrane potential = $-200$ mV). Inhibitors of the proton pump were therefore applied in order to investigate the role of the H$^+$ extrusion pump in maintaining this relatively alkaline pH$_i$. Pump inhibitors fall generally into two classes: indirect inhibitors, which act through reduction...
of the supply of ATP to the pump by metabolic blockade; and direct inhibitors, which act on the pump, but (ideally) not on any other metabolic processes.

**INDIRECT INHIBITION BY CYANIDE** Fig. 5A shows the effects of 1 mM NaCN on membrane potential and pH\textsubscript{i}, at pH\textsubscript{o} = 5.8. Rapid depolarization resulted from switching off the electrogenic H\textsuperscript{+} pump by ATP withdrawal (Slayman et al., 1973). A large (0.70 unit) but slow cytoplasmic acidification also occurred, in this example from pH\textsubscript{i} = 7.03 to 6.33. The effect of CN\textsuperscript{−} on pH\textsubscript{i} was usually biphasic, the two time constants in this case having values of 0.5 and 3.2 min. (Acidification cannot result from H\textsuperscript{+} transport into the cell as HCN, because that acid is too weak \([pK_a = 9.1]\) to release significant H\textsuperscript{+} at pH\textsubscript{i} = 7.2.) On removal of CN\textsuperscript{−}, both membrane potential and pH\textsubscript{i} recovered to the control values, but again membrane potential shifted faster than pH\textsubscript{i}.

![Graph showing effects of 1 mM NaCN on membrane potential and pH\textsubscript{i} at pH\textsubscript{o} = 5.8](image)

**FIGURE 5.** Cyanide-induced depolarization (lower trace) and cytoplasmic acidification (upper traces). External solutions: (A) standard DMG, pH 5.80; (B) standard HEPES, pH 8.40.

On the basis of the long-standing notion that plasma membranes of microorganisms are highly permeable to H ions (see above), it seemed likely that the CN\textsuperscript{−}-induced acidification might result from inward proton leakage uncompensated by normal pump-efflux. In the experiment of Fig. 5A, for example, 30 s after the introduction of CN\textsuperscript{−} there is an inwardly directed electrochemical potential difference for H\textsuperscript{+} \(\Delta \mu_{\text{H}^+} = 170\ \text{mV} (\text{pH}_{\text{i}} = 7.00, \text{pH}_{\text{o}} = 5.80; V_m = -100\ \text{mV})\). However, further experiments (Fig. 5B) showed that CN\textsuperscript{−}-induced acidification occurred essentially with the same time course even when the extracellular pH was kept at 8.40. Hence \(\Delta \mu_{\text{H}^+} = 0\) just after CN\textsuperscript{−} addition (pH\textsubscript{i} = 7.05, pHe\textsubscript{o} = 8.40; \(V_m = -80\ \text{mV})\). Because the absence of a net inward driving force on protons not only fails to halt the cytoplasmic acidification, but does not even modify its time course, an uncompensated
proton leak cannot be responsible for the CN\textsuperscript{−}-induced acidification. Obviously another source of cytoplasmic H\textsuperscript{+} must be sought.

**DIRECT INHIBITION BY ORTHOVANADATE** It has recently been shown that orthovanadate acts both as a potent inhibitor of the partially purified plasma membrane ATPase from *Neurospora* (Bowman and Slayman, 1979), and as a specific inhibitor of proton pumping, with little or no effect on the ionic leak pathways through the membrane (Kuroda et al., 1980). The effect of 1 mM orthovanadate\textsuperscript{2} on pH\textsubscript{i} was surprisingly small, sometimes producing a ~0.1-unit acidification, sometimes giving no discernable effect, and occasionally resulting in a slight pH increase, as shown in Fig. 6. Previous analysis (Kuroda et al., 1980), confirmed by the vanadate-induced depolarization in Fig. 6, indicates a threefold decrease of current flow through the proton pump; but despite that implied reduction of H\textsuperscript{+} efflux, pH\textsubscript{i} was maintained near 7.3 in the face of an inwardly directed Δ\textmu\textsubscript{H\textsuperscript{+}}, equal to \(-130\) mV. Strikingly, however,

\[\text{Vanadate}^2\]

\[\text{on pH}_i\text{ was surprisingly small, sometimes producing a ~0.1-unit acidification, sometimes giving no discernable effect, and occasionally resulting in a slight pH increase, as shown in Fig. 6. Previous analysis (Kuroda et al., 1980), confirmed by the vanadate-induced depolarization in Fig. 6, indicates a threefold decrease of current flow through the proton pump; but despite that implied reduction of H}^+\text{ efflux, pH}_i\text{ was maintained near 7.3 in the face of an inwardly directed Δμ}^+_{H\textsuperscript{+}}, \text{equal to ~130 mV. Strikingly, however,}\]

\[\text{This relatively high concentration must be used in order to obtain a sufficient amount of vanadate in the cytoplasm for rapid inhibition of the pump. Because of this access problem, the cells must also be phosphate starved to derepress the phosphate transport system that facilitates vanadate entry.}\]
CN⁻ still caused a 0.7-unit acidification, which could be reversed on removal of the respiratory inhibitor alone (Fig. 6). The overall effect of CN⁻ in the presence of the specific pump inhibitor was thus similar to that in its absence. (Hyperpolarization of the membrane on CN⁻ washout suggests that not all of the pump activity has been inhibited by vanadate, in agreement with the results of Kuroda et al. [1980]. After application of CN⁻, membrane resistance increases [Slayman, 1980] so that a rapidly rising [but small] pump current produces a large I-R drop, seen as membrane hyperpolarization.) These results strongly support the conclusions reached for application of CN⁻ alone; viz., proton leakage into *Neurospora* plays a minor role as a source of cytoplasmic H⁺. Thus, metabolic changes induced by CN⁻—whether or not the pump has previously been working—must constitute the major input to the cytoplasmic H⁺ pool.

How can vanadate specifically inhibit the proton-extruding pump in respiring *Neurospora* without allowing H⁺-producing processes to lower cytoplasmic pH? Because a major source of H⁺ must be oxidative metabolism, it would seem that vanadate must also inhibit respiration and glycolysis in order to keep the cytoplasmic proton pool constant. Fig. 7 demonstrates that respiration is indeed partially vanadate sensitive in intact hyphae of *Neurospora*. Oxygen consumption is reduced biphasically over a 10-min period to ~35% of the control value. The distinct presence of two components in the decline of respiration (time constants 0.4 and 5.4 min in Fig. 7) indicates that vanadate action may be metabolically complex, but because of the speed of the initial response, it is likely that this part of the respiratory inhibition results from blockade of the proton pump at times when the internal concentration of vanadate is still low. This interpretation was supported by experiments on mitochondria isolated from *Neurospora* (Fig. 7B). No inhibitory effects of 0.8 mM vanadate were observed either on electron transport, or on state 3 respiration. Since it takes 10 min for 0.8 mM vanadate to accumulate in the cytoplasm under these conditions (B. J. Bowman, personal communication), but the vanadate *K₁* of the partially purified membrane ATPase is 1.5 μM, we conclude that the action of vanadate on respiration in vivo results from secondary effects following pump inhibition. Such effects do not directly touch electron transport, since the respiratory inhibition in vivo can be abolished by uncouplers (data not shown). Therefore, it appears that a signal, whose role might be related to turning off H⁺ production by metabolism, is generated by the pump and acts on mitochondria at the level of oxidative phosphorylation. The concept of metabolic proton production can therefore provide an explanation for the observations in Fig. 6, because, under the influence of vanadate, output and input to the proton pool would be reduced, leaving only a small effect on pool size. Interrelations between the proton pump and oxidative metabolism are considered further in the Discussion.

**Intracellular Buffering Capacity**

To calculate the quantity of acid added to, or subtracted from, the cytoplasm during various manipulations, the intracellular buffering capacity (*Bᵢ*) must be known. It was measured in the experiments reported below.
FIGURE 7. Biphasic retardation of respiration by vanadate in suspension-cultured *Neurospora*. Cells were grown in shaking culture, harvested, and starved of phosphate for 3 h in P-free DMM. O$_2$ consumption was measured in the same medium at 25°C with a Clark oxygen electrode (Slayman and Slayman, 1970; Lambowitz and Slayman, 1971), and 1 mM orthovanadate was added at time zero from a 200-mM stock solution, pH 9.5. Respiratory rate was estimated at 10-s intervals from tangents drawn along the trace (inset A) of O$_2$ content of the chamber vs. time. Control rate = 44.8 μmol O$_2$/g dry wt·min. Data were fitted (solid line) by the equation

$$ r = 24.3 + 37.1 \cdot \exp(-t/0.423) + 38.6 \cdot \exp(-t/5.35) $$

where $r$ is respiration rate as percent control, and $t$ is time in minutes. Dotted line on inset A shows that for a separate batch of cells incubated in the presence of 20 mM phosphate (which competes with vanadate for entry), addition of vanadate was without effect. Inset B. Absence of inhibitory effect of vanadate on state 3 respiration of isolated mitochondria: oxygen electrode recording. Numbers in parentheses give the respiratory rate in microatoms of oxygen per milligram protein per minute. Respiration medium contained: 30 mM sucrose, 8 mM NaH$_2$PO$_4$, 5 mM MgCl$_2$, 0.7 mM EDTA, and 1 mM NADH, pH 7.2. 5 μl mitochondrial suspension (0.143 μg protein/ml), prepared according to Lambowitz et al. (1972), was added to the medium as indicated, and a basal respiratory rate was established. Where shown, ADP and vanadate were added to final concentrations of 0.67 and 0.8 mM, respectively.
Fig. 8 shows the effects of three different concentrations of butyrate (applied at pH₀ = 5.8) on pHᵢ. After a short delay, which reflects the time taken for new medium to reach the cell, pHᵢ decreased rapidly to a new stable value within 2.5 min. The new level could be sustained essentially as long as the recording lasted (in practice for periods of 10-15 min in butyric acid) with little or no tendency to return spontaneously to the control value. Upon washout of butyric acid, pHᵢ returned promptly to the control level, again without a large overshoot. As is expected from ordinary buffer considerations, the magnitude of the pH change induced by butyric acid was not proportional to the butyric acid concentration itself, but rather to the logarithm of the butyric acid concentration (Fig. 8, inset).

To shift pHᵢ in the alkaline direction, a weak base is required that can appreciably bind protons within the cell. Optimally, the pKᵢ of the base
should be higher than pH$_i$, and a correspondingly high external pH must be used in order to generate sufficient free base externally. Procaine ($pK_a \sim 9.0$), applied in millimolar concentrations at pH$_o = 8.2$, gave the predicted effect on pH$_i$, as shown in Fig. 9. The rise of pH$_i$ was $\sim 0.4$ unit with 5 mM procaine, and, like the response of pH$_i$ to butyric acid, was complete within 3 min. Unlike the results with butyric acid, there was a slight systematic tendency for

\[ \Delta \text{pH} = \text{log}([\text{procaine}]) \]

**Figure 9.** Elevation of pH$_i$ by the weak base procaine. Results are from three different hyphae exposed at time zero to various concentrations of procaine, indicated at left, in standard HEPES, pH 8.2. Bracketed numbers indicate pH$_i$ at the break in each trace. The lowest trace also shows the time course for restoring pH$_i$ to the original level upon washout of procaine. The inset displays the same data, replotted to show near linearity of the change in pH$_i$ with log (procaine concentration).

pH$_i$ to return to the control value in the continued presence of the base (trace B), and also for a small overshoot when the base was washed from the cell (not shown).

By applying Eq. 1 to several concentrations of butyric acid and procaine (producing many different values of pH$_i$), a general curve of $\beta_i$ vs. pH$_i$ could be calculated. The summary results of such calculations are plotted in Fig. 10.


where \( \beta_1 \) and \( \beta_2 \) are the buffer capacities contributed by the two dissociable groups, \( \text{H}^+ \) is the prevailing proton concentration, \( K_1 \) and \( K_2 \) are the dissociation constants for the two groups, and \( C_1 \) and \( C_2 \) are the corresponding group concentrations. The fitted values are as follows: \( K_1 = 6.9 \cdot 10^{-7} \text{ M (pK}_a = 6.16) \), \( K_2 = 3.4 \cdot 10^{-9} \text{ M (pK}_a = 8.47) \), \( C_1 = 159 \text{ mM}, C_2 = 103 \text{ mM} \). The significance of these values is discussed in the text.
At the normal pH$_i$ of 7.2–7.4, $\beta_i$ is 30–35 mM H$^+/\text{pH}$ unit. The general form of the data in Fig. 10 indicates that the pH$_i$ dependence of $\beta_i$ cannot arise from titration of a single ionizable group, which would produce a bell-shaped curve. However, the data can be fitted by the sum of two titratable groups, each with a pK$_a$ well removed from normal values of cytoplasmic pH. The optimal values for these pK$_a$'s appear from the fitted curve in Fig. 10 to be 6.16 and 8.40.

**DISCUSSION**

*Normal pH$_i$ of Neurospora*

A previous estimate of pH$_i$ in *Neurospora* from the distribution ratio of the weak acid 2,4-dinitrophenol (DNP) (at non-uncoupling concentrations) resulted in an absolute value of pH$_i$ of 6.4 at pH$_e$ = 5.8 (Slayman and Slayman, 1968). The present direct measurements find a value that is almost 1 pH unit higher, which suggests that in the case of DNP, the anion may be substantially permeant. An absolute value for pH$_i$ of 7.2–7.4, reported in the present work, is in close agreement with values obtained from a variety of animal and bacterial cells by the more reliable methods of recessed-tip pH electrodes (Roos and Boron, 1981) or $^3$P-NMR (Ugurbil et al., 1979; Slonczewski et al., 1981).

*pH$_i$ Changes Produced by Weak Acids and Bases: Measurement of $\beta_i$*

Our estimate of cytoplasmic buffering capacity at normal pH$_i$ is also in good agreement with estimates for other cells, obtained either by identical methods (giant barnacle muscle fiber: Boron, 1977; snail neurone: Thomas, 1976), by weak acid/base perturbation combined with $^3$P-NMR (yeast: A. Ballarin-Denti, unpublished data), or by direct titration of de-energized cells (Micrococcus: Scholes and Mitchell, 1970; Streptococcus: Malone, 1979). A noteworthy feature of all studies on whole cells, as well as those on isolated mitochondria (Mitchell and Moyle, 1967), is the finding that $\beta_i$ increases steeply as pH$_i$ is lowered. This presumably indicates that the major buffering groups have pK$_a$'s far more acidic than normal pH$_i$, and is consistent with the idea that amino acid side chains of proteins constitute the major cytoplasmic buffer. With the exception of the histidyl residue, all side chains have pK$_a$'s outside the pH range 6–8, which would give a minimum value of $\beta_i$ at the normal pH$_i$ and maxima at the pK$_a$'s of the dominant charged amino acids (i.e., at both higher and lower pH's). It follows from this that the remarkable uniformity of $\beta_i$ over the whole phylogenetic range from bacteria to mammals would be explainable on the basis of the fundamental similarity of protein composition.

**Role of the Cytoplasmic Buffer in pH$_i$ Stabilization**

Although the concentration of buffer-bound protons exceeds by more than five orders of magnitude the concentration of free protons, the buffer cannot be viewed as true pH regulator for anything more than very small acid or alkaline assaults. Suppose, for example, a normal, steady, pumped proton
efflux of 100 pmol cm\(^{-2}\) s\(^{-1}\), as previously calculated for Neurospora (Gradmann et al., 1978); sudden cessation of the pump, with all other metabolic processes continuing, would result in decline of pH\(_i\) at the rate of 0.6 unit min\(^{-1}\) for a cell of 14 \(\mu\)m diameter and buffering capacity of 40 mM H\(^+\)/unit.

Relative Insensitivity of pH\(_i\) to Changes in pH\(_o\)

Near-independence of pH\(_i\) from pH\(_o\) makes good sense for any free-living organism, such as Neurospora, which may need to survive and grow over a wide range of external pH (C. L. and C. W. Slayman, unpublished observations). Similar insensitivity has been demonstrated for yeast and bacteria (e.g., Navon et al., 1979; Slonczewski et al., 1981). The result is nevertheless surprising in view of the generally accepted transport model for plasma membranes of these and other nonanimal cells, which includes a large passive influx of protons (Harold, 1977; Slayman, 1977; Walker, 1980). At least two of the observations reported above are not in agreement with the simple H\(^+\) conductance model. First, even when the H\(^+\) pump is inhibited, H\(^+\) does not reach electrochemical equilibrium across the membrane, as would be expected if the leak to H\(^+\) were dominant. For example, in Figs. 5A and 6, \(\Delta \mu_{H^+}\) is \(-130\) mV in the presence of each inhibitor. Second, Fig. 5 shows that when the pump is inhibited, pH\(_i\) is still independent of pH\(_o\). Thus, the transmembrane leak of H\(^+\) into the cell must be small, and the major source of cytoplasmic H\(^+\) must be internal.

Given this conclusion, there is no a priori reason pH\(_i\) should respond to pH\(_o\). The relative lack of response of pH\(_i\) is impressive as a homeostatic phenomenon, but not necessarily indicative of feedback regulation of pH\(_i\). It is possible that the small dependence on pH\(_o\) that is seen results from changes in the activity of the H\(^+\) pump, and this idea is currently under investigation.

Origin of Cytoplasmic H\(^+\)

Under aerobic conditions, protons will be generated in the cytoplasm from several sources: CO\(_2\)/H\(_2\)CO\(_3\), protein synthesis (Raven and Smith, 1976a), and Krebs-cycle acids (which are secreted in large amounts by Neurospora; C. L. Slayman, unpublished observations). A question now arises concerning the origin of cytoplasmic H\(^+\) in the presence of CN\(^-\). It seems most likely that the production of acidic glycolytic end products is responsible, since this is the widely used method of regenerating oxidized pyridine nucleotide in aerobes deprived of oxygen as an electron acceptor. Similar cases of acidosis are seen in squid axons upon addition of metabolic inhibitors (also, in the presence of outwardly directed \(\Delta \mu_{H^+}\)) (Boron and De Weer, 1976); on removal of oxygen from mammalian muscle tissue (Garlick et al., 1979); in glucose-derepressed yeast (den Hollander et al., 1981); and in E. coli (Ugurbil et al., 1979).

The total production of acid equivalents in response to CN\(^-\) can be estimated as 35 mmol/kg cell water, taking for \(\beta_i\) an average value of \((-\)50 mM H\(^+\)/unit over the pH\(_i\) range 7.0–6.3. (Net acid secretion by the cell stops in the presence of CN\(^-\) [Slayman, 1970].) The initial rate of H\(^+\) production after application of CN\(^-\) is 12 mM/min (\(dpH_i/dt = 0.3\) unit/min, \(\beta_i = [-40\) m

\] mM H\(^+\)/unit at pH\(_i\) = 7.0). This is compatible with a normal rate of glucose
use of 2.7 mM/min (Slayman, 1973), which could yield as much as 16.2 mM H+/min for complete oxidation of the sugar. Under anaerobic conditions (CN⁻), the yield of H⁺ should be less than this.

**Metabolism, the Proton Pump, and Control of pHi**

Because H⁺ entry into the cell is small, control of pHi must result from the balance achieved between metabolism (the major producer of H⁺) and the operation of the H⁺ pump (the site of H⁺ ejection from the cell). Which of these two processes is regulated to stabilize pHi? At present we do not have sufficient information to give a rigorous answer to this question, although one observation reported here indicates a dominant role for metabolism: whether the H⁺ pump is working normally (Fig. 5A) or is severely curtailed (Fig. 6), recovery of pHi after CN⁻ treatment takes place at the same rate and to the same extent. This suggests that under some circumstances, metabolism can actually consume H⁺, as would occur, for example, if acidic glycolytic products were converted to neutral compounds.

On the other hand, when an acid load is imposed with butyric acid (Fig. 8), fairly large increases in pump activity have been recorded (Sanders et al., 1981), and there is little effect on oxidative metabolism under these conditions (D. Sanders, unpublished experiments). The nature of the response to an acid load therefore seems to depend on the particular form that the load takes. In other words, proton concentration is probably not the only error signal involved in regulation of the pump and metabolism.

**Control of pHi in Relation to Other Physiological Parameters**

The requirement for additional error signals can be rationalized by considering at least two functions of the proton pump and oxidative metabolism that might act to compromise the role of each in pHi control.

(a) ΔµH⁺. Uptake of many solutes in microorganisms is energized by ΔµH⁺, and the present work provides some evidence that ΔµH⁺ might itself be tightly controlled, at least in the pH range more acid than the normal growth pH. Fig. 11 is a replot of the data in Fig. 3, and shows that, with a slight lag, changes in membrane potential act to compensate, or even overcompensate, for the change in pHi. The result is a tendency for ΔµH⁺ to return to near the control value after a transient overshoot and undershoot. The time course of this slower change in membrane potential is shown in Fig. 3 to correspond with that for the small change in pHi, and on the basis of previous current-voltage measurements of the plasma membrane with acid pHi, probably originates from an increase in membrane leak conductance (Sanders et al., 1981).³

³The membrane potential can be replotted as a function of pHi (over the range where pHi has stabilized) to yield an apparent dependence of membrane potential on pHi of 120 mV/unit. This value is in reasonable agreement with that published earlier (Sanders et al., 1981) where pHi was lowered with butyric acid—a value of 170 mV/unit can be derived from those data.
(b) ATP. The requirement for stable intracellular ATP pools apparently must also involve modulation of both the H\(^+\) pump and oxidative metabolism, since the pump is a major consumer of ATP (Slayman et al., 1973). In this context, a pH-independent mechanism for feedback inhibition of respiration during blocking of the pump by vanadate (Fig. 6) is required, since [H\(^+\)]\(_i\) is not elevated and cannot serve as an error signal. The situation is reminiscent of that in animal cells, where the dominant electrogenic pump (the Na\(^+\)K ATPase) may exert negative feedback on oxidative metabolism, via the ATP/ADP ratio (Whittam, 1961; Blond and Whittam, 1964; Balaban et al., 1980).

It is probably, therefore, too oversimplified an approach to look for a unique regulator of pH\(_i\) in organisms where the H\(^+\) pump is intimately involved in other metabolic processes; ultimately the maintenance of constant pH\(_i\) will rely on the manner in which the pump is integrated into those processes. Kinetic investigations in the alga *Nitella* have already provided evidence for the existence of a feedback loop between metabolism and the proton pump (Hansen, 1978, 1980, 1982) and the challenge will now be to assign biochemical identity to such links.
APPENDIX

Correction of Recorded pH for Slow Electrode Response to \( V_m \) and pH

Background

In general, the pH traces \([\text{pH}_\text{traces}]\) that represent the primary data in most of the experiments described above should show three distinct components during experimental manipulation:

\[
\Delta \text{pH}_\text{traces} = \Delta \text{pH}_\text{true} = F_1[\Delta \text{pH}_\text{true}] + F_2[\Delta V_m(t)] - \Delta V_m(t),
\]

in which \( \Delta \text{pH}_\text{true} \) and \( \Delta V_m(t) \) represent the variations of internal pH and membrane potential, respectively, with time \( t \) after the onset of an experimental change. \( F_1 \) and \( F_2 \) are the fractions \((\leq 1)\) of \( \Delta \text{pH}_\text{true} \) and \( \Delta V_m(t) \) that are reported by the pH electrode; and \( \Delta \text{pH}_\text{true} = \Delta \text{pH}_\text{traces} \) is the measured change of the pH trace. The simple term \( \Delta V_m(t) \) is subtracted in Eq. A1, because the \( V_m \) electrodes (used as intracellular references in the differential recording procedure) were fast enough to report changing membrane potentials accurately in all of the experiments. In correcting for errors of \( F_1 \), the input function \([\Delta \text{pH}_\text{true}]\) must be determined, but in correcting for errors of \( F_2 \), the input function \([\Delta V_m(t)]\) is known from the \( V_m \) electrode. This means that correction for \( F_2 \) must be made before correction for \( F_1 \) can be. The correction procedure outlined below uses a segmental linear algorithm, rather than a mathematically analytic approach, because the functions \( pHi(t) \) and \( V_m(t) \) are complicated in usual practice. (The procedure can readily be adapted for computer use and “on-line” correction of traces if that is desired.)

Correction for \( F_2 \): pH Electrode Slow with Respect to \( \Delta V_m \)

For both correction procedures, we treat the input functions as piecewise linear functions. In the case of \( \Delta V_m(t) \),

\[
\Delta V_m(t) = V + \alpha_s t,
\]

which is transformed by the pH electrode into

\[
F_2[\Delta V_m(t)] = \left( V - \frac{\alpha_v}{\alpha_e} \right) [1 - \exp(-\alpha_v \Delta t)] + \alpha_e t.
\]

For the sake of simplicity in what follows, the notation for \( F_2 \) will be abbreviated slightly, letting \( F_2(t) = F_2[\Delta V_m(t)] \). Then, for any increment of time \((\Delta t)\) in which \( \Delta V_m(t) \) is linear, the following identity holds:

\[
F_2(t + \Delta t) = \left[ \Delta V_m(t) - F_2(t) - \frac{\alpha_v}{\alpha_e} \right] [1 - \exp(-\alpha_v \Delta t)] + \alpha_e \Delta t + F_2(t).
\]

This equation can be made the basis of an iterative correction procedure that commences at any point for which \( F_2(t) \) is known. Usually, \( F_2(t = 0) \) is known to be zero, and if from there uniform time intervals \((\Delta t)\) are selected, Eq. A4 can be rewritten in a more clearly iterative form:

\[
F_2[(n + 1)\Delta t] = \left[ \Delta V_m(n\Delta t) - F_2(n\Delta t) - \frac{\alpha_v}{\alpha_e} \right] [1 - \exp(\alpha_e \Delta t)]
\]

\[+ \alpha_e \Delta t + F_2(n\Delta t), \quad n = 0, 1, 2 \ldots \]
where $\alpha_e$ is an implicit function of $n\Delta t$ and should be written $\alpha_e(n\Delta t)$, and the
difference $\Delta V_m(n\Delta t) - F_2(n\Delta t)$ can be abbreviated as $V(n\Delta t)$. Eq. A5 says that
$F_2[\Delta V_m(t)]$ can be calculated in a point-to-point fashion by taking each successive
value of $F_2(n\Delta t)$ as the graph “origin” and estimating the next value, $F_2[(n + 1)\Delta t]$,
from the measured value and slope $(\alpha_e)$ of the voltage curve at $t = n\Delta t$, plus
the previously calculated value of $F_2(n\Delta t)$.

Returning to Eq. A1, the error in $\Delta pH_{\text{intra}}$, which is due to changes of membrane
potential, is $F_2[\Delta V_m(t)] - \Delta V_m(t)$, or simply $-V(n\Delta t)$. Letting the ensemble of $V(n\Delta t)$
be represented by $V(t)$, $\Delta pH_{\text{intra}}$ must be corrected for changes of membrane
potential by addition of $V(t)$. Thus,

$$\Delta_{\text{m}} pH_i \text{ (corrected for } \Delta V_m) = \Delta_{\text{m}} pH_i(t) + V(t). \quad (A6)$$

**Correction for $F_1$: pH Electrode Slow with Respect to $\Delta pH_i$**

In this description, it must be assumed that any required correction for membrane
potential has been made, so that Eq. A1 becomes

$$\Delta_{\text{m}} pH_i(t) = F_1[\Delta pH_i(t)], \quad (A7)$$

and the task is to estimate $\Delta pH_i(t)$ from $\Delta_{\text{m}} pH_i(t)$. As before, the input function is
treated as a piecewise linear function, in which each segment can be represented by

$$\Delta pH_i(t) = P + \alpha_p t, \quad (A8)$$

and is transformed by the pH electrode into

$$F_1[\Delta pH_i(t)] = \left( P - \frac{\alpha_p}{\alpha_e} \right) [1 - \exp(-\alpha_e t)] + \alpha_p t, \quad (A9)$$

with different values of $P$ and $\alpha_p$ [$P(n\Delta t)$ and $\alpha_p(n\Delta t)$] for each distinct segment. (Eqs.
A8 and A9 are completely analogous to Eqs. A2 and A3.)

Abbreviating $F_1[\Delta pH_i(t)]$ as $\tilde{F}_1(t)$, one can readily demonstrate from Eq. A9 that

$$P = \left. \frac{d\tilde{F}_1(t)/dt}{\alpha_e} \right|_{t=0} \quad (A10)$$

and

$$\Delta pH_i(t) = \tilde{F}_1(t) + \frac{d\tilde{F}_1(t)/dt}{\alpha_e}. \quad (A11)$$

A procedure for approximating the true time course of $\Delta pH_i$ from Eq. A11 is as
follows. Divide the time axis into a suitable number of segments, $\Delta t$ in length, over
which $\Delta pH_i(t)$ can be presumed linear. Determine the value $[\Delta_{\text{m}} pH_i(n\Delta t) = F_1(n\Delta t)]$
and the slope $[d\tilde{F}_1(n\Delta t)/dt]$ at the beginning of each segment. With $\alpha_e$ known from
control experiments, calculate $\Delta pH_i$ as

$$\Delta pH_i(n\Delta t) = F_1(n\Delta t) + \left. \frac{d\tilde{F}_1(t)}{dt} \right|_{t=n\Delta t} = F_1(n\Delta t) + P(n\Delta t), \quad (n = 0, 1, 2\cdots). \quad (A12)$$

This procedure (with Eq. A12) is conceptually the inverse of that used to calculate
$F_2(n\Delta t)$ in Eq. A5 above, but is remarkable in being much simpler to compute than
Eq. A5. It is, however, intrinsically less reliable than the computation of $F_2$, since small values of $\alpha_e$ tend to obliterate point-to-point variations of $\alpha_p$.

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