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The use of nuclear magnetic resonance for examining pH in living systems

Introduction

In what has become a classic paper, Moon and Richards (1973) demonstrated that phosphate compounds which occur naturally in cells could be used to measure intracellular pH (pH_i) non-invasively using ^{31}P nuclear magnetic resonance (NMR). The ability to probe a quantity as integral to cellular function as pH in living, unperturbed cells offered the promise of vast new insights into cellular metabolism. Indeed, hundreds of papers have been published as a direct result of Moon and Richards' original contribution, and the indirect impact of their work on the development of other applications of NMR to biology may be even greater. Today, NMR methods of pH measurement are common in clinical as well as in academic settings. In many cases, ^{31}P spectra are collected routinely as a means of assessing the energetic and acid–base status of a plant or animal sample during an experimental procedure. Many new NMR pH indicator compounds have been characterized, both endogenous and exogenous, which probe both pH_i and extracellular pH (pH_e). In addition, a wide variety of experimental protocols has been developed to examine acid–base balance non-invasively, including spectroscopy of isolated tissues and organs, in-vivo localized spectroscopy, and imaging methods.

This brief review discusses the principles of pH measurement using NMR and highlights many of the pH indicator compounds that are currently employed. The strengths and weaknesses of NMR measurement of pH are discussed, as are sources of error. Finally, applications to comparative animal and plant physiology are described to provide practical examples of the kinds of experiments that are possible and information that can be obtained using this methodology. This is in no way a comprehensive review of the literature, and is intended to provide an assessment of the current standing of NMR as an experimental tool in acid–base studies.

Practical considerations

The NMR signal is derived from the tiny current that is induced in a transceiver coil by atomic nuclei precessing about a strong magnetic field. The precessional, or resonance, frequency is determined by the magnetogyric ratio of the nucleus (highest for ^1H), and the field strength of the magnet. This frequency is also modified slightly by the electron shielding a nucleus experiences, such that nuclei in different chemical environments have peaks that appear at different positions in the NMR frequency spectrum. This chemical modification of resonance frequency is known as chemical shift. Chemical shift has units of Hz, but is usually normalized to parts per million (ppm).

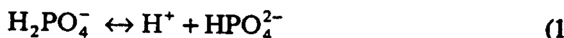
Because the detected current is weak, NMR is a very insensitive technique. This shortcoming means that spectra cannot usually be observed after a single excitation. Instead, a number of scans must be acquired and averaged together, which means that it may take several seconds to tens of minutes to acquire a final spectrum. For the purposes of measuring pH, the ability to acquire a well-resolved spectrum over a short time is highly desirable, particularly when the pH is changing rapidly. Since higher resonance frequencies equate to greater sensitivity, the use of nuclei with large magnetogyric ratios and of high field magnets improves time resolution for a given sample. However, sensitivity is also a function of the number of nuclei within the transceiver coil, which is directly related to the volume of the sample and the concentration of the metabolite being observed. Although it is difficult to put an absolute limit on the minimum volume and concentration which will yield a spectrum with an adequate signal-to-noise ratio (SNR), in general, sample volumes for biological applications must be of the order of a few cubic millimetres and concentrations must be greater than 0.1 mM.

Detailed descriptions of the fundamentals of NMR and its applications in biology have been covered in several recent reviews which focus on comparative physiology (Ellington & Wiseman, 1989; Wasser, Lawler & Jackson, 1996) and plants (Ratcliffe, 1994), as well as the more complete text by Gadian (1995).

Principles of NMR pH measurement

NMR pH measurement is possible because some resonances arise from chemical species that are in fast-exchange equilibrium between acid and base forms. Differences in electron shielding of the protonated and unprotonated nucleus result in the acid and base forms having slightly

different chemical shifts. In the case of the commonly used pH indicator inorganic phosphate (Pi), the equilibrium is as follows:



$$H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-} \quad (1)$$

The chemical shift of the acid form, δ_{acid}, is approximately 2.6 ppm less than that of the base form, δ_{base}. Because there is fast-exchange relative to the NMR time scale between the two forms, a single peak is observed for P_i which has a chemical shift, δ_{obs}, that is a function of the concentrations of the acid and base forms (Fig. 1A). The equilibrium concentrations of each form are determined by the pK_a (approximately 6.6 for P_i), and the pH is calculated from the Henderson–Hasselbalch equation:

$$pK_a = -\log_{10}\left(\frac{[H^+][HPO_4^{2-}]}{H_2PO_4^-}\right) \quad (2)$$

$$pH = pK_a + \log_{10}\left(\frac{[HPO_4^{2-}]}{[H_2PO_4^-]}\right) \quad (3)$$

For the NMR experiment, the Henderson–Hasselbalch equation is expressed in terms of chemical shift:

$$pH = pK_a + \log_{10}\left(\frac{\delta_{obs} - \delta_{acid}}{\delta_{base} - \delta_{obs}}\right) \quad (4)$$

The pH can then be calculated using NMR by measuring δ_{obs} in a spectrum (relative to the chemical shift of a pH-independent reference peak) and knowing the pK_a, δ_{acid}, and δ_{base} for the pH indicator species. In practice, the three unknowns in equation 4 are obtained empirically by measuring the chemical shift of the indicator species over a range of pH values in a model solution and fitting equation 4 to the experimental data (Fig. 1B). If two dissociable protons are present ($H_2A \leftrightarrow HA^- + H^+ \leftrightarrow A^{2-} + 2H^+$), the observed chemical shift is a function of the chemical shift of the fully protonated form, δ_1, the monoprotonated form, δ_2, and the unprotonated form, δ_3, and the two pK_a values, pK_1 and pK_2 (Robitaille et al., 1991):

$$\delta_{obs} = \frac{\delta_1}{1+10^{pH-pK_1}+10^{2pH-pK_1-pK_2}} + \frac{\delta_2}{1+10^{pK_1-pH}+10^{pH-pK_2}} + \frac{\delta_3}{1+10^{pK_2-pH}+10^{pK_1+pK_2-2pH}} \quad (5)$$

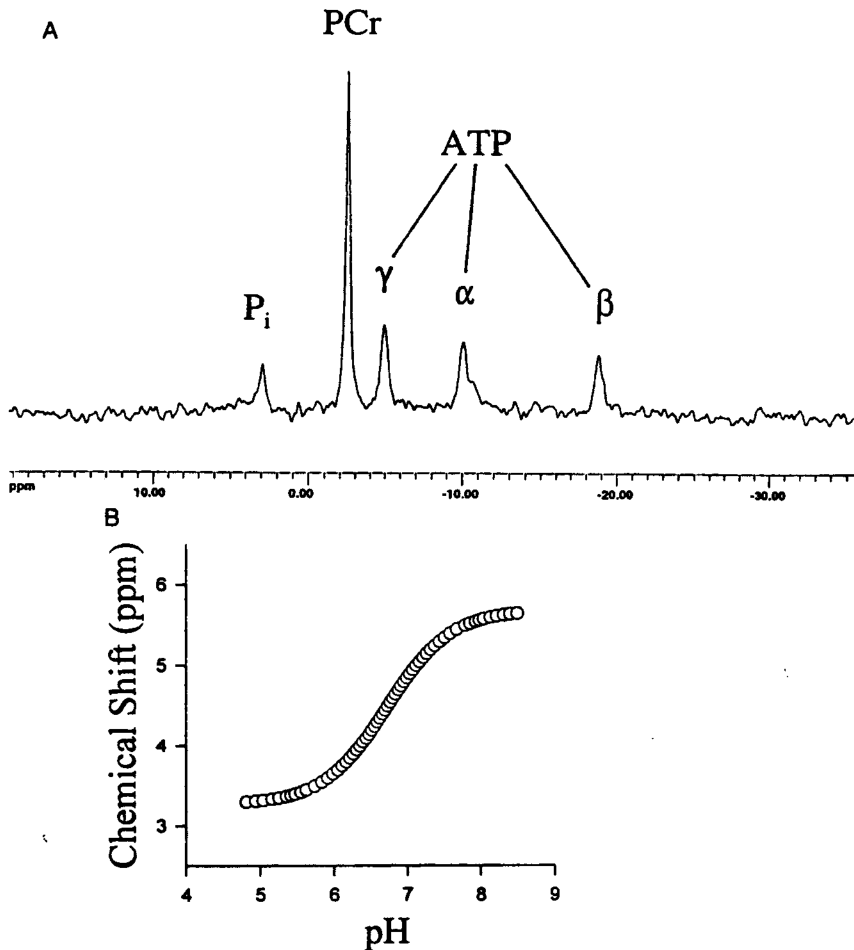


Fig. 1. **A**. Typical ^{31}P-NMR spectrum from skeletal muscle. The chemical shift of the P_i peak changes according to the pH_i (as does the chemical shift of the ATP peaks). The chemical shift of the phosphocreatine (PCr) peak is pH independent over the physiological pH range and is often used as an internal chemical shift reference. **B**. Relationship between pH and ppm for P_i calculated from equation 4 (see p. 00) using $\delta_{acid} = 3.27$, $\delta_{base} = 5.69$, and $pK_a = 6.72$ (Van Ginneken et al., 1995).

For most pH indicators used in biological applications, however, the complication of multiple pK_as can usually be avoided.

NMR pH indicators

The minimal criteria for a useful NMR pH indicator are: (1) adequate SNR, (2) pK_a in the physiological pH range, and (3) large dynamic range of chemical shift ($\delta_{acid} - \delta_{base}$). The SNR of the indicator must be high enough so that a well-resolved chemical shift can be measured without noise spikes contributing significantly to the peak shape (Madden et al., 1991). Also, the pH range to be examined should be within approximately ±1 pH unit from the pK_a because otherwise the pH-dependent changes in chemical shift become unacceptably small. Finally, accurate measurement of the indicator's chemical shift requires a suitable pH-independent chemical shift reference peak.

The most important and widely used endogenous indicator of pH_i is the resonance arising from P_i as observed in ^{31}P-spectra (Moon & Richards, 1973; reviewed in Rudin & Sauter (1992) and van den Thillart & van Waarde (1996); see Fig. 1). This naturally occurring pH_i probe has a pK_a of approximately 6.6 and it occurs in sufficient concentration to be observable in many tissue types. The phosphagen peak, which is phosphocreatine (PCr) in vertebrates, is generally used as a pH-independent chemical shift reference. A disadvantage of using P_i is the dependence of its chemical shift on the concentration of free Mg^{2+} (see below). Besides pH_i information, the ^{31}P-spectra of biological samples allows assessment of the energetic condition of the tissue of interest by measuring the relative peak areas (= relative concentrations) of ATP, PCr, and P_i, and by calculating the free $[Mg^{2+}]$ from the chemical shifts of the ATP peaks (Gupta, Benovic & Rose, 1978; Gupta & Moore, 1980; Kushmerick et al., 1986; Douman & Ellington, 1992; Combs & Ellington, 1995). Adverse physiological conditions almost always affect the relative concentrations of the high-energy phosphate compounds, either directly or indirectly, because ATP-dependent compensatory mechanisms are usually activated (for an extensive review of ^{31}P-NMR energetic studies see van den Thillart & van Waarde, 1996). This ability to assess the physiological 'health' of a tissue is a tremendous advantage of ^{31}P-NMR, particularly when perfused organs or tissues are being used.

The P_i-NMR signal is usually well resolved in vertebrate and plant tissues, but it has been shown to decrease to immeasurable levels following aerobic exercise in skeletal muscle (Pan et al., 1988). However, most physiological perturbations actually lead to an increase in the

intensity of the P_i resonance due to net phosphagen hydrolysis. In invertebrates which utilize phosphagens other than PCr (for example phosphoarginine), the equilibrium constant of the phosphotransferase reaction is substantially reduced relative to that of the creatine kinase reaction (Ellington, 1989), yielding a much smaller P_i peak at high ATP/ADP ratios which may be insufficient for pH measurement. Robitaille et al. (1991) conducted a thorough analysis of the pH characteristics of many alternative endogenous pH-sensitive phosphorus-containing compounds that can be observed with ^{31}P-NMR. However, the visibility of other phosphorus metabolites in ^{31}P-spectra is largely tissue specific.

In cases in which a suitable endogenous ^{31}P indicator resonance is not available, a useful alternative is the exogenous pH probe, 2-deoxyglucose-6-phosphate (2DG6P), as first suggested by Navon et al. (1977), which has a slightly lower pK_a (approximately 6.2) than P_i. From the perfusion medium, the substrate 2-deoxyglucose enters the cell via the glucose transporter and is phosphorylated by hexokinase to form 2DG6P, which is not further metabolized and accumulates in the cell. 2DG6P is an exclusively cytosolic pH indicator, unlike P_i, which may have a mitochondrial component (Garlick, Soboll & Bullock, 1992). However, pH measurements using both indicators have been in very good agreement since usually most of the P_i is cytosolic (Hamm & Yue, 1987; Wiseman & Ellington, 1989; Soto et al., 1996). A disadvantage of using 2DG6P is that it traps phosphate and causes a reciprocal reduction in the phosphagen concentration, and it inhibits glycolysis at high concentrations (Allen et al., 1985).

Measurement of pH using 2DG6P and the vast majority of phosphorus-containing compounds is hindered by their fairly low pK_as, which are not suitable for measuring pH in the alkaline physiological range (Robitaille et al., 1991). Notable exceptions include several phosphonate compounds. Szwergold, Brown and Freed (1989) demonstrated the utility of several 2-amino-phosphono-carboxylic acid compounds which have pK_as ranging from 6.9 to 7.6. Ideally, these compounds should be fit to equation 5 because of the presence of two titratable protons, but in practice a titration curve which does not extend to the alkaline extremes can be fit to equation 4 for a single pK_a. In addition, phosphonates which do not cross cell membranes can be used as pH_e probes, such as phenylphosphonate (Meyer, Brown & Kushmerick, 1985; Graham, Taylor & Brown, 1994), methylphosphonate (which is permeable in some cells; DeFronzo & Gillies, 1987) and 3-aminopropyl phosphonate (Gillies, Liu & Bhujwalla, 1994), all of which are non-toxic and have pK_as ranging from 6.9 to 7.6.

Pan et al. (1988) demonstrated that the imidazole protons on C-2 and

C-4 of carnosine (β-Ala-His) could be observed in ^{1}H-spectra as indicators of pH in vertebrate skeletal muscle. The high NMR sensitivity of protons makes this method advantageous when spectra need to be collected over a short time period, such as under conditions when pH is changing rapidly. Further, the chemical shifts of the imidazole resonances are not sensitive to the concentration of intracellular Mg^{2+}, giving them a distinct advantage over P_i (Pan et al., 1988). However, ^{1}H-spectroscopy requires the suppression of the intense water resonance, which will otherwise fill up the NMR digitizer and limit the dynamic range of the resonance of interest (and, hence, SNR). While soluble carnosine is present in fairly high concentrations in vertebrate muscle (Okuma & Abe, 1992), it is generally absent or present at very low concentrations in invertebrates (Morris & Baldwin, 1984; Wiseman & Ellington, 1989).

Recently, Aime et al. (1996) demonstrated that ^{1}H-NMR could be used to observe the pH sensitivity of paramagnetic complexes of the lanthanide, ytterbium, with the macrocyclic ligand DOTP (1,4,7,10-tetra-azacyclododecane-N,N′,N″,N‴-tetrakis methylenephosphonic acid). Nuclei that are bound to paramagnetic centers can undergo extremely rapid relaxation, or dramatic changes in chemical shifts that far outweigh the typical diamagnetic shifts observed in most spectra. The pH-sensitive Yb(DOTP) complex has multiple pK_as, but the relationship between chemical shift and pH is linear over a pH range of 5.0 to 7.5. Aime et al. (1996) proposed this compound as an exquisitely sensitive in-vivo pH_e indicator because of the large changes in chemical shift that are induced by the paramagnetic center for only slight changes in pH. The potential for enhanced sensitivity to pH changes makes this class of compounds intriguing, but their potential toxicity may ultimately limit their application in biology (Gadian, 1995).

^{13}C-NMR is being used increasingly to examine metabolism because specific carbons in a metabolite can be selectively enriched with the ^{13}C isotope by applying the proper substrate, and the fate of the label can be followed simultaneously through multiple metabolic pathways in living tissue (reviewed in Sherry & Malloy, 1996). Using ^{13}C-enriched substrates is usually necessary because of the extreme insensitivity of the ^{13}C nucleus (0.018 percent as sensitive as ^{1}H), which results from the low natural abundance of this isotope (1.1 percent of total carbon) and its small magnetogyric ratio. Chacko and Weiss (1993) showed that perfusion of a rat heart with glucose which was ^{13}C enriched at the C-1 position led to an enhanced peak in the ^{13}C spectrum for the C-3 of *sn*-glycerol-3-phosphate. This peak was pH sensitive, with a pK_a of 6.2. Measurement of pH_i using this peak allows the simultaneous measure-

ment of intermediary metabolism and pH_i (using the alanine methyl protons as a chemical shift reference). Further, because *sn*-glycerol-3-phosphate is a glycolytic product, it is exclusively cytosolic, and offers some advantage over the P_i resonance which may be observed in the cytosol and in the mitochondria.

^{19}F-NMR offers sensitivity nearly equal to that of protons (83 percent of ^{1}H), and has the advantage that no naturally occurring resonances exist which could cause peak overlap in the ^{19}F spectrum. The obvious disadvantage of using ^{19}F-labeled pH indicator compounds is that they must be loaded and trapped in the cell. Taylor and Deutsch (1983; 1988; 1989) have developed a number of pH-dependent fluorinated compounds, the most useful of which are the fluorinated α-methylamino acids. These compounds do not readily enter many cell types but can be loaded as methyl ester precursors, which are taken up by cells and hydrolysed by intracellular methyl esterases to yield the amino acid pH indicator (Taylor & Deutsch, 1983). Cells lacking methyl esterase activity have been shown to cleave the *p*-chloropH$_e$nyl ester precursor of one of these pH indicators, difluoromethylalanine (Taylor & Deutsch, 1988). This pH indicator is one of the most useful because its pK_a is 7.3, it has two peaks with a pH-dependent spacing (so no chemical shift reference is needed), and it shows a large dynamic range of chemical shift, making it very sensitive to pH changes. This class of compounds is also non-toxic to cells (Taylor & Deutsch, 1983).

Sources of error

The most important source of error in NMR pH measurement results from inaccurate calibration curves which are generated from titration solutions that do not reflect the conditions in the compartment of interest. This is particularly problematic when measuring pH_i because of the uncertainty associated with the composition of the cytosol in living cells. Inappropriate model solutions lead to errors in the empirically determined parameters, pK_a, δ_{acid}, and δ_{base}, which ultimately results in systemic errors in the calculation of pH_i. The principal factor affecting the relationship between chemical shift and pH_i for all NMR pH indicators is ionic strength, which in the cytosol is primarily a result of differences in the $[K^+]$ (Roberts, Wade-Jardetzky & Jardetzky, 1980; Taylor & Deutsch, 1983; Robitaille et al., 1991). For P_i, the free $[Mg^{2+}]$ also induces changes in the behavior of the chemical shift with pH (Roberts et al., 1980; Robitaille et al., 1991). Great care should therefore be taken in preparing a titration solution, particularly when experimental conditions might cause the ion concentration to change during pH_i

measurement, such as occurs for free Mg^{2+} during acidosis (Combs & Ellington, 1995). Because of the interaction between pH_i and free $[Mg^{2+}]$, Williams, Mosher and Smith (1993) developed a protocol for determining both quantities simultaneously from the chemical shift differences between the three ATP peaks. Temperature also has a strong effect on the pH–chemical shift relationship (Kost, 1990), but this is easily controlled by generating calibration curves at the experimental temperature.

Ackerman et al. (1996) created a general model for pH dependence of the chemical shift of the indicator resonance that also accounts for the pH dependence of the reference peak. The traditional model (equation 4) assumes no pH dependence of the reference species, and is invalid when the pH approaches the pK_a of the reference peak (which may occur during extreme pH excursions). The usual simplification is acceptable for cases in which the pH does not approach the reference pK_a, when truly non-titratable reference probes are used, or when external references are used (for example a reference within the NMR coil in a separate capillary tube).

Graham, Taylor and Brown (1994) demonstrated the error inherent in the non-linear relationship between pH and chemical shift. This problem is exacerbated as the spread in the true pH distribution increases and as the offset of the pH from the pK_a of the indicator increases. This effect is manifested as a skewing of the NMR peak as the true mean pH changes, or as a falsely indicated change in the NMR-derived pH as the standard deviation of the true pH distribution increases (without a change in the true mean pH). Graham et al. (1994) offered a remedy for this problem which consists of: (1) converting the ppm axis to pH using the Henderson–Hasselbalch equation, and (2) dividing the intensity at each point in the spectrum by the derivative of the Henderson–Hasselbalch equation with respect to chemical shift:

$$\frac{dpH}{d\delta_{obs}} = \frac{d}{d\delta_{obs}}\left[pK_a + \log\left(\frac{\delta_{acid} - \delta_{obs}}{\delta_{obs} - \delta_{base}}\right)\right] = \frac{\delta_{base} - \delta_{acid}}{(\delta_{acid} - \delta_{obs})(\delta_{obs} - \delta_{base})} \quad (6)$$

This latter treatment accounts for the non-linearity between ppm and pH and yields spectral peaks that accurately reflect the distribution of pH in a tissue.

It should be emphasized that, despite the uncertainties discussed above, evidence derived from comparisons with other pH measurement methods strongly suggest that NMR measurements of pH_i and pH_e usually have an absolute accuracy ranging from 0.05 to 0.1 pH units, and

changes in pH can be measured with a precision of ± 0.03 pH units (Madden et al., 1991; Gadian, 1995).

Applications in comparative physiology

NMR measurement of pH has been widely employed in comparative physiology, almost exclusively by using ^{31}P-spectroscopy and either the P_i or 2DG6P resonance as the pH indicator (reviewed in Ellington & Wiseman, 1989; van den Thillart & van Waarde, 1996; Wasser et al., 1996). Several experimental modes have evolved which depend on the type of spectrometer available and on the type of experiment: (1) spectroscopy of perfused or superfused tissues or organs, (2) spectroscopy of very small, homogeneous tissue samples, (3) whole-animal spectroscopy, including localized spectroscopy, and (4) spectroscopic imaging methods.

^{31}P-NMR has been used extensively to monitor pH_i transients in perfused or superfused molluscan muscle preparations during contractile activity (Chih & Ellington, 1985), environmental anoxia (Barrow, Jamieson & Norton, 1980; Ellington, 1983; Graham & Ellington, 1985), and experimentally induced acidifications (Ellington, 1985; Combs & Ellington, 1995). Changes in the intracellular free [Mg^{2+}] as a function of changes in pH_i have been examined in superfused muscles from horseshoe crab (Douman & Ellington, 1992) and from whelk (Combs & Ellington, 1995). Among vertebrates, pH_i has been examined in perfused turtle hearts during anoxia and lactic acidosis (Wasser et al., 1990; Jackson et al., 1991). These methodologies are useful for attaining global pH within a physiologically relevant whole tissue or organ, but cellular heterogeneity and the diffusive time lags associated with larger tissues elicit some disadvantages for certain types of experiments.

Dubyak and Scarpa (1983) developed a probe with a small microsolenoid transceiver coil (2.2 mm ID) which could be used to obtain phosphorus spectra from a single, cannulated barnacle (*Balanus nubilis*) depressor muscle fiber. This design removed potential variation resulting from cellular heterogeneity of multicellular tissue preparations. Hamm and Yue (1987) altered this design to incorporate a flow-through superfusion system to examine individual cells of the same muscle, and variations of this basic design have been used for examining pH in small tissue preparations or single cells (Wiseman & Ellington, 1989; Wiseman, Moerland & Kushmerick, 1993; Kinsey & Ellington, 1995; 1996; Combs & Ellington, 1995). The reduced diffusive distances across these small tissue preparations make them easy to keep well oxygenated, and more suitable for certain experimental

manipulations that require rapid equilibration of the medium across th tissue. This latter advantage of small preparations has been exploited t measure buffering capacity in molluscs by measuring pH_i transient during acidification with the weak acid DMO (5,5 dimethyloxazolidine-2,4-dione; Wiseman & Ellington, 1989; Kinsey & Ellington, 1995) and CO_2 (Zange, Grieshaber & Jans, 1990), as well a by employing NH_4Cl prepulses (Hamm & Yue, 1987; Zange et al 1990). These experiments can also be used to examine the rate of io exchange as measured by the slope of the recovery of pH_i followin acidification (Hamm & Yue, 1987; Zange et al., 1990; Kinsey & Elling ton, 1995).

Figure 2 shows examples of such experiments using DMO an NH_4Cl prepulse methods, and illustrates the need to acquire spectr

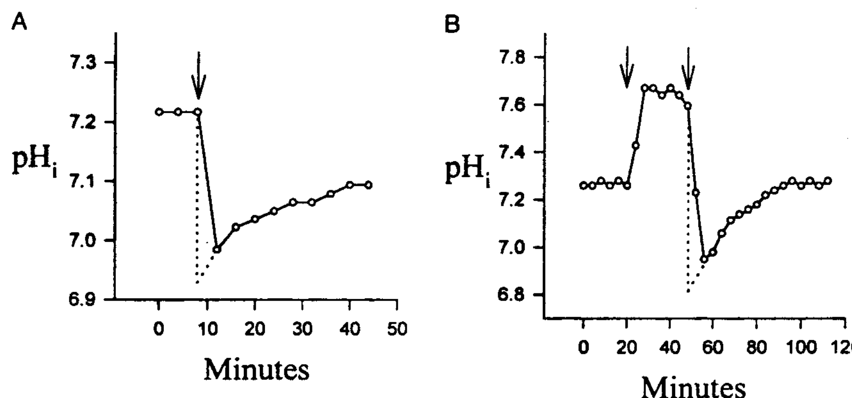


Fig. 2. **A.** DMO acid pulse experiment using a small strip of cardiac muscle (2 mm diameter) from the whelk *Fasciolaria tulipa*. The arrow indicates addition of 60 mmol DMO. **B.** NH_4Cl prepulse experiment in cardiac muscle from the whelk *Busycon contrarium*. The first arrow indicates the addition of 20 mmol NH_4Cl, which causes an alkalinization of the pH_i. The second arrow indicates clearance of extracellular NH_4Cl, which causes an intracellular acidification due to dissociation of NH_4^+ into NH_3 (which rapidly diffuses out of the cell) and H^+ (which stays in the cell). The rapid acidification in both experiments can be used to calculate buffering capacity, and the recovery of pH_i following acidification can be used to measure rates of 'proton pumping'. In both experiments, diffusive time lags are minimized by using small tissue preparations. However, the inability of NMR to collect data at a single point in time causes an underestimate of the actual acidification (dashed lines).

rapidly during periods of fast pH change, as well as to have rapid equilibration of the medium across the tissue. Even when using these very small preparations, there is an underestimate of the pH_i drop during the rapid acidification (dashed line) which results from the inability of NMR to sample a 'point in time'. As discussed earlier, the NMR signal (and hence the time resolution) is improved if either the sample size or the magnetic field strength is increased. Kinsey and Ellington (1996) made use of a very high-field spectrometer (14 Tesla or 600 MH for ^{1}H) to examine lactate transport and its effect on pH_i in individual muscle fibers from lobster. ^{1}H-spectra (for lactate measurement) and ^{31}P-spectra (for pH_i measurement – unpublished results) could be acquired every 15 seconds. Increased availability of high-field magnets, as well as continued improvements in microcoil design (Wiseman et al., 1993; Webb & Grant, 1996) should provide greater opportunities for examining small tissue preparations with excellent time resolution.

^{31}P-spectroscopy was used by Busa and Crowe (1983) to evaluate the relationship between cellular acidification and the onset of dormancy in suspensions of *Artemia* embryos. However, this interesting application is not suitable for most whole-animal studies because pH information is usually desired from a particular region or tissue in the body. One approach is to position the appropriate portion of the body within the sensitive volume of the probe's transceiver coil. Using this methodology, pH_i excursions were monitored in lugworm body wall muscles during environmental anoxia (Kamp & Juretschke, 1989) and changes in the pH of the medium (Juretschke & Kamp, 1990), in shrimp abdominal muscle during contraction (Thebault, Raffin & Le Gall, 1987), in fish muscle during movement (Chiba et al., 1990), and in salamander tail muscle during changes in ambient temperature (Johnson et al., 1993). One of the more interesting applications of this approach were the studies of Johnson et al. (1993) and Hitzig et al. (1994) which examined the fractional dissociation of imidazole (α-imidazole) in newt and lungless salamander tail muscle. These authors were testing the hypothesis that α-imidazole does not change with temperature as suggested by the alphastat hypothesis. Direct measurement of a-imidazole was achieved using ^{1}H-spectroscopy of the dipeptide carnosine (Pan et al., 1988) over a temperature range of 10–30 °C. Although pH_i changed with temperature, α-imidazole was found to be constant in the newt, as predicted by the alphastat hypothesis. This was not the case for the lungless salamander, which showed temperature dependent changes in α-imidazole.

A more precise method of attaining spectra from a localized region is to place a surface coil against the region of the body from which the

spectrum is desired. The major shortcoming of surface coils is that they do not excite nuclei homogeneously across the entire region sampled in the spectrum, meaning that the boundaries of the localized regions are 'fuzzy' and do not contribute equivalently to the final spectrum. However, surface coils have been used with great success. Rees et al. (1991) used this approach to demonstrate the acidification of tissues in the land snail *Oreohelix strigosa* which accompanies estivation. Fan, Higashi and Macdonald (1991) examined pH_i in the phasic adductor muscle of the bivalve *Mytilus edulis* during emergence-induced anoxia by using a surface coil placed on one of the animal's valves. Wegener, Bolas and Thomas (1991) used the NMR transceiver coil as a harness to hold a locust in place while they monitored pH_i in vivo from active flight muscles. Fishes make excellent NMR specimens if they can be properly immobilized, because they have large regions of muscle of homogeneous fiber type. Van den Thillart et al. (1989) developed a flow-through NMR probe for maintaining living fishes which contained an inflatable bladder inside the sample chamber to immobilize the fish by pressing it against the chamber wall. A surface coil positioned on the outside wall of the chamber was used to collect ^{31}P-spectra from homogeneous regions of epaxial muscle. This methodology has been used extensively to examine pH_i (as well as energetics) in fish muscle in vivo under a variety of experimental treatments (reviewed in van den Thillart & van Waarde, 1996).

More exact methods of obtaining localized pH measurements utilize magnetic field gradients and require imaging capability of the spectrometer. These methods allow the sample to be placed within a normal transceiver coil and have a spectrum collected from a small volume of the total sample (Ordidge et al. 1985; Ordidge, Connelly, & Lohman, 1986; Frahm, Merboldt & Hanicke, 1987). Similarly, chemical shift imaging allows the acquisition of spatially resolved spectra over the entire sample, and in the case of ^{31}P, pH maps can be constructed (Morikawa et al., 1993). The shortcoming of these methods is typically a low SNR, which becomes more problematic as the localized region becomes smaller and the concentrations of the metabolites in the spectrum become lower. Applications of these methods to comparative acid–base questions are limited. Skibbe et al. (1995; 1996) have used chemical shift imaging to acquire pH maps and observe pH gradients across the midgut of lepidopteran larvae. Although their results are the highest resolution to date for pH mapping, the voxel size of 2.0 mm^3 is still large relative to the size of the animal and spatial resolution is quite poor. Although localization using imaging gradients is appealing due to its elegance and ability to control precisely the localized region, the

SNR limitations currently make surface coils more useful for most applications involving small animals.

Applications in plant physiology

Although the literature on the NMR measurement of pH in plants is more modest than that for animals, a number of groups have used ^{31}P-NMR with great success (reviewed in Ratcliffe, 1991; 1994) The experimental considerations are much the same as described above, and methodologies have been developed for maintaining higher plants, excised tissues, and cell suspensions in good physiological condition (Ratcliffe, 1994). For experiments under illumination, adequate light must be supplied to the entire sample, which can be difficult for preparations such as tightly packed leaves or high-density photosynthetic cell suspensions. In the latter case, light is usually supplied with a fiber optic light guide, which may be inserted into the cell suspension. Of particular interest in NMR studies of plants are the separate peaks that occur in most ^{31}P-spectra for cytosolic and vacuolar P_i, which allows unequivocal multicompartmental analyses of pH regulation (Ratcliffe, 1994). As in most animal tissues, however, the cytosolic component of the P_i signal probably also contains contributions from organelles, which in plants include mitochondria, chloroplasts and amyloplasts. Hentrich et al. (1993) have demonstrated that P_i from chloroplasts can be observed in the alga *Chlamydomonas reinhardtii*, but in most cases a single peak is observed for cytosolic P_i.

^{31}P-NMR studies of the responses of pH_i to anoxia in plant cells have received considerable attention and have been recently reviewed (Ratcliffe, 1995; 1997). Attempts have been made to correlate metabolic proton production with changes in cytoplasmic and vacuolar pH during anoxia. Roberts et al. (1984; 1992) found that lactate production was closely correlated with intracellular acidification in anoxic maize root tips, while Saint-Ges et al. (1991) did not observe a tight coupling between lactate and protons in this tissue. Lactate efflux is known to be an important means of pH_i regulation in plants during anoxia, and Xia and Roberts (1994) demonstrated with ^{31}P-NMR that exposure of maize root tips to 3% O_2 prior to anoxia (so-called acclimation) led to an enhanced efflux of lactate and an increased ability to regulate pH_i. Cytoplasmic pH has also been measured in the context of the pH-stat model, which states that the switch from lactate to ethanol production following the imposition of anoxia is triggered by a drop in cytoplasmic pH (Davies, Grego & Kenworthy, 1974). NMR was first used to test this model by Roberts et al. (1984), who showed that experimental

reductions in pH_i in maize root tips prior to the onset of anoxia accelerated the switch to ethanol production. Fox, McCallan and Ratcliffe (1995) offered further evidence for the pH-stat model in the same tissue by observing that ethanol production was halted when anoxia was imposed under conditions which facilitated the recovery of pH_i to normal, aerobic values. These results showed that pH_i can reversibly trigger the switch from lactate to ethanol production. Similar NMR results have shown that the production of γ-aminobutyrate during hypoxia is also pH triggered in plants (Carroll et al., 1994; Ford, Ratcliffe & Robins, 1996).

^{31}P-NMR has also been used to examine cytoplasmic and vacuolar pH in plants during changes in the external ionic environment. Hyperosmotic conditions caused a substantial increase in vacuolar pH, while cytoplasmic pH continued to be tightly regulated in barley roots (Martinez & Läuchli, 1993) and in mung bean root tips (Nakamura et al., 1992). In both studies, high external [Ca^{2+}] was found to diminish vacuolar alkalinization. Spickett, Smirnoff and Ratcliffe (1992) monitored cytoplasmic and vacuolar pH, as well as phosphorus metabolite levels in ^{31}P-spectra of maize root tips during hyperosmotic shock. These authors observed treatment-induced increases in pH of both compartments, increases in the levels of phosphocholine and vacuolar phosphate, and transient increases followed by recovery to initial levels of cytoplasmic phosphate.

The high surface area to volume of cell suspensions makes them ideal for examining membrane transport phenomena. Sakano, Yazaki and Mimura (1992) monitored the cytoplasmic and vacuolar acidification associated with inorganic phosphate uptake in cultured cell suspensions of *Catharanthus roseus*. Gout, Bligny and Douce (1992) examined the effects of changes in pH_e on transmembrane proton movement in sycamore cell suspensions. In this study, ^{13}C-NMR was used to monitor the pH-sensitive resonances of the CH_2-linked carboxyl groups of citric acid and the ^{13}C-enriched carbon of bicarbonate in order to measure pH at the acid and basic extremes, respectively, while ^{31}P-NMR was used to measure pH at intermediate values.

Conclusions

NMR is well established as an accurate, and in many ways a straightforward, method of measuring pH in living systems. The tremendous advantages offered by the non-invasive nature of NMR promise to maintain it as a powerful tool, while its insensitivity secures it as one of many methods for examining acid–base balance. The advances in

hardware made in each new generation of spectrometers, and the increasing availability of high-field magnets to physiologists, are reassuring signs that NMR methods of studying pH in living systems will continue to improve. In fact, the development of magnetic susceptibility-matched or superconducting transceiver coils may be the next major advancement for biological studies. These improvements are also sure to widen the applicability of imaging and localized spectroscopy methods to plant and animal acid–base studies. Although multicompartmental pH analyses are routine in NMR studies of plants, this important area has yet to be satisfactorily addressed in animals, but the further characterization of pH indicators may make this possible in the future.

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