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Intracellular pH Measurements by ^{31}P Nuclear Magnetic Resonance. Influence of Factors Other Than pH on ^{31}P Chemical Shifts†

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ABSTRACT: Titration curves plotting chemical shift vs. pH for inorganic phosphate and glucose 6-phosphate in solutions of various composition are presented. Physiological concentrations of K^+ (0.1 M) and Mg^{2+} (5 mM) are shown to significantly shift the titration curve. The Mg^{2+} effect can be partly or completely reversed by addition of sufficient quantities of adenosine triphosphate or organic acids. The acidic protein bovine serum albumin and soluble maize root tip protein have no noticeable effect on the titration curves, whereas the basic protein protamine exerts a profound effect. The results clearly indicate that knowledge of intracellular ionic strength and free Mg^{2+} concentrations in the sample are required if the determination of intracellular pH by ^{31}P NMR is to be considered accurate within ±0.05–0.1 pH unit.

Since 1973 (Moon & Richards, 1973) in vivo ^{31}P nuclear magnetic resonance has been increasingly and extensively used to estimate intracellular pH [see Burt et al. (1979) and Radda & Seeley (1979) for reviews]. The method relies on the fact that the chemical shift (δ, resonance frequency relative to a standard) of ^{31}P in many phosphates is strongly dependent on pH; if the titration curve of a given phosphate compound in the intracellular milieu is known, a determination of pH from a measured δ is possible. Inorganic phosphate (P_i) and sugar phosphate resonances are the most suitable for measurement of intracellular pH (Burt et al., 1976), because their pK_a's lie near neutrality, and they are often present at high intracellular concentrations. The accuracy of the pH measurement (disregarding limitations imposed by the quality of the spectrum) depends on our understanding the extent to which factors other than pH influence δ. Analogous problems apply to other methods for measuring intracellular pH (Radda & Seeley, 1979).

The majority of articles describing application of the NMR method either fail to discuss whether or not factors other than pH affect the δ of P_i or state that factors such as Mg^{2+} (Burt et al., 1976, 1979; Navon et al., 1979; Colman & Gadian, 1976; Hollis, 1980) and ionic strength (Burt et al., 1976, 1979; Hollis, 1980) have no effect on the titration curve of P_i or that the effects can be ignored.

Recently an effect of ionic strength has been reported (Gadian et al., 1979; Ugurbil et al., 1979; Ogawa et al., 1978); we also reported (Roberts et al., 1980) that Mg^{2+} and certain concentrated polyelectrolytes shift the apparent pK_a of P_i.

Here we describe details of these and other effects, for both P_i and glucose 6-phosphate (Glc-6-P). Two observations indicate that at least some of these effects are significant: first, titration of P_i in undiluted, filtered homogenates of maize tissue (root tips or coleoptiles plus primary leaf) yield points that clearly do not lie on the titration curve of 5 mM KP_i. Second, when the cytoplasmic pH of maize root tip cells was induced to fall, the δ for the Glc-6-P resonance indicated a slightly larger drop (0.1 pH unit) than that indicated by the cytoplasmic P_i resonance, suggesting that one or both of the titration curves we were using to estimate pH did not exactly apply to the cytoplasm of these cells (unpublished results).

Materials and Methods

Preparation of Homogenates. Maize (*Zea mays* L.) root tips (1 mm long) from 2-day-old plants or coleoptiles and primary leaves from 4-day-old dark-grown plants were collected on ice, and ground in an ice-cold mortar. The homogenate was filtered through two layers of miracloth to remove the largest particles and so minimize suspension effects in the pH measurement using electrodes (Westcott, 1978). The pH was adjusted with 0.5 M NaOH or HCl.

Titration of P_i and Glc-6-P ^{31}P δ's. Although titration curves for P_i and Glc-6-P are plotted together, they were titrated as separate solutions—except those involving protamine and phospholipids. At least two solutions, separately made up, were titrated for each mixture. The pH was adjusted either by mixing different proportions of K_2HPO_4 and KH_2PO_4 solutions or by adding 0.5 M NaOH or HCl. Protamine (free base) was acidified with concentrated HCl.

Potentiometric Titrations. Potassium phosphate or Na_2Glc-6-P (100 mL, 5 mM) was adjusted to pH 2.5–3.0 with 0.5 M HCl, and titrated with 0.5 M NaOH, the pH being

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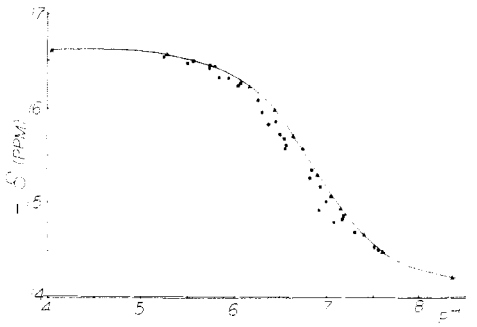


FIGURE 1: Titration curve of 5 mM potassium phosphate (▲), also showing points obtained on titration of P_i present in undiluted filtered maize root tip (●) or maize coleoptile plus primary leaf (■) homogenates.

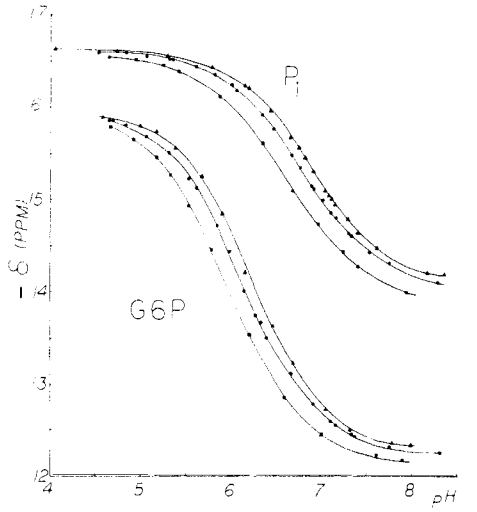


FIGURE 2: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 0.1 M KCl (●) or in the presence of 0.5 M KCl (■).

measured with a combination electrode standardized against pH's 4 and 7. The symbols used in the figures indicate the magnitude of error in curve placement.

Preparation of Lipid Dispersions. Dry phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol (all from soybean; Sigma chemicals), and phosphatidylserine (from bovine brain; Sigma) in 20:10:3:2 proportions, respectively, were placed in a test tube with P_i and Glc-6-P. Water was added to make the lipid concentration 4 mg/mL. The tube was then sonicated for 5 min in a bath sonicator.

Preparation of Maize Root Tip Soluble Protein. Chilled (4 °C) root tips were homogenized in an ice-cold mortar, and the brei was centrifuged for 2 h at 130000*g*. The supernatant was dialyzed (tubing molecular weight cut-off 3500) for 3 days. The volume change was negligible.

Spectra of the solutions were obtained in a modified Varian XL100 spectrometer, operating at 40.5 MHz, at 24 °C. Chemical shifts are expressed relative to methylenediphosphonic acid (0.5 M in Tris, pH 8.9).

Results

Figure 1 shows the titration curve of 5 mM KP_i together with points obtained from titration of the P_i in undiluted, filtered homogenates of maize root tips and maize coleoptiles with primary leaves. It is clear that the pK_a of P_i in the homogenates is 0.1–0.2 pH unit lower than that of 5 mM KP_i.

Increasing the ionic strength (0.1 or 0.5 M KCl) of a 5 mM solution of potassium phosphate or Glc-6-P causes the titration curves to be displaced downward over the pH range 4–8

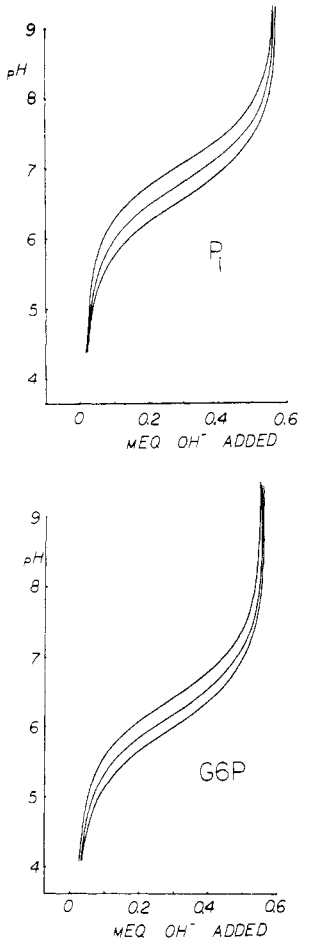


FIGURE 3: Potentiometric titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (upper curves) or in the presence of 0.1 M KCl (middle curves) or in the presence of 0.5 M KCl (lower curves).

Table I: pK_a Values of Potassium Phosphate and Glc-6-P in the Presence of Various Concentrations of $MgCl_2$ and KCl, Determined from Potentiometric Titrations or Estimated from Titration of ^{31}P δ Values

solution	by potentiometry	by NMR
5 mM KP_i[a]	7.0	6.95
+0.1 M KCl	6.875	6.85
+0.5 M KCl	6.65	6.62
+5 mM $MgCl_2$	6.8	6.7
+50 mM $MgCl_2$	6.25	6.25
+5 mM $MgCl_2$ + 0.1 M KCl	6.675	6.65
5 mM Glc-6-P	6.4	6.3
+0.1 M KCl	6.2	6.2
+0.5 M KCl	6.0	6.0
+5 mM $MgCl_2$	6.2	6.1
+50 mM $MgCl_2$	5.7	5.6
+5 mM $MgCl_2$ + 0.1 M KCl	6.1	6.1

[a] Potassium phosphate.

(Figure 2). The displacement is due to a lowering of the phosphate pK_a's, since pK_a's determined from potentiometric titrations (Figure 3) agree closely with those estimated from titration of phosphate δ's (Table I). The effect of KCl is not changed if 5 mM potassium phosphate, Glc-6-P, ATP, and UDPG are titrated together (data not shown).

Figure 4 shows that Mg^{2+} (5 and 50 mM $MgCl_2$) shifts the titration curves of potassium phosphate and Glc-6-P upward and to the left. Part of the shift is due to Mg^{2+} lowering the pK_a's of those phosphates, for pK_a's estimated from these

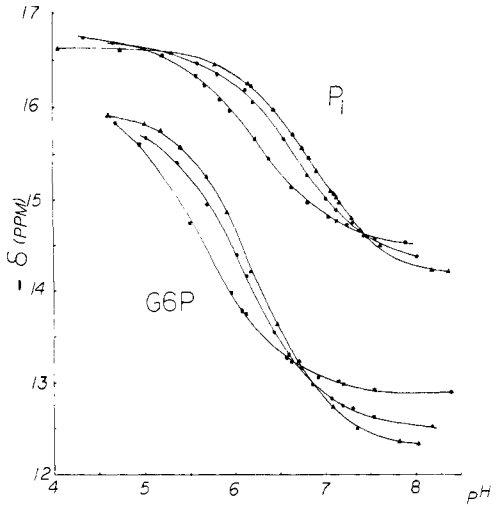


FIGURE 4: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 5 mM $MgCl_2$ (●) or in the presence of 50 mM $MgCl_2$ (■).

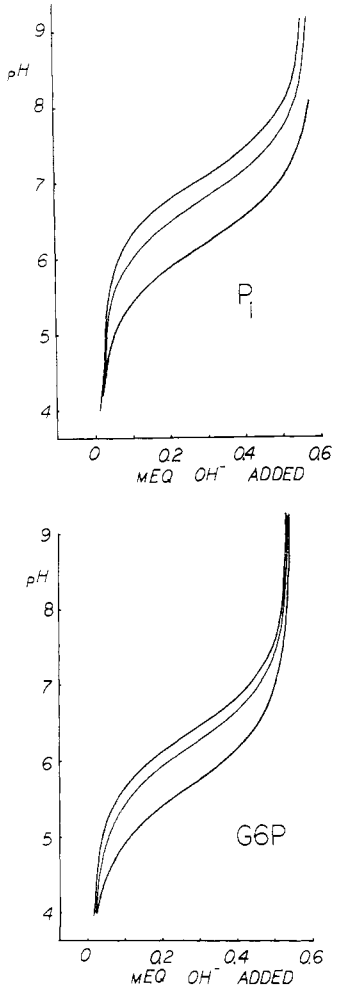


FIGURE 5: Potentiometric titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (upper curves) or in the presence of 5 mM $MgCl_2$ (middle curves) or in the presence of 50 mM $MgCl_2$ (lower curves). The lower curve in the titration of potassium phosphate stops where $MgPO_4$ precipitation began.

titration curves (Table I) agree quite well with those determined from potentiometric titrations (Figure 5). However, there are clearly specific Mg^{2+} binding effects on ^{31}P δ's of Glc-6-P and P_i; these effects are particularly evident at the wings of the titration curves (Figure 4) and are discussed below.

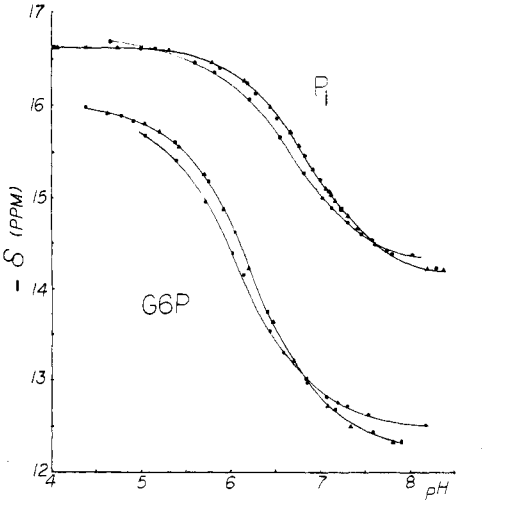


FIGURE 6: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 5 mM $MgCl_2$ (●) or in the presence of 5 mM $MgCl_2$ plus 5 mM ATP (■).

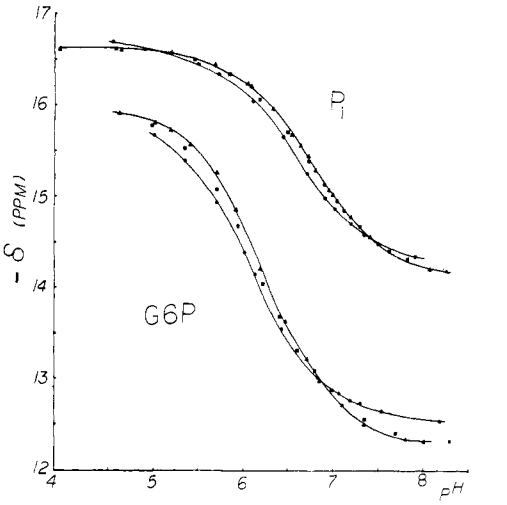


FIGURE 7: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 5 mM $MgCl_2$ (●) or in the presence of 5 mM $MgCl_2$ plus 5 mM citrate (■).

Cells generally contain compounds other than P_i and Glc-6-P that could interact with Mg^{2+} and so reduce the Mg^{2+} effect. Thus, we found that the effect of 5 mM $MgCl_2$ on the titration curves of potassium phosphate and Glc-6-P could be completely reversed if 5 mM ATP was added (see Figure 6), ATP sequestering the magnesium and so reducing the free Mg^{2+} concentration essentially to zero. Citrate (5 mM) also reduced the 5 mM $MgCl_2$ effect (Figure 7), though not completely. In contrast, titration of potassium phosphate and Glc-6-P together or with 5 mM or 50 mg/mL or 4 mg/mL phospholipid dispersion caused either no or a barely detectable diminution of the Mg^{2+} effect (data not shown).

As can be seen from Figure 8, 5 mM $MgCl_2$ and 0.1 M KCl have a competitive effect on Glc-6-P- and potassium phosphate ^{31}P δ's at the wings of the titration curves, high ionic strength reducing the strength of Mg^{2+} binding to phosphates. However, near the middle of the titration curves (Figure 8), 0.1 M KCl does not reverse the Mg^{2+} effect; this is accounted for by the observation that Mg^{2+} and ionic strength in combination lower the pK_a of those phosphates more than when added alone (Figure 9 and Table I).

Bovine serum albumin (50 mg/mL) and maize root tip protein did not affect the titration curves of Glc-6-P and potassium phosphate in the presence or absence of 5 mM $MgCl_2$ (data not shown) (the root tip protein could only be titrated

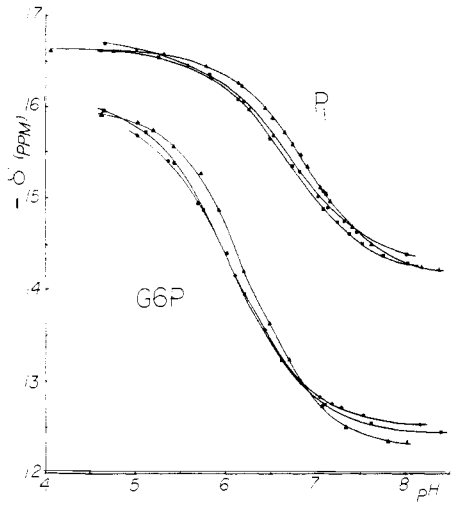


FIGURE 8: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 5 mM $MgCl_2$ (●) or in the presence of 5 mM $MgCl_2$ plus 0.1 M KCl (■).

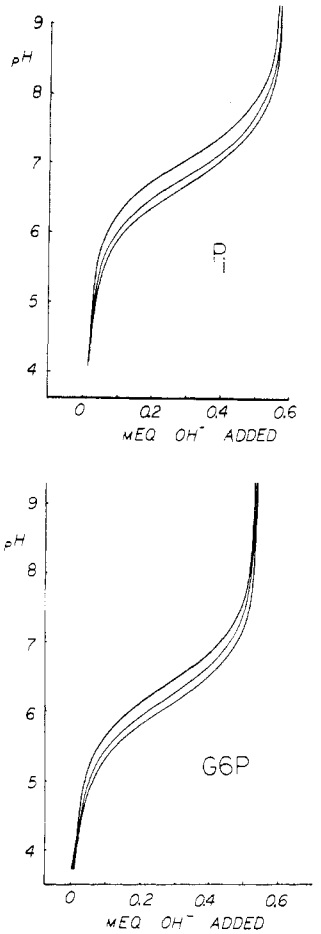


FIGURE 9: Potentiometric titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (upper curves) or in the presence of 5 mM $MgCl_2$ (middle curves) or in the presence of 5 mM $MgCl_2$ plus 0.1 M KCl (lower curves).

with potassium phosphate, as it rapidly hydrolyzed Glc-6-P). The basic protein protamine (10 or 50 mg/mL) does, however, greatly decrease the pK_a of potassium phosphate and Glc-6-P (Figure 10).

Discussion

These experiments demonstrate that the δ values of inorganic and sugar phosphates are dependent on factors other than pH. Some of these factors have significant effects at

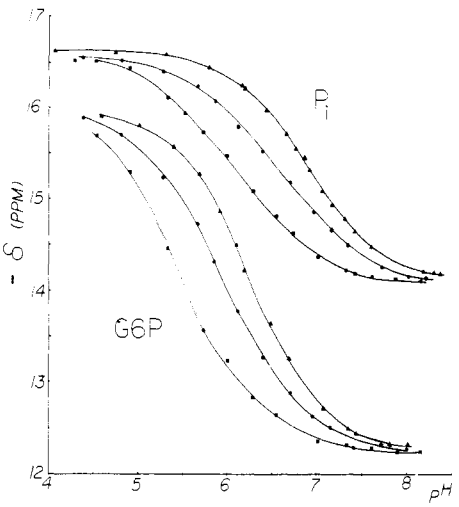
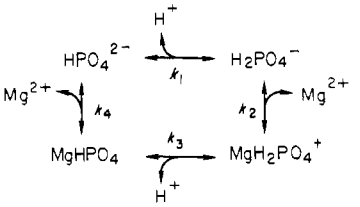


FIGURE 10: Titration curves of 5 mM potassium phosphate and 5 mM Glc-6-P alone (▲) or in the presence of 10 mg/mL protamine (●) or in the presence of 50 mg/mL protamine (■).

Scheme I



usual physiological concentrations (viz., 0.1 M KCl, 5 mM $MgCl_2$), whereas others (e.g., the effect of protamine) might only be relevant to intracellular pH determination on cells of unusual composition. Glc-6-P and P_i titration curves are affected in a qualitatively and quantitatively very similar manner.

The alterations in form and position of the titration curves can be expected to be due to a combination of pure electrostatic effects (the positive charges of Mg^{2+}, K^+, and protamine competing with H^+ for the negative charges of phosphate groups and lowering the pK_a of the latter) and more specific binding effects (altering the magnetic shielding tensor of the phosphorus atom). Protamine appears to act mainly by an electrostatic mechanism, whereas K^+ and, in particular, Mg^{2+} also appear to exert specific binding effects.

Such specific binding effects are evident from the different ^{31}P δ's at the wings of the titration curves obtained at different Mg^{2+} concentrations (Figure 4); i.e., $\delta_{HPO_4} \neq \delta_{MgHPO_4}$, and $\delta_{H_2PO_4} \neq \delta_{MgH_2PO_4}$. These Mg^{2+}-dependent titration curves cannot be analyzed in terms of a simple Henderson–Hasselbach-type equation, used by Gadian et al. (1979) and Hollis (1980). In a phosphate solution with a pH not very far from neutrality containing Mg^{2+}, four equilibria must be considered (see Scheme I). In these equations, $K_1 = (H^+)(HPO_4^{2-})/(H_2PO_4^-)$; $K_2 = (Mg^{2+})(H_2PO_4^-)/(MgH_2PO_4^+)$; $K_3 = (H^+)(MgHPO_4)(MgH_2PO_4^+)$, and $K_4 = (Mg^{2+})(HPO_4^-)/(MgHPO_4)$. At extremes of pH, equilibria involving the PO_3^{3-} ion or the uncharged species H_3PO_4 also need to be taken into account. The parentheses indicate activities. The observed ^{31}P chemical shift, δ_o, of solutions of these rapidly exchanging phosphates is given by

$$\delta_o = \frac{[HPO_4^{2-}]}{[P_i]}\delta_{HPO_4^{2-}} + \frac{[H_2PO_4^-]}{[P_i]}\delta_{H_2PO_4^-} + \frac{[MgHPO_4]}{[P_i]}\delta_{MgHPO_4} + \frac{[MgH_2PO_4^+]}{[P_i]}\delta_{MgH_2PO_4^+} \quad (1)$$

where $\delta_{HPO_4^{2-}}$, etc., indicate the δ's of HPO_4^{2-}, etc., P_i is the total phosphate, and the brackets indicate concentrations, since the weighting factors for chemical shift averaging are determined by concentrations rather than activities. Where activities can be equated with concentrations, one may write

$$\delta_o = \frac{K_1K_3K_4}{\alpha}\delta_{HPO_4^{2-}} + \frac{K_3K_4[H^+]}{\alpha}\delta_{H_2PO_4^-} + \frac{K_1K_3[Mg^{2+}]}{\alpha}\delta_{MgHPO_4} + \frac{K_1[H^+][Mg^{2+}]}{\alpha}\delta_{MgH_2PO_4^+} \quad (2)$$

where $\alpha = K_1[H^+][Mg^{2+}] + K_1K_3K_4 + K_3K_4[H^+]$.

This expression can be rearranged to give

$$[H^+] = \frac{K_1K_3K_4(\delta_{HPO_4^{2-}} - \delta_o) + K_1K_3[Mg^{2+}]\delta_{MgHPO_4}}{K_1[Mg^{2+}](\delta_o - \delta_{MgH_2PO_4^+}) + K_3K_4(\delta_o - \delta_{H_2PO_4^-})} \quad (3)$$

As $[Mg^{2+}] \rightarrow 0$, $[H^+] \rightarrow K_1(\delta_{HPO_4^{2-}} - \delta_o)/(\delta_o - \delta_{H_2PO_4^-})$, or

$$pH = pK_1 + \log\frac{\delta_o - \delta_{H_2PO_4^-}}{\delta_{HPO_4^{2-}} - \delta_o} \quad (4)$$

the equation used by Gadian et al. (1979) and Hollis (1980). Analogous expressions apply to titration curves of Glc-6-P and to interactions with other ions. More complicated expressions become necessary if more than four equilibria need to be considered.

The following statements can be made in the light of this analysis: (i) K_a's of $H_2PO_4^-$ and $MgH_2PO_4^+$ (i.e., K_1 and K_3) are dependent on ionic strength (Figures 2 and 3), as reported by Gadian et al. (1979), Ogawa et al. (1978), and Ugurbil et al. (1979). (ii) The competitive interaction between Mg^{2+} and ionic strength (Figure 8), noted under Results, indicates that the binding constants of magnesium phosphates (i.e., K_2 and K_4) are dependent on ionic strength. (iii) The free Mg^{2+} concentration in cells, in addition to being a function of K_2 and K_4, depends on the concentration of nucleotide phosphates, such as ATP, and other agents that can sequester Mg^{2+}, such as citrate, because these compounds can considerably reverse Mg^{2+} effects on phosphate ^{31}P δ's (Figures 6 and 7). These observations suggest that a variety of titration curves, described by eq 1, apply to the various intracellular conditions experienced in vivo because most parameters in that equation are influenced by factors other than pH.

The quantitative usefulness of the above equations is limited, however, because accurate δ values for species that cannot be observed in the absence of free phosphate ions such as δ_{MgHPO_4} and $\delta_{MgH_2PO_4^+}$ are extremely difficult to obtain. Extrapolation is of limited value because of the concentration dependence of stability constants. In addition, at higher concentrations, where concentrations and activities cannot be equated, ratios of activity coefficients will appear in eq 2. Since these activity coefficients are likely to be concentration dependent, the number of unknowns will be much larger than the number of experimentally measurable parameters, precluding an unequivocal analysis.

In view of these effects, one can say that without knowledge of the concentration of substances such as K^+, Mg^{2+}, ATP, ADP, and organic acids in the various intracellular compartments, considerable uncertainty must be placed on intracellular pH determinations using in vivo ^{31}P NMR. If the composition of the medium is unknown only within the most usual physiological limits (0.1–0.2 M ions), the uncertainty is of the order of 0.2–0.5 pH unit. However, it can be as high as 1 pH unit in specialized cells which permit a wider range of ionic strength and divalent ion concentration, such as halophilic bacteria and plants. Examination of the titration curves presented suggests that the uncertainty is similar in magnitude over all regions of the pH range 4–8. In contrast, the intrinsic accuracy of the ^{31}P NMR method is greatest near the middle of the titration curve (where δ's are most sensitive to pH), and less accurate toward the wings. Knowledge of intracellular ionic strength and free Mg^{2+} levels would reduce the uncertainty to approximately 0.1 pH unit—as these factors appear to exert the most significant effects.

When *changes* in intracellular pH are to be examined by means of ^{31}P NMR spectroscopy, it is important to consider the possibility that changes in intracellular conditions other than pH may affect δ values. In this regard, we have used ^{31}P NMR to observe cytoplasmic and vacuolar pH in maize root tip cells induced to extrude large amounts of H^+ (Roberts et al., 1981); resonances assigned to the cytoplasmic compartment indicate that the cytoplasmic pH may be slightly lower in cells extruding H^+. Concomitant with H^+ extrusion, these cells accumulate K^+ (>10 μequiv g^{-1} h^{-1}); hence the ionic strength in one or all intracellular compartments must be increasing. Figure 2 shows that increasing ionic strength at constant pH induces δ to become more negative which on a given titration curve would suggest a pH *rise*. Hence the apparent cytoplasmic pH drop we see cannot be attributed to the accumulation of K^+. Another example is changes in intracellular conditions during anaerobiosis: depletion of MgATP that occurs under such conditions can be expected to increase intracellular free Mg^{2+} concentrations; higher free Mg^{2+} levels have been estimated to be present in anaerobic human erythrocytes compared to aerobic cells (Gupta et al., 1978).

Although this report sounds a cautionary note concerning the accuracy of in vivo ^{31}P NMR for intracellular pH determination, using a single standard titration curve, comparison of intracellular pH measurements by ^{31}P NMR and other techniques (e.g., Gadian et al., 1979; Navon et al., 1977) suggests that the NMR method is not less accurate than the alternatives. Furthermore, independent determinations of the ionic strength and ionic composition of the intracellular medium do make it possible to choose the appropriate standard titration curve and reduce the limits of uncertainty to ±0.05–0.1 pH unit.

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pH-Induced Conformational Transitions of Ferricytochrome *c*: A Carbon-13 and Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: The acid and alkaline unfoldings of ferricytochrome *c* have been studied by ^{13}C and ^{2}H NMR spectroscopy employing native cytochrome *c* and cytochrome *c* in which the methyl groups of the methionyl residues have been enriched by 45% ^{13}C or ^{2}H. A p*K* of 3.4 ± 0.2 for the acid transition was determined from the natural abundance ^{13}C NMR spectra by following the collapse of a sharp singlet due to 10 threonine residues upon raising the pH from 2 to 5. This same transition was monitored by following the ^{2}H resonance due to Met-80 in deuterium-labeled cytochrome *c*. The progressive broadening of the ^{2}H resonance line width and decrease in the paramagnetic shift together with the ^{13}C NMR results indicate that the displacement of Met-80 as an axial heme iron ligand occurs concomitantly with the loss of the characteristic cytochrome fold. The presence of a paramagnetically shifted ^{2}H resonance due to Met-80 below pH 2, however, indicates that cytochrome *c* at low pH can form a loosely folded structure that allows Met-80 to come into close proximity with the heme iron. The alkaline transition was also monitored selectively by following the appearance in the region of diamagnetic ^{13}C shifts of the Met-80 resonance in ^{13}C-enriched cytochrome *c*. The ^{2}H resonance due to Met-80 in the deuterium-enriched protein disappears from its hyperfine-shifted upfield position without line broadening and reappears coincident with the Met-65 methyl resonance. The disappearance of both the Met-80 [^{13}C]- and [^{2}H]methyl resonances parallels the disappearance of the 695-nm absorption band with a p*K* of ~9. The ^{2}H resonance line widths proved to be sensitive indicators of the mobility of the methionyl residues.

Ferricytochrome *c* is known to form at least five stable conformational states which depends upon the ionization states of various functional groups within the protein (Dickerson & Timkovich, 1975). The pH-induced transitions between these conformations have been extensively studied by various spectroscopic methods including optical spectroscopy (Greenwood & Wilson, 1971; Drew & Dickerson, 1978; Davis et al., 1974; Babul & Stellwagen, 1972), EPR[1] (Lambeth, et al., 1973; Brautigan et al., 1977), and proton NMR (Gupta & Koenig, 1971; Morishima et al., 1977). Since the overall folding of the protein and the axial ligands of the heme iron mediate the oxidative-reductive properties of the protein, much attention has been focused on the unfolding of the native conformation which involves the displacement of the axial ligands Met-80 and His-18.

In this study, we have taken advantage of the sensitivity of the chemical shifts of ^{13}C NMR resonances in proteins to changes in tertiary structure to study the acid and alkaline unfolding of ferricytochrome *c*. In addition, we have prepared cytochrome *c* in which the methyl groups of Met-65 and Met-80 are isotopically enriched with either ^{13}C or ^{2}H. This has allowed the critical Met-80 group to be followed through the unfolding process by ^{13}C and ^{2}H NMR without interference from natural abundance background resonances.

Experimental Procedures

Materials. The cytochrome *c* employed for the natural abundance ^{13}C spectra was Sigma Type VI obtained from horse heart. The isotopically enriched ferricytochromes were prepared by established methods (Ando et al., 1966; Jones et al., 1975; Schejter & Aviram, 1972) with only minor modifications. Sigma Type VI protein was treated with either 90% ^{13}C- or ^{2}H-enriched methyl iodide at pH 3.0 and 25 °C for 24 h. At pH 3 the protein is partially unfolded thus allowing greater access to Met-80 which is normally buried within the protein interior. Only Met-65 and Met-80 are methylated since there are no free sulfhydryl groups, and free amines such as lysine are protonated at low pH. Demethylation of the methionyl residues was accomplished by incubation with dithiothreitol (DTT) at pH 10.5 for 48 h followed by elution on Sephadex G-25 with 0.02 M ammonium bicarbonate at pH 9-9.5. For final purification the samples were eluted on Amberlite CG-50 to remove forms of enriched cytochrome *c* that were not demethylated.

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[1] Abbreviations used: DTT, dithiotreitol; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Me_4Si, tetramethylsilane; CM, carboxymethyl.