Measurements of Cytoplasmic and Vacuolar pH in *Neurospora* Using Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy†

Timothy L. Legerton, Keiko Kanamori, Richard L. Weiss,* and John D. Roberts*

ABSTRACT: The nitrogen-15 chemical shift of the N1 (τ)-nitrogen of ^{15}N-labeled histidine and the half-height line widths of proton-coupled resonances of the δ- and ω,ω'-nitrogens of ^{15}N-labeled arginine and of the α-nitrogens of ^{15}N-labeled alanine and proline were measured in intact mycelia of *Neurospora crassa* to obtain estimates of intracellular pH. For intracellular ^{15}N-labeled histidine, the N1 (τ)-nitrogen chemical shift was 200.2 ppm. In vitro measurements showed that the chemical shift was slightly affected by the presence of phosphate, with which the basic amino acids may be associated in vivo. These considerations indicate a pH of 5.7–6.0 for the environment of intracellular histidine. The half-height line widths of the δ- and ω,ω'-nitrogens of [^{15}N]arginine were 15 and 26 Hz, respectively. In vitro studies showed that these line widths also are influenced by the presence of phosphate, and, after suitable allowance for this, the line widths indicate pH 6.1–6.5 for intracellular arginine. The half-height line widths for intracellular alanine and proline were 17 and 12 Hz, respectively, which are consistent with an intracellular pH of 7.1–7.2. Pools of histidine and arginine are found principally in the vacuole of *Neurospora*, most likely in association with polyphosphates. Proline and alanine are cytoplasmic. The results reported here are consistent with these localizations and indicate that the vacuolar pH is 6.1 ± 0.4 while the cytoplasmic pH is 7.15 ± 0.10. Comparisons of these estimates with those obtained by other techniques and their implications for vacuolar function are discussed.

Neurospora crassa accumulates substantial pools of a variety of amino acids when grown in minimal medium. Some of these amino acids, such as arginine and histidine, have been shown to be almost exclusively localized within a subcellular organelle, the vacuole. Others, such as alanine and glutamine, are principally cytoplasmic (Weiss, 1973; Vaughn, 1980). When arginine is supplied exogenously, the intracellular pool of this amino acid expands rapidly to about 4 times the level found in mycelia growing in minimal medium. However, even under these conditions, at least 80% of the intracellular arginine is in the vacuole (Subramanian et al., 1973; Weiss, 1976). The histidine pool is similarly expanded by exogenous supply, although it has not yet been shown what proportion of this expanded pool is in the vacuole.

The intravacuolar physical environment contrasts significantly with the cytoplasmic environment. Thus, from ^{15}N NMR, it is clear that the motion of arginine in the vacuole is much more restricted than its motion in aqueous solution. Furthermore, cytoplasmic amino acids, such as glutamine and alanine, have much more freedom of motion than does vacuolar arginine (Kanamori et al., 1982a). We have now extended these observations on the intracellular environment and used in situ ^{15}N NMR to measure the pH of the cytoplasmic and vacuolar compartments in *N. crassa*.

Measurements of intracellular pH have been attempted by several methods, and the entire subject has been recently reviewed (Roos & Boron, 1981). Three general approaches have been used. The first relies upon the observation that biological membranes are relatively permeable to many uncharged chemical species and impermeable to many ionic species. A weak acid or base, which is impermeable in its ionized form and freely permeable in its un-ioinized form, is allowed to equilibrate across such a membrane. Its distribution across the membrane is determined by the pH on either side, provided the compound is not metabolized and that it is not toxic to the cell. A second approach is the use of glass microelectrodes which are inserted into the cell. These can provide estimates of intracellular pH provided that damage to the cell by the probe is minimized. More recently, ^{31}P NMR has been successfully used to make pH determinations of intracellular compartments. This method relies on measurement of the chemical shifts of inorganic phosphates and organophosphate metabolites localized within the subcellular compartments and correlation of these chemical shifts with those obtained at various pHs in vitro (Navon et al., 1979; Shulman et al., 1979). One limitation of this technique is that phosphate chemical shifts are dependent upon ionic strength in addition to pH. Furthermore, association with basic amino acids such as arginine causes a 0.5-ppm downfield shift of the ^{31}P resonance of methyl hydrogen phosphate at pH 6.5 (Katz et al., 1974). Because we cannot accurately estimate the ionic strength within a cellular compartment, it has been estimated that the uncertainty of pH measurements made in this way is approximately 0.2–0.5 pH unit (Roberts & Jardetzky, 1981). In addition, the technique is limited to those subcellular compartments known to contain significant amounts of phosphorus-containing compounds.

Other molecules contain nuclei which can show pH-dependent chemical shifts in the physiological range. Brown et al. (1977) detected C2 proton resonances of histidyl residues of hemoglobin in erythrocytes by the spin-echo method and, from their chemical shifts, estimated the pH in erythrocytes. The N3 (π)- and N1 (τ)-nitrogens of histidine show very substantial chemical shift changes in the range from pH 4 to 8 (Blomberg et al., 1977). The shift changes are especially relevant to the present research because free histidine is found principally in the vacuole of *Neurospora*, and the chemical shifts of the N3 (π)- and N1 (τ)-nitrogens of histidine are most sensitive to pH in the pH range from 5 to 7 which corresponds

† From the Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024 (T.L.L. and R.L.W.), and Contribution No. 6750 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91125 (K.K. and J.D.R.). *Received July 30, 1982.* This work was supported by Grant PCM78-19030 from the National Science Foundation (to R.L.W.), by a Biomedical Research Support grant to the University of California, Los Angeles, by U.S. Public Health Service Research Grant GM-11072 from the Division of General Medical Sciences, and by the National Science Foundation (K.K. and J.D.R.). T.L.L. was supported by U.S. Public Health Service National Research Award 07104.

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closely to estimates of vacuolar pH. As a result, the ^{15}N shifts of histidine provide a potentially more suitable probe of vacuolar pH than do ^{31}P phosphate shifts. The problem with ^{31}P signals from vacuolar phosphate is that the chemical shift of phosphate is most sensitive to pH in a higher pH range (6–8) than the ^{15}N shifts of histidine which makes it less suitable to probe the somewhat acidic environment of the vacuole. In addition, ^{31}P signals from vacuolar phosphate are difficult to distinguish from other intracellular phosphate species as well as the substantial signal of the phosphate in the medium.

Another NMR parameter that can be used as a probe of intracellular pH is the line width of proton-coupled ^{15}N resonances of α-amino groups of amino acids and the guanidino group of arginine. In aqueous solution, the protons attached to these nitrogens undergo base-catalyzed exchange with water protons, and the rates of exchange, $1/\tau_e$ (where τ_e is the average lifetime between proton transfer), increase with pH. Thus, for the $N_{\omega,\omega'}$ of arginine, the proton-coupled ^{15}N resonance is a sharp triplet with a J_{N-H} of 92 Hz at pH <5, where the exchange is slow on the NMR time scale ($\tau_e J \gg 1$), but the resonance broadens and coalesces to a singlet as the exchange rate approaches the order of magnitude of the coupling constant ($\tau_e J \approx 1$) at pH 6–7.5 (Blomberg et al., 1976; London et al., 1977; Yavari & Roberts, 1978). For the α-amino nitrogens of alanine and proline, which are less basic, the proton-coupled ^{15}N resonance is a broad singlet at pH <6.0, indicating that the rate is intermediate on the NMR time scale ($\tau_e J \approx 1$), and the resonance gradually sharpens over pH 6–8 as the rate of exchange becomes fast ($\tau_e J \ll 1$). Because the line widths of the ^{15}N resonances are sensitive to changes in pH in the physiological range for both the guanidino nitrogens of arginine, which is in the vacuole, and the α-amino nitrogens of proline and alanine, which are in the cytoplasm, these line widths should be useful as probes of pH of the respective subcellular compartments.

The exchange rate at a given temperature depends only on pH for dilute (<50 mM) aqueous solutions of amino acids, and for intracellular amino acids too, proton exchange with cellular water is expected to be most important. However, at higher amino acid concentrations (1 M), direct proton transfer between two amino acid molecules has been shown to make some contribution to the exchange rates of α-amino protons (Sheinblatt & Gutowsky, 1964). The presence of bases other than hydroxide ions, if present in high concentrations and closely associated with the amino acid, may also affect the exchange rates. Therefore, the proton exchange rates for amino acids were studied in model systems which simulate the intracellular environment.

We report here the chemical-shift and line-width measurements of ^{15}N-labeled amino acids which enable estimation of the cytoplasmic and vacuolar pHs of intact mycelia of *Neurospora crassa*.

Experimental Procedures

Strains, Media, and Growth. *Neurospora crassa* strains LA1 (wild type, 74A) and LA6 (*aga*, allele UM906) were from the collection of one of the authors (R.L.W.). The mutant strain, LA6, lacks arginase activity and cannot degrade arginine. The growth medium was Vogel's minimal medium N supplemented with 1.5% sucrose (Vogel, 1964). Nitrogen-free medium is the same medium without ammonium nitrate.

Cultures were inoculated with an aqueous suspension of washed conidia to a final concentration of 1×10^7 conidia/mL. Conidia were germinated in 1-L baffled flasks containing 500 mL of minimal medium for 12–14 h at room temperature or until growth of the culture was logarithmic as measured by culture turbidity. Aeration was provided by gyrotatory shaking. Where specified, cycloheximide [4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione] was added to the culture medium to a final concentration of 20 μg/mL to prevent the incorporation of ^{15}N-enriched amino acids into proteins (Legerton et al., 1981).

For preparation of mycelia containing intracellular [^{15}N]-alanine and [^{15}N]proline, strain LA1 was germinated as described above in medium containing 0.2% $^{15}NH_4Cl$ as a sole nitrogen source. After sufficient biosynthesis of the amino acids had occurred (approximately 12 h), the mycelia were collected by filtration, washed extensively with water, and resuspended in approximately 18 mL of minimal medium for NMR measurements.

For preparation of mycelia containing uniformly ^{15}N-labeled histidine, strain LA1 was germinated on minimal medium as described above. When the culture was growing logarithmically and had reached a satisfactory density, cycloheximide was added. After 5 min, approximately 20 mg of uniformly ^{15}N-labeled histidine was added to the culture. When most of the labeled amino acid had been taken up by the mycelia (2–4 h), they were collected by filtration and prepared for NMR measurements as described above.

Mycelia containing uniformly ^{15}N-labeled arginine were prepared as previously described (Kanamori et al., 1982a).

Chemicals. ^{15}N-Substituted ammonium chloride (95% enriched in ^{15}N) was purchased from MSD isotopes. DL-[^{15}N]Alanine used for extracellular line-width measurements was purchased from Bio-Rad Laboratories. Uniformly ^{15}N-labeled arginine and histidine were prepared as previously described (Kanamori et al., 1982a). ^{15}N-Labeled proline used for extracellular line-width measurements was prepared as described below. Sodium phosphate glass (average chain length 5.5) was purchased from Sigma Chemical Co. and treated with Chelex to remove paramagnetic ions. All other chemicals were reagent grade or equivalent.

Isolation of [^{15}N]Proline. *N. crassa* was grown on 0.25% $^{15}NH_4Cl$ as a sole nitrogen source, and an acidic and neutral amino acid fraction was obtained from a total cell hydrolysate as previously described (Kanamori et al., 1982a). This fraction was evaporated to a dark syrup, brought to a final volume of 10 mL with water, and adjusted to pH 7.0, and 90 mL of absolute ethanol was added. The suspension was stirred for 30 min at room temperature and then filtered through Whatman 1 filter paper, and the filtrate was evaporated to dryness. This sample was estimated by amino acid analysis on a Beckman Model 119C amino acid analyzer to contain approximately 30 mg of proline as well as small quantities of alanine, ammonia, and some trace contaminants. The sample was then thoroughly dried, a minimum volume of absolute ethanol (5 mL) was added to dissolve the proline, and the whole solution was stirred for 30 min at room temperature. The suspension was filtered and the filtrate evaporated to dryness. The resulting sample contained approximately 20 mg of proline contaminated principally with ammonia.

NMR Measurements. The mycelial suspensions used for chemical-shift and line-width measurements were prepared as previously described (Kanamori et al., 1982a). Measurements of chemical shifts and line widths from extracellular amino acids were carried out as described below. The ^{15}N spectra of intracellular [^{15}N]histidine in *N. crassa* and of histidine in aqueous solution were obtained with a Bruker WM 500 spectrometer operating at 50.65 MHz. All other ^{15}N

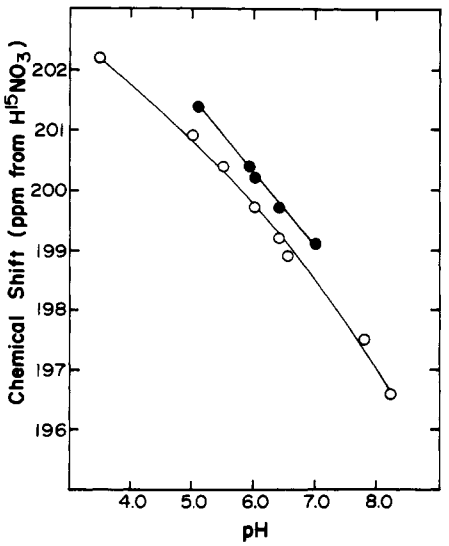

FIGURE 1: Plots of the ^{15}N chemical shift of the N1 (τ)-nitrogen of histidine vs. pH for 0.6 M aqueous solutions of histidine without (●) and with (○) 0.6 M sodium phosphate.

spectra were obtained with a Bruker WH-180 spectrometer operating at 18.25 MHz. All of the histidine spectra were obtained with proton decoupling during the acquisition period. Chemical shifts are reported in parts per million (ppm) upfield of 1 M HNO_3 and are accurate to ±0.05 ppm. Spectra for arginine, alanine, and proline were obtained with proton coupling during acquisition and decoupling during delay to take advantage of the nuclear Overhauser effect and still retain the proton couplings. Line widths at half-height were measured in expanded-scale spectra to within ±1 Hz. The ^{15}N line widths for all of the amino acids, intra- or extracellular, under full proton decoupling, were 6 ± 1 Hz. The sample temperatures were maintained at 10 ± 1 °C unless otherwise stated. As previously noted, the conditions for NMR measurements do not appear to adversely affect mycelial viability as judged by growth rate and the ability to take up amino acids from the medium.

Results and Discussion

An approximately 0.6 M aqueous solution of histidine was used to obtain in vitro chemical shift values for the N1 (τ)-nitrogen as a function of pH. This concentration is probably a reasonable approximation of the intravacuolar concentration of histidine in mycelia used for in vivo measurements. Vacuoles of both yeast and *Neurospora* have previously been shown to contain large quantities of polyphosphates which may associate with basic amino acids (Durr et al., 1979; Urrestarazu et al., 1977; Cramer et al., 1980; Vaughn & Davis, 1981). Because of this, and because the chemical shift of the N1 (τ)-nitrogen of histidine is somewhat dependent upon ionic strength, in vitro measurements were performed in the presence and absence of 0.6 M phosphate. The histidine solution contained 0.6 M histidine and 0.3 M Cl^- (μ = 0.3) and the histidine–phosphate solution contained 0.6 M histidine, 0.3 M Cl^-, 0.6 M K^+, and 0.6 M $H_2PO_4^-$ (μ = 0.9). This permitted estimation of the change in pK_a of histidine as a result of ionic strength effects and/or phosphate association. Figure 1 shows the results wherein the downfield shift of the N1 (τ)-nitrogen with increasing pH results from deprotonation of the imidazolium cation which has a pK_a of 6.0 for free histidine at 25 °C. There is a small but real dependence of the chemical shift and of the pK_a of the N1 (τ)-nitrogen of histidine on the presence of phosphate.

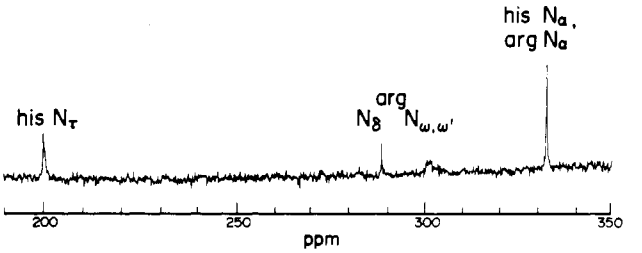

FIGURE 2: ^{15}N spectrum of uniformly ^{15}N-labeled histidine in cultures of intact *N. crassa* mycelia obtained at 50.65 MHz with gated proton decoupling, using a repetition time of 10 s. Peaks for intracellular [^{15}N]arginine are also observed because the [^{15}N]histidine added to the culture medium for incorporation by mycelia contained trace amounts of [^{15}N]arginine.

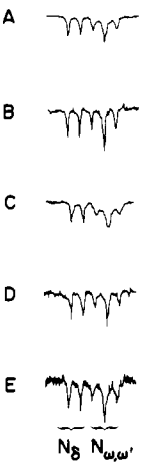

FIGURE 3: Proton-coupled ^{15}N spectra of arginine. (A) 1 M arginine in nitrogen-free medium, pH 6.2; (B) 1 M arginine in nitrogen-free medium + 0.2 M sodium pentaphosphate, pH 6.2; (C) 1 M arginine in nitrogen-free medium, pH 6.5; (D) 1 M arginine in nitrogen-free medium + 0.2 M sodium pentaphosphate, pH 6.5; (E) intracellular uniformly ^{15}N-labeled arginine in *N. crassa*.

Figure 2 shows the spectrum of intracellular uniformly ^{15}N-labeled histidine obtained with gated proton decoupling. Sharp resonances were obtained for both the α- and N1 (τ)-nitrogens with chemical shifts of 334.8 and 200.2 ppm, respectively. The N3 (π)-nitrogen resonance was not observed, possibly because of its longer relaxation time, or more likely as the result of line broadening caused by traces of paramagnetic ions (Bachovchin & Roberts, 1978). Comparison of the N1 (τ)-nitrogen chemical shift value with the data shown in Figure 1 suggests that the intravacuolar pH is between 5.7 and 6.0, the range depending upon the importance of the contributions of phosphate and ionic strength in vivo. Line broadenings were measured for the $^{15}N_\delta$- and $^{15}N_{\omega,\omega'}$-nitrogens of uniformly ^{15}N-labeled arginine, another vacuolar amino acid, in an attempt to verify this pH estimate for the vacuole. The proton-coupled spectra obtained for intracellular arginine and for arginine in nitrogen-free medium at pH 6.2 and 6.5 in the presence and absence of 0.2 M sodium pentaphosphate are shown in Figure 3. Table I summarizes the relevant line-width data. In the absence of polyphosphate, the line widths for the N_δ- and $N_{\omega,\omega'}$-nitrogens of intracellular arginine correspond most closely to an intravacuolar pH of 6.1. The presence of polyphosphate produces some peak sharpening and gives a close approximation to the intracellular values at pH 6.5. A possible explanation for the slower proton exchange rate in the presence of polyphosphate is that the guanidino nitrogens are hydrogen bonded to phosphate oxygens and the guanidino protons are then relatively less reactive in

Table I: Half-Height Line Widths for δ- and ω,ω'-Nitrogens of Arginine

medium	arginine concn (M)	pH	line widths (Hz)[a] N_δ	$N_{\omega,\omega'}$
N free	1	5.85	12	22
N free	1	6.2	17	27
N free + 0.2 M pentaphosphate	1	6.2	11	14
N free	1	6.5	22	48
N free + 0.2 M pentaphosphate	1	6.5	12	22
N free	1	7.0	105[b]	128[b]
N free + 0.2 M pentaphosphate	1	7.0	30	44
intracellular			15	26

[a] Line widths for the center peak of the resolved triplet for $N_{\omega,\omega'}$ and the average line widths of the resolved doublet for N_δ, unless specified otherwise. [b] The line widths of the coalesced singlet.

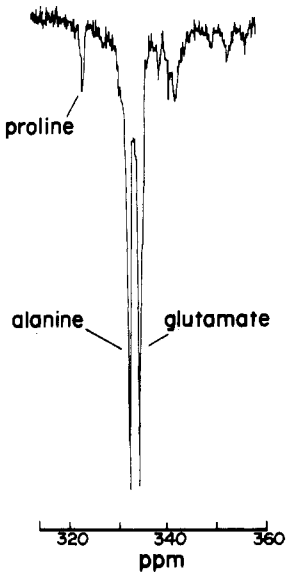

FIGURE 4: ^{15}N proton-coupled spectrum of intact mycelia of *N. crassa* germinated on nitrogen-free medium with $^{15}NH_4Cl$ as the sole nitrogen source.

proton exchange with water. A similar effect has been observed for alkylammonium ions hydrogen bonded to anions (Grunwald & Ralph, 1975). Above pH 6.5, the peaks broaden rapidly in both the presence and absence of phosphate (Table I), in the latter case, coalescing into broad singlets at pH 7. The best estimate of intravacuolar pH based on both histidine and arginine seems to be about 6.0–6.1 if one ignores possible effects of phosphate, which introduce an uncertainty of the order of 0.4 pH unit.

Alanine is found principally in the cytoplasm of mycelia growing in minimal medium (Vaughn, 1980). Proline is similarly distributed (T. L. Legerton, unpublished experiments). Proton-coupled spectra for the α-nitrogens of intracellular ^{15}N-labeled alanine and proline are shown in Figure 4. Figure 5 shows the relationship between the proton-coupled line widths of these resonances and the pH in nitrogen-free medium, and it is clear that the line widths are relatively independent of the alanine concentrations from 10 to 40 mM, which are representative of the intracellular concentrations under the growth conditions employed in these experiments. The line widths for 10 mM alanine in pure water at pH 5.85, 6.0, 6.5, and 7.0 were close to those for N-free medium containing, as major ingredients, potassium phosphate (28 mM), sodium

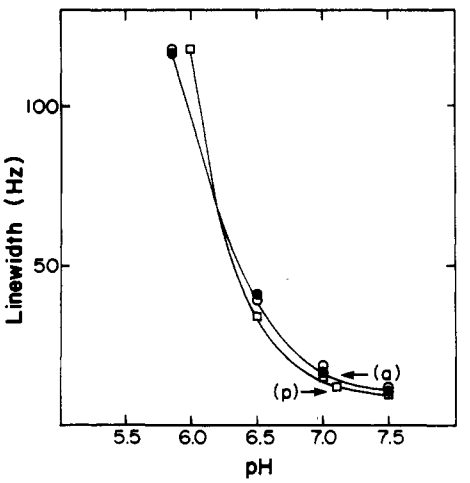

FIGURE 5: Plots of the line width at half-height against pH for 0.01 M alanine in nitrogen-free medium (●), 0.04 M alanine in nitrogen-free medium (○), and 0.005 M proline in nitrogen-free medium (□).

citrate (8 mM), and sucrose (43 mM). This fact indicates that these carbon sources and inorganic salts, which are also expected to be present in the cytoplasm, have little effect on the pH-dependent rates of proton exchange at these concentrations. Arrows in Figure 5 show the line widths determined from the intracellular spectra for alanine (a) and proline (p). These two values agree in corresponding to an estimated cytosolic pH of 7.1–7.2. Line broadening of the proton-coupled ^{15}N resonances of intracellular arginine, alanine, and proline is caused predominantly by proton exchange and not by paramagnetic ions in the cell, because (1) with proton decoupling the ^{15}N resonances of all of these amino acids, intra- or extracellular, were sharp ($\nu_{1/2}$ = 6 ± 1 Hz) (Experimental Procedures), and (2) the nuclear Overhauser enhancement values of $^{15}N_{\omega,\omega'}$ of arginine and $^{15}N_\alpha$ of alanine in *Neurospora* were found to be 90% of the maximum value which indicates that the relaxations of the nuclei are predominantly due to dipolar interactions with proton (Kanamori et al., 1982a).

While the pH measurements reported here are subject to some uncertainties because of phosphate and ionic strength effects, there seems to be a clear difference of perhaps 1 pH unit between the environment of the vacuolar and cytoplasmic amino acids. This conclusion is in accordance with previous estimates of cytoplasmic and vacuolar pHs. Thus, the pH of the *Neurospora* cytoplasm has been determined with glass microelectrodes to be 7.19 (Sanders & Slayman, 1982), and experiments with ^{31}P NMR in *Neurospora* have tentatively identified two compartments with pHs of 7.1–7.3 and ≤6.0 (C. Slayman, unpublished experiments) which probably represent cytoplasm and the vacuole, respectively. The pH of the yeast vacuole has been estimated to be 6.5 by ^{31}P NMR of intravacuolar polyphosphate (Navon et al., 1979). These measurements are in close agreement with the values which we report here.

All of these results provide further evidence for the intracellular localizations of arginine, histidine, alanine, and proline in *Neurospora*. Moreover, the observation that polyphosphates in yeast and basic amino acids in *Neurospora* are both found in an acidic location further substantiates the notion that these compounds are cosequestered within the same organelle in these organisms.

The existence of a pH gradient across the vacuolar membrane is consistent with the presence of a proton-translocating ATPase in this membrane (Bowman & Bowman, 1982). The ATPase and the H^+ gradient so formed are probably re-

sponsible for the energy-linked movement of arginine (and presumably other substances). In addition, the acidity of the vacuole relative to that of the cytoplasm is consistent with the lysosomal nature of this organelle and the acidic pH optima of proteases and nucleases associated with the organelles (Matile, 1971).

^{15}N NMR is emerging as a powerful probe of the intracellular environment. Utility has been demonstrated for monitoring in vivo metabolism of many important nitrogen-containing compounds (Legerton et al., 1981; Kanamori et al., 1982b). It has also been shown that ^{15}N NMR can be employed to examine subtle features of intracellular organization such as the microviscosity of intracellular compartments and the association of small molecules in vivo (Kanamori et al., 1982a). The work reported here demonstrates the feasibility of using ^{15}N NMR to estimate the pH of subcellular compartments.

Registry No. L-Histidine, 71-00-1; L-arginine, 74-79-3; L-alanine, 56-41-7; L-proline, 147-85-3; nitrogen-15, 14390-96-6.

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