Characterization of Methylphosphonate as a $^{31}$P NMR pH Indicator*

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The $^{31}$P NMR pH indicator, methylphosphonate, has been extensively characterized, and the uncertainty in pH determination by its chemical shift has been analyzed. The $pK_a$ decreases by 0.003 pH unit/°C and 0.06 pH unit/100 mM ionic strength. The $pK_a$ appears not to be sensitive to Ca$^{2+}$ but is sensitive to Mg$^{2+}$, resulting in an uncertainty of 0.05 pH unit. Substituting 300 mM Na$^+$ for 300 mM K$^+$ causes the $pK_a$ to decrease by 0.1 pH unit. Taking the effects of temperature, ionic strength, and cation identity into account, the overall estimated uncertainty in pH determination can be as high as ±0.1 pH unit.

Methylphosphonate was tested as a pH indicator in Ehrlich ascites tumor cells. Our data indicate that both the unchanged and monoanion forms of methyl phosphonate are very permeable, rendering this compound unsuitable as a pH indicator in this system. However, the sensitivity of this compound’s chemical shift to pH and the relative insensitivity to other parameters suggest that phosphonates, as a group, may be applicable as pH indicators by $^{31}$P NMR.

$^{31}$P NMR has been used extensively to measure both intracellular and extracellular pH in living cells and tissues. P$_i$ and 2-dGlu-6-P$^+$ are the two most commonly used indicators of pH in $^{31}$P NMR studies. The advantage of using P$_i$ is that it is endogenous to all cells. The disadvantages are that P$_i$ levels are generally low and change with the metabolic state of the cell (1, 2), and the intra- and extracellular P$_i$ peaks are indistinguishable from each other unless the pH gradient across the cell membrane is greater than about 0.27 pH unit (1). The indicator 2-dGlu-6-P$^+$ is not metabolized but inhibits glycolysis and, therefore, can be toxic to cells. In addition, factors other than pH, such as ionic strength and Mg$^{2+}$, can affect the chemical shift of both P$_i$ and 2-dGlu-6-P$^+$ (3). Because of the problems associated with these indicators, other indicators have been sought that are more ideal: highly sensitive to pH in the physiological region; unaffected by factors other than pH; nontoxic; and, therefore, can be toxic to cells. In addition, factors other than pH, such as ionic strength and Mg$^{2+}$, can affect the chemical shift of both P$_i$ and 2-dGlu-6-P$^+$ (3). Because of the problems associated with these indicators, other indicators have been sought that are more ideal: highly sensitive to pH in the physiological region; unaffected by factors other than pH; nontoxic; and, therefore, can be toxic to cells.

Methylphosphonate was first used as an intracellular pH indicator in Escherichia coli by Slonczewski et al. (4) who found that ionic strength had a negligible effect on its $pK_a$. Studies by Gillies et al. (1) confirmed a negligible effect of ionic strength and also suggested that methylphosphonate was relatively impermeable to several mammalian cell types. More recently, methylphosphonate has been used by Lin et al. (5) as an external pH indicator in frog skin studies where it is impermeable and by Labotka and Kleps (6) and Stewart et al. (7) as an intracellular pH indicator in erythrocytes, where it apparently equilibrates rapidly across the membrane. Stewart et al. (7) have also suggested that the chemical shift of methylphosphonate is somewhat dependent on ionic strength.

In the present communication, we analyze the behavior of this indicator in vitro and in Ehrlich ascites tumor cells. We analyze the uncertainty in pH determined by the chemical shift of methylphosphonate as a function of temperature, ionic strength, Mg$^{2+}$, Ca$^{2+}$, and Na$^+$ and K$^+$. We show methylphosphonate to be nontoxic to EAT cells, yet it is not an ideal indicator of intracellular pH in this system due to its high permeability to cellular membranes.

MATERIALS AND METHODS

Cell Culture—Ehrlich letré carcinoma cells, strain E, were obtained from American Type Culture Collection and were cultured in suspension using minimum essential medium-autoclavable (GIBCO) supplemented with 10% fetal bovine serum (HyClone partially characterized, Logan, UT). Cultures were initially inoculated at approximately 1 x 10$^6$ cells/ml. For loading cells, methylphosphonate was added to a final concentration of 20 mM in this growth medium by dissolving the appropriate amount of methylphosphonic acid (Alfa Products, Danvers, MA) into serum-free medium, titrating to pH 7.2 with 10 M NaOH, and filtering through a Sterivex-GS 0.22-μm filter unit (Millipore, Bedford, MA) directly into the culture. Cells were grown at 37 °C in 5% CO$_2$, 95% air to a density of 2-5 x 10$^5$ cells/ml (3-5 days) prior to harvest.

For harvesting, the cells were first placed at 4 °C for 1 h/liter of cells. After this time, they were collected by centrifuging for 7 min at 1000 x g in 250-ml polystyrene tubes (Corning Glass Works, Corning, NY). The cell pellets were resuspended (typically 1 ml of pelleted cells/liter of suspension was obtained), pooled, and washed with 250 ml of cold (4 °C) buffer A: (in mM) 5 KCl, 140 NaCl, 1.2 CaCl$_2$, 2 MgSO$_4$, 4.2 or 20 NaHCO$_3$, 1 KH$_2$PO$_4$, and 50 or 25 PIPES at pH 6.7. The cells were centrifuged again and resuspended in buffer A to cell densities of 20-30% in either a 10- or 20-mm outer diameter NMR tube (Wilcock Glass Co., Inc., Buena, NJ) containing 25 μl of Antifoam B emulsion (Sigma). The suspensions were kept in an ice bath for transfer to the spectrometer (about 5 min). At 37 °C, buffer A pH becomes 7.0.

NMR Spectroscopy—Depending on accessibility, either a Nicolet NT-380 with a 20-mm probe operating at 145.78 MHz or a Bruker WP-200 with a 10-mm broadband probe operating at 81.02 MHz was used for the in vivo experiments. These performed on the Nicolet were accomplished using a hollow fiber bioreactor (2). This bioreactor contains a fiber basket for perfusion and aeration and an air-driven impeller to keep the cells suspended. Experiments have shown that 10$^6$ cells/ml (30% cell density) can be maintained in this reactor for up to 10 h (2). In the in vitro experiments performed on the Bruker WP-200 with a 20-mm probe operating at 145.78 MHz or a Bruker WP-200 with a 10-mm broadband probe operating at 81.02 MHz was used for the in vivo experiments. These performed on the Nicolet were accomplished using a hollow fiber bioreactor (2). This bioreactor contains a fiber basket for perfusion and aeration and an air-driven impeller to keep the cells suspended. Experiments have shown that 10$^6$ cells/ml (30% cell density) can be maintained in this reactor for up to 10 h (2). In the in vivo experiments performed on the Bruker WP-200 with a 20-mm probe operating at 145.78 MHz or a Bruker WP-200 with a 10-mm broadband probe operating at 81.02 MHz was used for the in vivo experiments. These performed on the Nicolet were accomplished using a hollow fiber bioreactor (2). This bioreactor contains a fiber basket for perfusion and aeration and an air-driven impeller to keep the cells suspended. Experiments have shown that 10$^6$ cells/ml (30% cell density) can be maintained in this reactor for up to 10 h (2).
rate of about 2 bubbles/s. In vivo spectra were initially referenced to the α-phosphate of ATP at -10.05 ppm or, when appropriate, to either diethylmethyl phosphonate at +36.07 ppm or GPC at +0.49 ppm relative to 85% phosphoric acid. All in vitro experiments were performed on the Bruker instrument. The titration spectra were referenced to GPC and/or diethylmethyl phosphonate. Control studies have shown that the chemical shift of diethylmethyl phosphonate is not affected by changes in Ca²⁺, Me⁺, Z⁺, temperature, Na⁺, or K⁺ and that it is nontoxic to EAT cells at concentrations of 10 mM. Spectra were obtained with acquisition parameters as indicated in the figure legends.

Titrations—Intracellular environment titrations were accomplished using a buffer containing methylphosphonate. The BSA was treated with chelating resin (Dow) and extensively dialyzed against ultrapure water (Millipore) to remove contaminating metal ions. The bench titrations were accomplished using a Beckman pH 71 meter, a Beckman 68200 glass electrode, and a Microscribe 4500 strip chart recorder. The samples were maintained at the appropriate temperatures using a Precision 254 circulating water bath. The pKₐ values were determined graphically by the base end points.

RESULTS

Effect of Temperature—pKₐ data obtained from titrations at various temperatures on two ionic strengths are illustrated in Table I. These data indicate that the pKₐ decreases consistently with increasing temperature. At I = 306 mM the ΔpKₐ/°C is -0.005 with a linear correlation coefficient of 0.96, whereas for I = 93 mM it is -0.003 (r = 0.79). Similar results were obtained for KCl with 0.2 molar ratio Mg²⁺/0.08 molar ratio Ca²⁺ or for 300 mM NaCl, in which the ΔpKₐ/°C were -0.005 and -0.003, respectively. Titration of KCl at I = 93 mM on the NMR yielded pKₐ values of 7.65 and 7.62 at 25 (not shown) and 37 °C (Table II), respectively. These data are consistent with a change of about -0.004 pH unit/°C increase.

Effect of Ionic Strength—Table I also illustrates the effect of ionic strength on the pKₐ of methylphosphonate. The two extremes of ionic strength were chosen to encompass ionic strength values found in vivo. A change in the pKₐ of less than -0.12 pH unit occurs over the 213 mM change in ionic strength tested. Assuming the change in the pKₐ to be linear with respect to changes in ionic strength, which is a valid approximation of a form of Davie’s equation discussed in Perrin and Dempsey (8), a maximum change in the pKₐ of -0.06 pH unit/100 mM change in ionic strength occurs. NMR titrations provided similar results. KCl solutions of 93 and 306 mM ionic strengths at 37°C result in pKₐ values of 7.62 and 7.47, respectively (Table II), a difference in pKₐ values of 0.15 over the same range.

The first three lines of Table III show the direct effect of ionic strength on the chemical shift of methylphosphonate at pH 7.5 and 37 °C. A plot of these data gives an effect of -0.135 ppm/100 mM change in ionic strength. Since the sensitivity of methylphosphonate is 2.2 ppm/pH unit at pH 7.5 (see below), these data correspond to an effect of 0.061 pH unit/100 mM ionic strength.

### Table I

| Temperature (°C) | I  | N | pKₐ | ΔpKₐ | ΔpKₐ/100 mM I |
|-----------------|----|---|-----|------|--------------|
| 37              | 306| 8 | 7.52 ± 0.02 | -0.003 |              |
| 37              | 93 | 4 | 7.59 ± 0.07 | -0.07 ± 0.05 |              |
| 37              | 325| 8 | 7.46 ± 0.04 | -0.06 ± 0.03 |              |
| 37              | 306| 7 | 7.50 ± 0.05 | -0.08 ± 0.04 |              |
| 22              | 306| 7 | 7.57 ± 0.03 | -0.08 ± 0.04 | -0.004      |
| 22              | 93 | 4 | 7.65 ± 0.04 | -0.08 ± 0.04 | -0.004      |
| 22              | 325| 4 | 7.54 ± 0.01 | -0.03 ± 0.02 |              |
| 22              | 306| 3 | 7.47 ± 0.02 | -0.10 ± 0.04 |              |
| 15              | 306| 4 | 7.59 ± 0.07 | -0.12 ± 0.07 | -0.006      |
| 15              | 93 | 4 | 7.71 ± 0.07 | -0.12 ± 0.07 | -0.006      |

*Calculated at the pKₐ.  
*Number of samples.  
*Mean with standard deviation.  
*Difference from 306 mM KCl.  
*Also contains 5 mM Mg²⁺/2 mM Ca²⁺.  
*NaCl instead of KCl.

### Table II

| Condition | Chemical shift of | pKₐ  |
|-----------|-------------------|------|
| HA        | A⁻               | pKₐ  |
| Methylphosphonate | 93 mM KCl | 24.93 | 20.93 | 22.94 | -0.72 |
|           | 306 mM KCl       | 24.93 | 20.93 | 22.94 | 0.07  |
| I.E. + 0.04 molar ratio Mg²⁺ | 24.88 | 21.03 | 22.96 | 0.75  |
| I.E. + 1.0 molar ratio Mg²⁺ | 24.88 | 20.97 | 22.93 | 0.75  |
| I.E. + 2.0 molar ratio Mg²⁺ | 24.85 | 21.12 | 22.91 | 0.75  |
| 300 mM KCl | 24.93 | 20.93 | 22.94 | 0.75  |
| 150 mM KCl, 150 mM NaCl | 24.93 | 21.00 | 22.92 | 0.75  |
| 300 mM NaCl | 24.93 | 21.03 | 22.98 | 0.75  |
| P₁        | I.E. + 0.08 molar ratio Mg²⁺ | 0.73 | 3.32 | 2.03 | 0.68 |
|           | I.E. + 2.0 molar ratio Mg²⁺ | 0.73 | 3.32 | 2.03 | 0.68 |
|           | I.E. + 4.0 molar ratio Mg²⁺ | 0.73 | 3.28 | 2.01 | 0.68 |
| 150 mM KCl, 150 mM NaCl | 0.67 | 3.37 | 2.02 | 0.68 |
| 300 mM NaCl | 0.75 | 3.37 | 2.06 | 0.58  |

*a HA, chemical shift of acidic end point.  
*a A⁻, chemical shift of basic end point.  
*a I.E., intracellular environment buffer (see “Materials and Methods”).

### Table III

| Sample | I | Methylphosphonate | Diethylmethyl phosphonate |
|--------|---|-------------------|---------------------------|
|        | mM | ppm   | ppm     | ppm   |
| 300 KCl | 338 | 22.81 ± 0.04 | 36.05 |
| 150 KCl | 190 | 22.98 ± 0.02 | 36.05 |
| 50 KCl  | 90  | 23.15      | 36.05 |
| 300 KCl/10 Ca²⁺ | 367 | 22.73 | 36.05 |
| 300 KCl/25 Ca²⁺ | 412 | 22.73 | 36.05 |
| 300 KCl/10 Mg²⁺ | 368 | 22.71 ± 0.04 | 36.03 ± 0.04 |
| 300 KCl/25 Mg²⁺ | 414 | 22.43 | 36.13 ± 0.04 |
| 300 KCl/10 Ca²⁺/10 Mg²⁺ | 397 | 22.67 | 36.05 |
| 300 KCl/25 Ca²⁺/25 Mg²⁺ | 22.49 | 36.13 ± 0.04 |
| 300 NaCl²⁺ | 333 | 22.87 | 36.05 (set) |
| 300 NaCl | 338 | 22.77 | 36.05 |

*Mean values and standard deviations. Standard deviations of 0 are not shown.  
*Contained 10 mM methylphosphonate and no GPC. Chemical shifts are referenced to diethylmethyl phosphonate set to 36.05 ppm.  
*Titrated with KOH.
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**Ca$^{2+}$ and Mg$^{2+}$ Effect**—Since cells can contain varying amounts of either Ca$^{2+}$ or Mg$^{2+}$, several experiments were designed to investigate the effects of these divalent cations on the titration curves and chemical shift of methylphosphonate. Table I lists $pK_a$ values at 22 and 37 °C with or without both 0.08 molar ratio (2 mM) Ca$^{2+}$ and 0.2 molar ratio (5 mM) Mg$^{2+}$. Correcting for the change in ionic strength from 306 to 325 mM with both cations present (which would account for a $pK_a$ change of approximately -0.011) the $pK_a$ values are observed to change by -0.02 at 22 °C and -0.04 pH unit at 37 °C.

Changes in NMR chemical shift values for methylphosphonate in solutions at 37 °C, pH 7.5, and containing Ca$^{2+}$ and Mg$^{2+}$ both at 0.4 molar ratio (10 mM) or 1.0 molar ratio (25 mM) are -0.14 and -0.32 ppm, respectively (Table III).

For Ca$^{2+}$ alone, Table III shows an apparent effect of -0.08 ppm change for either 0.4 or 1.0 molar ratio Ca$^{2+}$. However, in another experiment, NMR titrations with Ca$^{2+}$ of a 300 mM KCl solution at 37 °C showed no change in the chemical shift of methylphosphonate up to a molar ratio of 0.4. When the effect of ionic strength is taken into account, the change in chemical shift becomes -0.04 ppm for a molar ratio of 0.4 and +0.02 ppm for a molar ratio of 1.0 resulting in -0.1 ppm/molar ratio and +0.02 ppm/molar ratio, respectively. Thus, it appears that the effects for Ca$^{2+}$ alone are inconsistent and small.

For Mg$^{2+}$ alone, Table III indicates chemical shift changes of -0.1 ppm for a molar ratio of 0.4 and -0.38 ppm for a molar ratio of 1.0. Taking into consideration the effect of ionic strength on chemical shift, these values become -0.06 and -0.28 ppm, respectively, which correspond to a Mg$^{2+}$ effect -0.15 to -0.28 ppm/molar ratio.

**Na$^+$ versus K$^+$ Effect**—Table I shows a change in the $pK_a$ of -0.1 pH unit when 300 mM Na$^+$ is substituted for 300 mM K$^+$ at either 22 or 37 °C. NMR titration data (Table II) provide similar results indicating that a difference in the $pK_a$ values of 0.05 pH unit occurs between successive samples. NMR data in Table III indicate a -0.1 ppm change at pH 7.5. Similar results were obtained for P$_i$, where the $pK_a$ appeared to decrease about 0.05 pH unit between the two tested samples (Table II).

**Intracellular Environment Titrations**—Buffers approximating the intracellular environment of EAT cells were titrated at 37 °C to yield calibration curves for intracellular pH determinations. Data for 0.04 molar ratio, 1 molar ratio, and 2 molar ratio (0.4, 10, and 20 mM, respectively) Mg$^{2+}$ are shown in Table II. A titration at 0.5 molar ratio Mg$^{2+}$ produced a curve coincident with the 0.04 molar ratio curve (not shown). Comparing the 1 and 2 molar ratio data, in the worst case the $pK_a$ decreased by 0.1 pH unit with the increase in Mg$^{2+}$. Table II also shows the response of P$_i$ in the same titrations. The Mg$^{2+}$ effect is similar in that the maximum effect occurs near the $pK_a$. The effect on P$_i$, however, is not observed until Mg$^{2+}$ is raised to a molar ratio of 4, which decreases the $pK_a$ by 0.15 pH unit.

**Methylphosphonate as an Intracellular pH Indicator in EAT Cells**—Adding methylphosphonate directly to a cell suspension while acquiring $^{31}$P spectra fails to give rise to a methylphosphonate peak corresponding to intracellular pH within 5 h (data not shown). Therefore, we have attempted to load methylphosphonate into cells by growing cultures in its presence. Methylphosphonate can be loaded into EAT cells by incubation in the growth media as described under "Materials and Methods." Growth assays are illustrated in Fig. 1 and indicate that concentrations as high as 20 mM methylphosphonate do not inhibit growth. When cells are grown in the presence of 20 mM methylphosphonate for several days, exhaustively washed, and then acid-extracted, $^{31}$P NMR spectra such as those shown in Fig. 2 are obtained. Similar results have been obtained in as little as 24 h of incubation. Taking differential saturation into account, the concentrations of intracellular methylphosphonate are about 5 mM when cells are incubated in 20 mM methylphosphonate. In vivo $^{31}$P spectra of methylphosphonate-loaded cells in a buffer containing 1 mM methylphosphonate at pH 6.8 show a methylphosphonate peak that corresponds to pH = 7.14 (Fig. 3). However, as illustrated in the time course of Fig. 4, after 15 min a second peak appears, corresponding to pH = 6.78. After 25 min, this downfield peak has increased while the upfield peak has virtually disappeared. At times after 25 min there appears to be only the downfield peak.

The above results indicate that the methylphosphonate is leaking out of these cells. This hypothesis was tested by placing washed methylphosphonate-loaded cells for 1 h in a methylphosphonate-free buffer in an NMR tube through which was bubbled carbogen. $^{31}$P spectra of the resulting supernatant showed a methylphosphonate peak of intensity equivalent to about 0.08 mM as determined by standard addition. This amount corresponded to the expected total amount in the cells, further indicating that it had leaked out during incubations.

**DISCUSSION**

**Uncertainty of pH Measurement**—In NMR measurements, pH is inferred from the chemical shift of a suitable indicator. Hence, a relevant question is: "What is the uncertainty in pH from the measured chemical shift?" In the present study, uncertainty arises from two sources, i.e. in assigning chemical shift and in relating chemical shift to pH.

Uncertainty in assigning chemical shift is a function of both signal-to-noise ratio and line widths. The absolute error in assigning chemical shift can be estimated to be one-half of the peak width at one noise level below the peak height. For methylphosphonate in vivo, this value is about ±25 Hz, corresponding to errors of up to 0.16 pH unit in assigning pH at the $pK_a$. On the other hand, the standard error of the mean,
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FIG. 2. 81.02 MHz $^{31}$P NMR spectrum of perchloric acid cell extract. This spectrum is the Fourier transform of 78,160 free induction decays arising from a 57° tipping pulse applied every 0.82 s at 22 °C. The free induction decay was subjected to 1 Hz exponential line broadening prior to Fourier transformation. Chemical shifts are referenced to GPC at 0.49 ppm.

FIG. 3. 145.75 MHz in vivo $^{31}$P NMR spectrum of EAT cells. Methylphosphonate-loaded EAT cells in the bioreactor were at 30% cell density (1 X 10⁶ cells/ml) and 37 °C. Spectrum is the result of 15 min accumulation with a 70° pulse applied every 1.3 s. Exponential multiplication of the free induction decay was accomplished with 30-Hz line broadening. Chemical shifts of peaks were assigned as the average between the shifts at half-height.

arrived at by repetitive sampling, is ±7 Hz or 0.04 pH unit.

Uncertainty in relating pH to chemical shift is illustrated in Fig. 5, which shows two titration curves, A and B, representing an indicator under two different conditions and thus two different $pK_a$ values. At a measured chemical shift ($\delta_m$) there are two possible values of pH ($pH_{1m}$ or $pH_{2m}$) that can be estimated. Using the median pH value between the two curves as the determined pH, an uncertainty of ±$\frac{1}{2}$ ($pH_{1m} - pH_{2m}$) results. At some point, one has to decide what level of uncertainty is tolerable. Generally, an uncertainty in assigning intracellular pH of ±0.05 pH unit is tolerable, since most other measurements of intracellular pH are not more accurate (3, 9).

Temperature Effect—Titration data have shown that a −0.004 pH unit/°C change in the $pK_a$ of methylphosphonate occurs with temperature (Table I). This effect can be considered negligible as long as the temperature is maintained within ±10 °C, which is easily attainable. This is borne out by NMR experiments which have measured no significant differences between the relative chemical shifts of methylphosphonate at 20 and 37 °C.

Ionic Strength Effects—The $pK_a$ of methylphosphonate is dependent on ionic strength both in theory (8) and in practice (our data and Ref. 7). The effect of ionic strength on the $pK_a$ of methylphosphonate is similar to that observed for P, and 2-dGlu-6-P (3) in that the end points of the titrations are not affected by ionic strength, yet the $pK_a$ is affected. This effect on $pK_a$ is translated into an effect on the chemical shift. Our data indicate a change in chemical shift of −0.994 ppm/100 mM ionic strength, while Stewart et al. (7) shows −0.829 ppm/“ionic strength unit.” However, the dependence of chemical shift on ionic strength at a particular pH is not the relevant parameter. The parameter of interest is the uncertainty in the pH determined by a particular chemical shift, as discussed above. Titration curves must be performed at ionic strengths which encompass those estimated to occur physiologically. In the present study, such data indicate a maximum deviation in estimated pH of 0.12 pH unit. This maximum occurs at pH
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**Fig. 4.** Sequential 5-min regional $^{31}P$ spectra of EAT cells preincubated in 20 mM methylphosphonate. Cells were cultured for 4 days in the presence of methylphosphonate, harvested, and resuspended as detailed in the text. At $t = 0$, cold cell suspension was placed in a Bruker 200-MHz magnet that was prewarmed to 37 °C. Spectra were collected in 5-min blocks thereafter. Data indicate loss of upfield peak and subsequent appearance of downfield peak.

**Fig. 5.** Uncertainty in pH measurement and calibration curve. The above curves were computer-generated from the Henderson-Hasselbach equation under two theoretical conditions, A and B, resulting in two different $pK_a$ values. The chemical shift end points are from the intracellular environment experiments. Note that the $pK_a = 7.5$ curve is a calibration curve for methylphosphonate in EAT cells.

Values near the $pK_a$. Therefore, the maximum uncertainty in absolute pH determination is ±0.06 pH unit. The uncertainties for $P$, and 2-dGlu-6-P are ±0.08 and ±0.1 pH unit, respectively (1). The uncertainties of determining changes in pH are much less, since cells presumably do not change their ionic strength by large amounts. Therefore, although the chemical shift of methylphosphonate is dependent upon ionic strength, the effect of ionic strength is negligible in determining the pH.

**Ca$^{2+}$ Effect**—The effect of Ca$^{2+}$ on the chemical shift of methylphosphonate is inconsistent and small. The worst case, at 0.4 molar ratio, indicated a ~0.1 ppm/molar ratio change. Increasing the molar ratio to 1.0 causes a change in the chemical shift in the opposite direction. We interpret this inconsistency, combined with the small changes observed, as Ca$^{2+}$ having a negligible effect on the pH determined by the chemical shift of methylphosphonate.

**Mg$^{2+}$ Effect**—Our data suggest that Mg$^{2+}$ has some effect on the $pK_a$ and, hence, chemical shift of methylphosphonate. At 1.0 molar ratio, neglecting effects of ionic strength, Mg$^{2+}$ has a ~0.3 ppm/molar ratio effect on the chemical shift of methylphosphonate at its $pK_a$. However, in the presence of other compounds which stimulate the intracellular environment, no effect of Mg$^{2+}$ was observed up to 1.0 molar ratio. The reason for this difference is currently unexplainable. It is possible that the BSA or HEPES in the intracellular environment buffer may be chelating some of the Mg$^{2+}$. BSA is an unlikely candidate, however, because titrations with unchelated and chelated BSA are identical. Also, Roberts et al. (3) showed that BSA at 50 mg/ml had no effect on the titration curves of $P$ or 2-dGlu-6-P in the presence or absence of 5 mM MgCl$_2$. Nonetheless, the relevant analysis involves the uncertainty of pH as discussed above. From this type of analysis, Mg$^{2+}$ at a molar ratio of 2.0 in the intracellular environment causes an uncertainty of ±0.05 pH unit in the pH determined by the chemical shift of methylphosphonate. The data suggest that a 2:1 complexation of Mg$^{2+}$ to methylphosphonate is occurring. However, this is not conclusive since it appears from data in Table III that molar ratios less than 2 also have an effect.

It appears from the above data that the major effect of divalent cations on the chemical shift of methylphosphonate is due mostly to Mg$^{2+}$ and that Ca$^{2+}$ has little or no effect. This is also suggested by the following observations. (a) The change in chemical shift by Mg$^{2+}$ alone accounts for the combined Mg$^{2+}$/Ca$^{2+}$ effect (Table III); and (b) NMR titration curves containing 0.2 molar ratio, Mg$^{2+}$ with and without 0.08 molar ratio Ca$^{2+}$ are coincident (not shown). These data imply that either there is no effect due to Ca$^{2+}$ or that the Ca$^{2+}$ effect is masked by the Mg$^{2+}$ effect.

**The Effect of Monovalent Cations**—The differences in $pK_a$ between methylphosphonate in solutions of 300 mM K$^+$ and 300 mM Na$^+$ is as large as 0.1 pH unit. This would represent an uncertainty due to monovalent cations of ±0.05 pH unit. Under physiological conditions, this uncertainty would be much less, since the extra- and intracellular concentrations of both of these ions are well known and relatively constant.

**Fig. 6.** Sensitivities of the $^{31}P$ NMR pH indicators $P$, 2-dGlu-6-P, and methylphosphonate. The curves were determined from computer-generated data points of the Henderson-Hasselbach equation using experimental chemical shift end points and $pK_a$ values at 37 °C and $I$ of 150 mM, the estimated intracellular ionic strength of EAT cells. Note: data for $P$ are from Ref. 1 and data for 2-dGlu-6-P from Ref. 11.
This difference has relevance, however, since methylphosphonate can be used as either an intracellular (4, 6, 7) or an extracellular (5) pH indicator.

Uncertainty in pH Determined by Methylphosphonate—

Titration of methylphosphonate in a buffer simulating the intracellular environment yields curve B shown in Fig. 5 with \( pK_a = 7.5 \). At present, it appears that the maximum uncertainty in pH as determined by the chemical shift of methylphosphonate is \( \pm 0.1 \) pH unit. This uncertainty has been estimated primarily by the added effects of \( Mg^{2+} \) and ionic strength on the \( pK_a \) of methylphosphonate. However, it is unlikely that these effects will be additive, in which case the uncertainty would be less. Additionally, if ionic strength and \( Mg^{2+} \) are known and can be monitored, the uncertainty could be reduced further.

Comparison of Methylphosphonate-, \( P_i \), and 2-Deoxyglucose-6-P—Fig. 6 shows the relative sensitivities of the three \( ^{31}P \) NMR pH indicators methylphosphonate, \( P_i \), and 2-dGlu-6-P. Near physiological pH 7.2 (the vertical line on the graph), the sensitivities are 2.00, 1.23, and 0.59 ppm/pH unit, respectively. Methylphosphonate is considerably more sensitive to pH changes than either \( P_i \) or 2-dGlu-6-P near physiological pH, thus giving more accurate pH values as determined by the chemical shift.

Determination of Intracellular pH in Vivo—The experiments illustrated in Figs. 3 and 4 indicate that methylphosphonate is leaking out of EAT cells at an appreciable rate. This in contrast to the long equilibration time required for methylphosphonate to enter these cells, as observed in NMR spectra from both extracted and in vivo samples. One relevant observation that sheds light on this apparent discrepancy is the fact that, even after several days in 20 mM methylphosphonate, EAT cells contained only 5-10 mM internal methylphosphonate. This phenomenon has been observed a number of times at different time periods and suggests that the concentrations inside the cell are less than the external concentrations at equilibrium. If these are equilibrium values, the only way this could occur is if either methylphosphonate is actually exported from the cell or if the monovalent anion is permeable. Assuming a membrane potential of \(-30 \) mV (10), the equilibrium concentration ratio (out/in) would be 3.29 for a permeant monovalent anion and 10.83 for a permeant dianion. Since the observed ratio is about 2.67 (20/7.5), we feel our data strongly suggest that the monovalent anion is permeable.

Nevertheless, it has been difficult to ascertain the intracellular concentrations of methylphosphonate in vivo. The \( T_1 \) of methylphosphonate phosphorous is about 5 s, which contributes to a poor signal-to-noise ratio for methylphosphonate, since saturation of the peak occurs when using Ernst angle pulsing for the ATP peaks. This poor signal-to-noise ratio contributes to the inaccuracy of pH determination with methylphosphonate in these cells.

It appears that methylphosphonate is not suitable as an intracellular pH indicator in EAT cells due to leakage. However, we are in the process of using a different maintenance system which may be able to overcome the poor signal-to-noise ratio discussed above. The use of methylphosphonate in cell systems other than those noted above has yet to be explored.

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