^{31}P Nuclear Magnetic Resonance of Phosphonic Acid Analogues of Adenosine Nucleotides as Functions of pH and Magnesium Ion Concentration†

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ABSTRACT: The ^{31}P NMR proton-decoupled spectra of α,β-methylene-ATP [Ap(CH_2)pp], β,γ-methylene-ATP [App(CH_2)p], and α,β-methylene-ADP [Ap(CH_2)p] were measured as functions of pH and Mg^{2+} concentration. Each ATP analogue yielded three resonances: two doublets and one doublet-of-a-doublet. Assignments of resonances were based upon spin-coupling multiplets, their coupling constant magnitudes (24–27 Hz for –P–O–P– and 4–10 Hz for –P–CH_2–P–), and the magnitude of the chemical shift movement during proton titration or its direction of movement. All phosphonyl resonances are substantially downfield compared to phosphoryl resonances. The chemical shifts of terminal phosphonyl units moved upfield with increasing pH or rising Mg^{2+} concentration. The chemical shifts of phosphonyl and phosphoryl anhydride plus ester units usually either moved downfield during proton titration and addition of Mg^{2+} or remained constant. Accurate pK_a' values were readily determined from chemical shift movements as a function of pH: 3.05 ± 0.04 and 8.80 ± 0.05 for App(CH_2)p, 7.34 ± 0.06 for Ap(CH_2)pp, and 8.29 ± 0.02 for Ap(CH_2)p. Addition of Mg^{2+} or Tris produced an acidic shift of the alkaline pK_a' values. Addition of Mg^{2+} at pH 7.0 to the nucleotides caused large movements in the chemical shifts of their terminal two phosphorus atoms.

Cohn & Hughes (1960, 1962) published the first ^{31}P NMR spectra of adenosine nucleotides. More extensive studies of the ^{31}P NMR spectra for natural nucleotides have been published (Tran-Dinh et al., 1975; Labotka et al., 1976). ^{31}P NMR studies have been reported for β,γ-imido-ATP (Tran-Dinh & Roux, 1977) and thiophosphate analogues of adenosine nucleotides (Jaffe & Cohn, 1978). Shriver & Sykes (1981) have obtained spectra of the complexes formed from myosin's subfragment 1 derivative with β,γ-imido-ATP and with ADP. However, no thorough studies of the phosphonyl analogues of ATP and ADP have appeared to date.

The first phosphate-modified adenosine nucleotide analogues to be synthesized were App(CH_2)p[1] (Myers et al., 1963) and Ap(CH_2)p (Myers et al., 1965). A second methylene analogue of ATP, Ap(CH_2)pp, is now commercially available, although Yount (1975) noted that no detailed description of its synthesis has been published. Although these analogues bind to ATP- or ADP-utilizing enzymes, the P–CH_2–P bond is not split by most enzymatic reactions as is the P–O–P bond. Consequently they have been used to study the ATPase of myosin and actomyosin (Moos et al., 1960; Yount et al., 1971; Mannherz et al., 1973), RNA polymerase (Simon et al., 1965), glutamine synthetase (Wellner & Meister, 1966), mitochondrial ADP/ATP translocase (Klingenberg et al., 1971; Duee & Vignais, 1968), adenylate cyclase (Krug et al., 1973), and intestinal 5'-nucleotidase (Burger & Lowenstein, 1975).

We were interested in the use of ^{31}P NMR to study the interaction of nonhydrolyzable analogues of ATP to myosin, actomyosin, and other muscle proteins. Before such studies could be undertaken, we felt it necessary to understand the effect of pH and Mg^{2+} on the chemical shifts and coupling constants of each phosphorus atom in the analogues. This report describes the ^{31}P NMR properties of Ap(CH_2)pp, App(CH_2)p, and Ap(CH_2)p as functions of pH and Mg^{2+} concentration. A preliminary report of a portion of these results has been made (Burt et al., 1979).

Materials and Methods

The sodium salt of App(CH_2)p and the tetralithium salt of Ap(CH_2)pp were purchased from P-L Biochemicals, Inc. A portion of the tetralithium salt of Ap(CH_2)pp was passed through a Dowex-50 acid form column to replace the Li^+ with H^+, taken to dryness by freeze-drying, and dissolved in distilled H_2O. The free acid form of Ap(CH_2)p was synthesized by the procedure of Myers et al. (1965). All other chemicals were purchased from local supply houses.

^{31}P NMR Measurement. The NMR spectrometer was a Bruker HFX-5 with ^2D stabilization operating at 36.43 MHz for ^{31}P. It contained facilities for most modes of Fourier transform signal-averaging and broad band, continuous wave hetronuclear ^1H decoupling. Chemical shift data are reported as parts per million (ppm) relative to 85% inorganic orthophosphoric acid; downfield chemical shifts are positive, and upfield chemical shifts are negative. All NMR spectra were obtained from solutions that also contained 7–10% D_2O.

^{31}P NMR Titrations of Nucleotides. Acid or base was added to constant quantities of each nucleotide. They were then diluted to 2–3 mL with H_2O followed by 0.10 volume of D_2O. Some studies had excess $MgCl_2$, and one experiment had 91 mM Tris. The pH was measured at 23–25 °C on a research pH meter.

Determination of Nucleotide Concentration. Nucleotide concentrations were determined either by weighing out the nucleotide to be dissolved in a measured volume of H_2O or by measuring the nucleotide's absorbancy in 0.10 M Tris-HCl buffer at pH 7.4 using an extinction coefficient of 14.2 mM^{-1} cm^{-1} (P-L Biochemicals, 1979–1980 catalog).

Results

Assignment of Resonances. The β resonances of App(CH_2)p and Ap(CH_2)pp give typical doublet-of-a-doublet

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[1] Abbreviations: Ap(CH_2)pp, α,β-methylene-ATP; App(CH_2)p, β,γ-methylene-ATP; Ap(CH_2)p, α,β-methylene-ADP; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Table I: Coupling Constants of Methylene Analogues of Adenine Nucleotides

pH	nucleotide	coupling constants (Hz) $J_{\alpha-\beta}$	$J_{\beta-\gamma}$
4.45	Ap(CH_2)pp, 4.2 mM	9.8	25.6
8.35	Ap(CH_2)pp, 4.2 mM	8.5	24.4
7.70	App(CH_2)p, 3.6 mM	26.9	7.4
11.60	App(CH_2)p, 3.6 mM	26.9	4.9
3.43	Ap(CH_2)p, 10.0 mM	9.8	
9.62	Ap(CH_2)p, 10.0 mM	9.8	

structures. Since phosphonate resonances occur substantially downfield compared to phosphate resonances, this allows the γ phosphorus of App(CH_2)p and the α phosphorus of Ap(CH_2)pp to be assigned. The remaining resonance of each analogue is then assigned to its remaining phosphorus atom.

Since both doublet resonances of Ap(CH_2)p are downfield, proton-coupled spectra and the pH titration behavior of chemical shifts are used to make assignments. Terminal phosphonate units move downfield with decreasing pH [see Figures 1 and 2 and Lubansky (1978)]. This behavior is observed in the more upfield resonance of Ap(CH_2)p (see Figure 3) identifying it as the β phosphorus. This assignment is confirmed with a proton-coupled spectrum where only the upfield resonance is split into sharply defined doublet-of-a-triplet peaks caused by coupling to the two methylene protons and the α-phosphorus atom. The α phosphorus is assigned to the remaining downfield resonance. This assignment is confirmed with the proton-coupled spectrum where the downfield resonance is broad and consists of many overlapping peaks caused by coupling to two methylene protons, the β phosphorus and the two C-5 ribosyl protons.

All spectra of the nucleotides yielded only their predictable chemical shifts, showing that the nucleotides were pure by ^{31}P NMR analysis. Table I summarizes the coupling constants for the phosphonic acid analogues.

Titration of Ap(CH_2)pp. A portion of Ap(CH_2)pp was converted into the free acid and titrated with 0.1 M tetrabutylammonium hydroxide, because its cation is not chelated by nucleotides. Figure 1 shows the effect of increasing pH on the chemical shifts of the three phosphorus atoms. The α-phosphorus chemical shift showed a 1 ppm rise from pH 6.4 to 8.8. The γ phosphorus showed one large simultaneous increase in the chemical shift, 3.8 ppm, from pH 6.00 to 10.00.

The behavior of the β-phosphorus chemical shift is unusual. At pH 3.15, its chemical shift is 7.1 ppm. As the pH rises to 8.8, there is a shallow decrease to 5.1 ppm. Because this abnormal chemical shift movement could not be related to a proton titration, we carried out a ^{31}P NMR titration of the tetralithium salt of Ap(CH_2)pp (Figure 1). The β-phosphorus chemical shift is now constant between pH 2.9 and 13.00 at 7.0 ppm. The chemical shift movement of the γ phosphorus in the presence of 16.8 mM Li^+ (4.9 ppm) is greater than the movement observed during titration of the free acid form of the nucleotide (3.8 ppm). Also the chemical shift movements of the α- and γ-phosphorus atoms in the presence of Li^+ occur at a slightly lower pH range than during the titration of the free acid nucleotide. Smith & Alberty (1956) showed that Li^+, Na^+, and K^+ but not tetraalkylammonium cations are chelated by nucleotides. This suggests that nucleotide interaction with a nonchelatable cation may produce anomalous movement. As a result all of our succeeding titrations used NaOH for titration.

In a limited study, we determined the chemical shifts of Ap(CH_2)pp (5 mM) at pH 7.0 without and with 15 mM $MgCl_2$. The chelation of Mg^{2+} by App(CH_2)p increased the

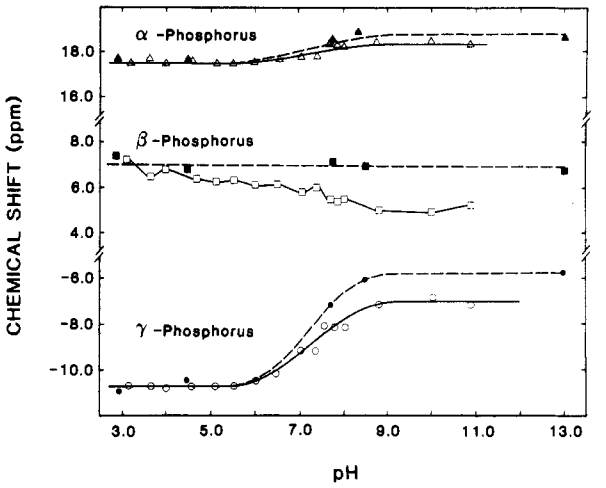

FIGURE 1: Chemical Shifts of the phosphorus atoms of Ap(CH_2)pp as a function of pH. Solutions of the free-acid form of Ap(CH_2)pp (open symbols) and of the tetralithium salt of Ap(CH_2)pp (closed symbols) in 10% (v/v) D_2O were adjusted to varying pH values with tetrabutylammonium hydroxide and HCl solutions. The final nucleotide concentrations were 3.6 mM for the free acid and 4.2 mM for the tetralithium salt. The solid lines represent the titration curves of the free-acid nucleotide titration assuming a pK_a' of 7.34. The broken line represents the titration curve for the tetralithium salt assuming a pK_a' of 7.06. (○, ●) γ-Phosphorus resonance; (□, ■) β-phosphorus resonance; (△, ▲) α-phosphorus resonance.

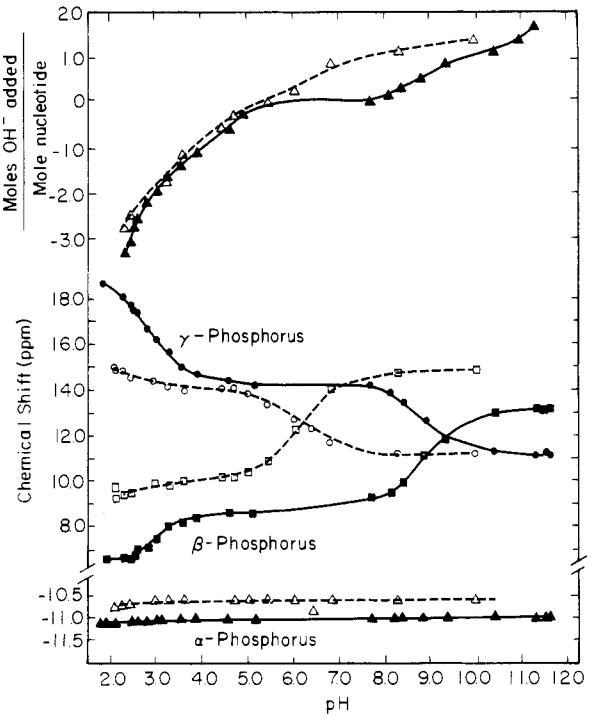

FIGURE 2: Chemical shifts of the phosphorus atoms of App(CH_2)p as a function of pH. App(CH_2)p was titrated with 0.1008 N NaOH and 0.1004 N HCl to varying pH values in 2.20 mL. After their ^{31}P NMR spectra were obtained, 0.020 mL of 1.23 M $MgCl_2$ was added to each solution. The new pH values and ^{31}P NMR spectra in 11 mM $MgCl_2$ were measured. The top plot gives moles of base added or neutralized per mole of App(CH_2)p. The lower plots give the chemical shifts of the phosphorus atoms as a function of pH. All closed symbols represent chemical shifts measured without divalent cation and all open symbols represent chemical shifts measured in the presence of $MgCl_2$. (○, ●) γ Phosphorus; (□, ■) β phosphorus; (△, ▲) α phosphorus.

chemical shifts of only the γ phosphorus (from −9.6 to −5.4 ppm) and β phosphorus (from 6.1 to 8.1 ppm).

Titration of App(CH_2)p. The upper part of Figure 2 shows a plot of moles of acid–base added per mole of App(CH_2)p

Table II: Summary of ^{31}P NMR Titration Analyses

analogue	additions	phosphorus atom	$n \pm$ SE[c]	p$K_a' \pm$ SE[c]	max rise of chemical shift with increasing pH
β,γ-methylene-ATP	none	γ	0.81 ± 0.04	2.93 ± 0.02	−4.15
		β	0.65 ± 0.06	3.22 ± 0.04	1.79
			0.74 ± 0.06[d]	3.05 ± 0.04[d]	
		γ	0.95 ± 0.03	8.81 ± 0.03	−3.14
		β	1.17 ± 0.05	8.79 ± 0.04	4.58
			1.04 ± 0.06[d]	8.80 ± 0.05[d]	
	11 mM $MgCl_2$	γ	1.06 ± 0.04	6.13 ± 0.03	−2.74
		β	1.02 ± 0.02	6.19 ± 0.01	4.76
			1.04 ± 0.05[d]	6.16 ± 0.03[d]	
	91 mM Tris-HCl	γ	1.72 ± 0.09	7.97 ± 0.05	−3.22
		β	2.02 ± 0.19	8.25 ± 0.09	4.58
			1.85 ± 0.30[d]	8.12 ± 0.11[d]	
	91 mM Tris-HCl, 11 mM $MgCl_2$	γ	1.18[a]	5.89[a]	−2.71
		β	1.28 ± 0.15[b]	5.80 ± 0.13[b]	4.89
α,β-methylene-ATP	none	γ	0.98 ± 0.06	7.34 ± 0.06	3.76
α,β-methylene-ADP	none	β	1.01 ± 0.03	8.27 ± 0.02	−3.56
		α	1.00 ± 0.02	8.31 ± 0.01	4.00
			1.01 ± 0.03[d]	8.29 ± 0.02[d]	
	56 mM $MgCl_2$	β	1.12 ± 0.06	5.84 ± 0.05	−3.23
		α	1.07 ± 0.03	5.74 ± 0.02	4.13
			1.09 ± 0.07[d]	5.78 ± 0.05[d]	

[a] Only two titration points occurred between δ_A and δ_B for the γ-phosphorus atom. This is enough to calculate values for the slope and pK_a', but it does not permit a calculation of their standard error. [b] These results for the β phosphorus are based on three points that occurred between δ_A and δ_B. [c] A linear regression analysis was performed to calaulate the pK_a' and Hill coefficient (Li, 1957). [d] An analysis of covariance was used to calculate the adjusted mean values from the values for the two above phosphorus atoms (Li, 1957).

to give the indicated pH. There are two weakly ionizing protons that titrate above pH 3.5. The pK_a' of each titration can be estimated as the pH at which 50% of the protons are titrated. The two pK_a' values estimated from acid–base consumption are 4.7 and 8.8. Addition of $MgCl_2$ causes the proton titration to shift toward lower pH values with estimated pK_a' values of 4.4 and 6.4. These values may be compared to those determined by chemical shift movement (Table II) of 8.80 without $MgCl_2$ and 6.16 with $MgCl_2$. The lower part of Figure 2 shows the chemical shifts as a function of pH. In the absence of Mg^{2+} the β- and γ-phosphorus atoms have two pH regions of significant chemical shift movements, pH 1.8–3.9 and pH 7.7–10.4. The movement at pH 7.7–10.4 corresponds to the alkaline proton titration. There is no movement of either chemical shift from pH 3.9 to 5.2, since the ionizable proton with a pK_a' of 4.7 (estimated from acid–base consumption) occurs on the purine ring (Labotka et al., 1976).

Addition of $MgCl_2$ caused the chemical shift titration curves for the β- and γ-phosphorus atoms to move in an acidic direction. The alkaline chemical shift movement now occurs at pH 4.7–8.3, similar to its acid–base consumption plot. There are no significant chemical shift movements below pH 4.7.

Titration of Ap(CH_2)p. The upper part of Figure 3 presents a plot of the moles of base added per mole of Ap(CH_2)p to give the indicated pH. Two weakly ionizable protons are seen with estimated pK_a' values of 4.3 and 8.5. Addition of excess $MgCl_2$ shifts the curve in an acidic direction. The pK_a' values are now 3.5 and 5.4. The pK_a' values of the alkaline ionizable proton estimated from base consumption are essentially equal to their pK_a' values of 8.29 without $MgCl_2$ and 5.78 with $MgCl_2$ obtained from chemical shift movement (Table II).

The lower part of Figure 3 presents the chemical shifts of the two phosphorus atoms as a function of pH. As the pH rises, the α-phosphorus chemical shift has a small increase from pH 2.1 to 4.0 and a large increase at pH 7.1 to 11.00. The chemical shift of the β phosphorus shows two large decreases

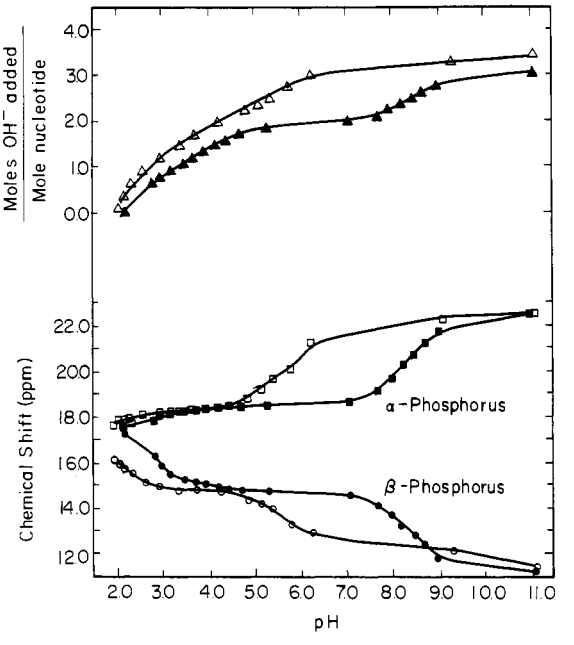

FIGURE 3: Chemical shifts of the phosphorus atoms of Ap(CH_2)p as a function of pH. Ap(CH_2)p was titrated with 0.1008 N NaOH to varying pH values in 2.20 mL. Their ^{31}P NMR spectra were measured. One titration series of solutions did not contain any divalent cation and is represented by closed symbols; a separate series of solutions contained 56 mM $MgCl_2$ and is represented by open symbols. The top plots describe the moles of base added per mole of Ap(CH_2)p as a function of pH. The lower plots give the chemical shifts of the phosphorus atoms as a function of pH. (○, ●) β Phosphorus; (□, ■) α phosphorus.

at pH 2.1–4.0 and at pH 7.1–11.00. Again there is no movement between pH 4.0 and 7.1 since the pK_a' of 4.3 for base titration represents a proton bound to the purine ring.

Addition of $MgCl_2$ causes the two curves to be displaced in an acidic direction. The atoms have their largest chemical

shift movements at pH 4.2–9.1. The β phosphorus has a moderate decrease of its chemical shift as the pH rises from 1.9 to 3.4. The α phosphorus now has no significant movement at this pH region.

Effect of Mg^{2+} Concentration on the Chemical Shifts of $App(CH_2)p$ and $Ap(CH_2)p$. The effects of $MgCl_2$ on the chemical shifts of $App(CH_2)p$ at pH 7.0 in 0.2 M Tris-HCl buffer were determined. The γ-phosphorus chemical shift decreased from 13.8 to 11.4 ppm, and the β-phosphorus chemical shift rose from 9.8 to 14.2 ppm as the molar ratio of $[Mg^{2+}]$:$[App(CH_2)p]$ rose from 0:1 to 6:1. These two chemical shifts intersect at a molar ratio of 0.4:1. The α-phosphorus chemical shift rose from −11.1 to −10.5 ppm as the molar ratio rose from 0:1 to 1:1, and then the chemical shift decreased slightly to −10.8 ppm as the molar ratio increased to 6:1. When the molar ratio is 1:1, the chemical shift movements are 67% complete for the γ phosphorus and 91% complete for the β phosphorus.

The chemical shifts of $Ap(CH_2)p$ as a function of Mg^{2+} at pH 7.0 were also determined. The β-phosphorus chemical shift decreased from 14.1 to 12.3 ppm, and the α-phosphorus chemical shift rose from 19.2 to 21.9 ppm as the molar ratio of $[Mg^{2+}]$:$[Ap(CH_2)p]$ rose from 0:1 to 6:1. At a molar ratio of 1:1, the α- and β-phosphorus chemical shift movements were 74% and 72% complete, respectively.

Discussion

Markley (1973) showed that the movement of an atomic nucleus chemical shift during titration of a conjugate acid can be used to obtain the pK_a' and Hill coefficient (n); if δ_A is the acidic asymptotic chemical shift, δ_B is the basic asymptotic chemical shift, and δ is the chemical shift observed during the proton titration, then $pH = pK_a' + n \log (\delta_A - \delta)/(\delta - \delta_B)$. A normal conjugate acid should yield a Hill coefficient of 1.0. As a practical matter, we concluded that $|\delta_A - \delta_B|$ must be at least 1.5 ppm in order to obtain reliable results with our experimental conditions.

Table II summarizes the pK_a' and Hill coefficient values for the three nucleotides. $App(CH_2)p$ yields two separate pK_a' values of 3.05 and 8.80. The alkaline proton titration yielded the expected Hill coefficient of 1.0, but the acidic proton titration yielded an unexpectedly low value of 0.74. This may be due to either underestimating the absolute value of δ_A or an abnormality of this acidic proton. Addition of excess $MgCl_2$ to $App(CH_2)p$ gives a new pK_a' for the alkaline ionizable proton of 6.16 with a normal Hill coefficient. Thus $MgCl_2$ caused the pK_a' to drop by 2.74 pH units.

Addition of Tris to $App(CH_2)p$ decreased the pK_a' of only the alkaline ionizable proton from 8.80 to 8.12. However, Tris produced an abnormally high Hill coefficient of 1.85 ± 0.20, suggesting that Tris-H^+, whose pK_a' is 8.3 (Good et al., 1966), competes with the last ionizable proton for the same binding sites on the nucleotide. This competition by Tris-H^+ would produce a lower pK_a'. Since the Tris and the nucleotide have titratable protons in nearly the same pH range, the Hill coefficient must be increased to 2. The presence of $MgCl_2$ produces a small drop of the pK_a' from 6.16 without Tris to 5.80 with Tris, and its Hill coefficient with Tris is much closer to 1.

Only the γ phosphorus of $Ap(CH_2)pp$ had a sufficiently large chemical shift movement to apply the above equation. Its pK_a' in the absence of Li^+ is 7.34, and its Hill coefficient is 1.0. Only one chemical shift value for the tetralithium salt was obtained within the pH range of the alkaline proton titration. Assuming a Hill coefficient of 1.0, this point yields a pK_a' of 7.06.

The pK_a' of only the alkaline ionizable proton of $Ap(CH_2)p$ can be determined from the movement of chemical shifts. The pK_a' values are 8.29 without any addition and 5.78 in the presence of $MgCl_2$, and the Hill coefficients were both normal.

Myers et al. (1965) reported the pK_a' values of $App(CH_2)p$ from the acid–base titration to be 4.2 and 8.4. These values are slightly less than the pK_a' values for $App(CH_2)p$ reported here of 4.8 and 8.80. This difference is probably due to differences in ionic strength and the presence of chelatable monovalent cations. Myers et al. (1963) reported the pK_a' values for $Ap(CH_2)p$ to be 4.00 and 8.15. These values are very close to our pK_a' values of 4.2 and 8.29.

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