Dimethyl Methylphosphonate (DMMP): A ^{31}P Nuclear Magnetic Resonance Spectroscopic Probe of Intracellular Volume in Mammalian Cell Cultures†

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Received August 13, 1992; Revised Manuscript Received January 26, 1993

ABSTRACT: Dimethyl methylphosphonate (DMMP), when added to a suspension of erythrocytes, has been reported to have a lower frequency chemical shift inside of cells than outside. This work further investigates the same phenomenon in hollow-fiber bioreactor cultures of six mammalian cell lines and describes the application of DMMP as a measure of intra- versus extracellular volumes in mammalian cell cultures. No toxic effects of the DMMP were observed at the concentrations used here. The dependence of the shift of intracellular DMMP on intracellular protein content was shown to be similar for cultured mammalian and red blood cells. Also consistent with shifts in erythrocytes, an increase in the intracellular protein concentration due to a reduction in cultured cell volume increased the magnitude of the shift to lower frequency. Longitudinal relaxation (T_1) values for intra- and extracellular DMMP were measured so that partially saturated DMMP peaks in ^{31}P NMR spectra of mammalian cell cultures can be corrected to give the relative volumes of the intra- and extracellular compartments; this information provides a relative measure of culture growth. Intracellular volume measured by this method can also be used to quantify intracellular metabolites such as ATP during the growth of the culture. To explore the mechanism behind the intracellular shift, we have also addressed the three possible contributions to the chemical shift of DMMP: hydrogen-bonding interactions, magnetic susceptibility, and ionic strength. Data is presented which eliminates the latter two mechanisms and strongly supports the hypothesis that the observed intracellular shift is due to a reduction in hydrogen bonding between water and DMMP in the cytoplasm.

Dimethyl methylphosphonate (DMMP,[1] $(CH_3O)_2CH_3PO$) is a small, neutral molecule that, in a suspension of red blood cells, partitions evenly between intra- and extracellular compartments. In such suspensions, the intracellular ^{31}P nuclear magnetic resonance (NMR) of DMMP occurs at lower frequency than the extracellular resonance. These observations led to a series of studies characterizing transfer of DMMP across erythrocyte membranes (Kirk & Kuchel, 1986; Potts et al., 1989), describing its use for measuring erythrocyte volume (Kirk & Kuchel, 1988; Raftos et al., 1988), and investigating the mechanism behind the shift in the intracellular resonance (Kirk & Kuchel, 1988a,b; Xu et al., 1991). To date, all work describing the ^{31}P NMR spectroscopy of DMMP has been performed on erythrocytes. However, erythrocytes differ in many respects from other mammalian cells: they lack organelles and endomembrane systems, have a very low membrane potential and a very large hemoglobin content, and do not replicate.

The work presented here is two-pronged: to examine the intracellular shift of DMMP in mammalian cell cultures and to provide additional evidence for the mechanism giving rise to the intracellular shift. For the former, we report intracellular DMMP shifts in a number of mammalian cell lines cultured in NMR-compatible hollow-fiber bioreactors and demonstrate the use of DMMP as a measure of intra- and extracellular volumes in these cultures. The intracellular/extracellular volume ratio provides a measure of relative cell density during the growth of a culture. Work was recently completed in this laboratory using ^{31}P NMR spectroscopy of DMMP to measure the volume of bioreactor cultures of C6 glioma cells (Lien et al., 1992). With additional information and a few simple assumptions, DMMP can also be used to quantify the absolute concentrations of metabolites in the ^{31}P NMR spectra of mammalian cell cultures.

To probe the mechanism behind the intracellular shift of DMMP, Kirk and Kuchel (1988) measured ^{31}P NMR shifts of DMMP in a number of pure solvents having different hydrogen-bonding characteristics than water. These investigators also reported small shifts of DMMP to lower frequency in the presence of either lysozyme or diamagnetic hemoglobin. In both cases, magnetic susceptibility effects were accounted for through the use of a spherical bulb reference. These workers hypothesized that the observed shifts in DMMP are due to changes in the hydrogen-bonding interaction between DMMP and the solvent.

In the current work, we have attempted to verify this hypothesis by addressing the three possible contributions to the chemical shift of DMMP: hydrogen-bonding interactions (i.e., solvation), bulk magnetic susceptibility, and ionic strength. ^{31}P NMR experiments were performed on DMMP over a range of solvent concentrations for mixtures of water with three organic solvents which hydrogen bond with varying strengths. The observed shifts in DMMP were corrected for contributions from susceptibility, and ^{31}P NMR spectra of DMMP in high ionic strength solutions verified that the shifts

† This research was supported by NIH R01 GM43046 and American Cancer Society CN-57 (to R.J.G.).

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified essential medium; DMMP, dimethyl methylphosphonate; EAT, Erlich ascites tumor; FBS, fetal bovine serum; FID, free induction decay; GPC, glycerophosphorylcholine; NMR, nuclear magnetic resonance; NAD, nicotinamide adenine dinucleotide; PCho, phosphorylcholine; PCr, phosphocreatine; PEth, phosphorylethanolamine; P_i, inorganic phosphate; TFE, trifluoroethanol; UDP, uridine diphosphate.

0006-2960/93/0432-4665$04.00/0

are not affected by that property of the cytoplasm. The residual DMMP shifts as a function of the mole fraction of solvent verify that the chemical shift of DMMP depends on hydrogen bonding between DMMP and the solvent.

The chemical shift of DMMP was also measured in varying concentrations of the protein albumin and compared to intracellular shifts in three mammalian cell lines in which the intracellular protein concentration was determined. These observations suggest that the presence of protein alters the hydrogen-bonding equilibrium of water to intracellular solutes and may have significance for the thermodynamics of protein dynamics.

MATERIALS AND METHODS

Mammalian Cell Cultures. The NMR-compatible bioreactor circuit is described in detail elsewhere (Gillies et al., 1991). Briefly, the circuit continually circulates incubated oxygenated medium through ~1400 cellulose acetate fibers in a 25-mm-diameter hollow-fiber bioreactor containing 10^8–10^{10} cells in the extracapillary spaces between the porous fibers. The flow rate through the circuit was varied from about 80 to 170 mL/min during the growth of the culture, as needed to deliver sufficient oxygen to the cells.

All culture media were prepared from powdered media and sodium bicarbonate from Sigma Chemical Co. (St. Louis, MO). Penicillin-G (60 mg/L) and streptomycin sulfate (100 mg/L) (Sigma) were added to all media; 2.5 or 5 mM DMMP (97%, Aldrich Chemical Co., Milwaukee, WI) was added to media used in bioreactor cultures. The C6 rat glioma cells were obtained from the American Type Culture Collection (Beltsville, MD) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT). The 9L rat glioma cells were cultured in DMEM supplemented with 10% FBS. Both glioma lines were cultured first as monolayers and then as suspensions in spinner flasks, where they grew as spheroids. The spheroids were transferred to the bioreactor when the cells reached 10^8–10^{10} in number. Chinese hamster ovary (CHO) cells were cultured as monolayers in McCoy's 5A medium supplemented with 10% FBS; the cells were a gift from Dr. E. W. Gerner (University of Arizona). The cells were seeded onto macroporous gelatin beads (CultiSphere-G, HyClone Laboratories, Inc., Logan, UT) for growth in suspension, where they were adapted to DMEM supplemented with 5% FBS; ~10^8 cells were transferred to the bioreactor. Erlich ascites tumor (EAT) cells, strain A-31 (CCL 77), were purchased from the American Type Culture Collection and cultured in minimal essential medium (MEM) supplemented with 10% FBS. The EPO 8F7 cells are an Sp-2 mouse-derived hybridoma which secrete the F12 monoclonal antibody against human erythropoietin. They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Nu-serum (Collaborative Research, Inc., Bedford, MA); they were provided as a generous gift from Amgen, Inc. Both the EAT and the EPO cells are anchorage-independent lines which were grown in suspension to a density of 10^5–10^6 cells/mL in 1–2 L before being transferred to the bioreactor. RN1a cells (mouse 3T3 fibroblasts transfected with the yeast PMA-1 gene) were obtained from Dr. R. Perona (Inst. Invest. Biomed., Madrid) and cultured as monolayers in high-glucose DMEM supplemented with 10% Nu-serum. For inoculation into the bioreactor, the cells were grown in suspension on CultiSphere-G gelatin beads until their number reached ~10^9.

The rat C6 glioma cells were subjected to hyperosmotic shock on the fourth day of bioreactor culture by adding NaCl to the culture medium to increase the sodium content from 160 to 240 mM. The response of the cells was monitored by acquiring ^{31}P NMR spectra every 30 s beginning 5 min prior to, and for 30 min following, the addition of NaCl.

NMR of Bioreactor Cell Cultures. For acquisition of ^{31}P NMR spectra, the bioreactor was placed in a Bruker AMX400 wide-bore spectrometer fitted with a 25-mm probe built by Bruker/Spectrospin (Zürich) and tuned to 162 MHz. For all spectra other than spin–lattice relaxation experiments (see below), a one-pulse sequence was used with a 25–30-μs phase (~30°), a sweep width of 100 ppm, and a recycle time of 1–2 s; WALTZ-16 broad-band decoupling was applied at the ^{1}H frequency during acquisition. Typically a free induction decay (FID) consisted of 4K or 8K points and between 3600 and 7200 scans.

DMMP Toxicity Studies. The toxicity of DMMP on CHO cells was measured in 24-well culture plates inoculated with 5×10^3 cells in each well in McCoy's 5A medium. The DMMP was added 4 h later to concentrations of 0, 0.5, 2.5, 5.0, 10.0, and 20.0 mM, with four wells per DMMP concentration. One set of plates were fixed with glutaraldehyde after 71 h of growth; the others, after 108 h. The cells were stained with 0.1% crystal violet, and the relative cell numbers were determined by measuring absorbance at 590 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer (Gillies et al., 1986).

Spin–Lattice Relaxation (T_1) Measurements. The spectral parameters used to acquire ^{31}P NMR spectra of cell cultures are normally optimized for the β-ATP resonance and use a recycle time of only about 1 s, which is many times shorter than the T_1 of DMMP (~13 s in a bioreactor with flow). Therefore the DMMP peaks in these spectra will always be partially saturated and thus considerably less intense than in a fully relaxed spectrum. In order to use DMMP as an indicator of the relative volume of cells occupying a bioreactor, T_1 values for the intra- and extracellular compartments are necessary to correct the peak areas from the partially saturated spectra to give areas representing fully relaxed peaks. For a peak acquired with a recycle time t_R, the area of the partially saturated peak, $A(t_R)$, relative to the fully relaxed peak area, A_o, can be calculated from the equation for the exponential rate of growth of the longitudinal magnetization (Freeman, 1988):

$$A(t_R)/A_o = (1 - e^{-t_R/T_1}) \cos \theta \quad (1)$$

where θ is the tip angle of the excitation pulse (30° in our experiments).

The T_1 value for extracellular DMMP in the absence of flow at 37 °C was measured on a bioreactor containing culture medium but no cells. A progressive saturation pulse sequence was used with a relaxation delay of 150 μs, an excitation pulse of 55 μs, and 104 acquisitions; 12 variables delays were used, ranging from 2 ms to 120 s. WALTZ-16 decoupling was applied during acquisition, and no line broadening was applied to the FID.

Four T_1 measurements on DMMP were made on two separate CHO bioreactor cultures, with two measurements each using inversion–recovery and progressive saturation pulse sequences. The progressive saturation experiments were performed as described above, except that 128 and 256 scans were acquired in each experiment and a 1-Hz line broadening was used. The inversion–recovery experiments were similar, except that a relaxation delay of 120 s and a 90° pulse of 50 μs were used and 16 scans were acquired. The intra- and extracellular peaks were deconvoluted with standard Bruker

software, and the data were analyzed with a nonlinear least-squares fit to a single exponential for each peak using SigmaPlot software (Jandel Scientific, Corte Madera, CA) on an IBM PC.

The Effect of Solvent on the Chemical Shift of DMMP. The chemical shift of DMMP was measured in a range of solvent systems in which varying amounts of water were replaced by either 1,4-dioxane, 2,2,2-trifluoroethanol (TFE), or ethanol. The fluorines on TFE render the hydroxyl hydrogen much more acidic than that on ethanol; therefore TFE hydrogen bonds more strongly than ethanol but less strongly than water. Dioxane undergoes little or no hydrogen bonding. Deuterated water (99.9%) was purchased from Aldrich Chemical Co.; 1,4-dioxane (99%), from Fisher Scientific (Pittsburgh, PA); TFE (100%), from Pfaltz-Bauer, Inc. (Waterbury, CT); and ethanol (100%), from Midwest Grain Product Co. (Weston, MO).

To make these measurements, two DMMP solutions were measured in the NMR spectrometer at once, using a coaxial NMR tube (Wilmad Glass Co., Buena, NJ) in a 10-mm NMR tube. For the TFE and ethanol experiments, the inner tube contained 15 mM DMMP in 1:2 H_2O/D_2O, while the outer tube contained 6 mM DMMP in varying solvent systems in which 0% to 100% of the water was replaced with TFE or ethanol. For dioxane, 8 mM DMMP in 2:1 H_2O/D_2O was placed in the inner tube, while the outer tube contained 2.0 mL of 4 mM DMMP in which the dioxane was varied from 0% to 100%. The chemical shift of DMMP is equal in H_2O and D_2O (Kirk & Kuchel, 1988).

The proton-decoupled ^{31}P NMR spectra were acquired on a Bruker AMX400 wide-bore spectrometer with a 10-mm broad-band probe tuned to 162 MHz. A single-pulse sequence was used with a pulse width of 5.3 μs (30°). The recycle times and sweep widths were 3.0 s and 30 ppm for TFE, 3.5 s and 56 ppm for dioxane, and 4.7 s and 10 ppm for ethanol. In all cases, 1500 transients were collected and inverse-gated WALTZ-16 decoupling was applied during acquisition. An exponential line broadening of 0.1 or 0.5 Hz was applied before Fourier transformation. The temperature of the samples was maintained at 25 °C with the variable temperature unit on the spectrometer.

The Effect of Ionic Strength on the Chemical Shift of DMMP. ^{31}P NMR spectra were acquired of 6 mM DMMP in three solutions having high ionic strength, relative to 15 mM DMMP in 1:2 H_2O/D_2O in a coaxial tube. The solutions were (a) 50 mM $CaCl_2$ (Sigma Chemical Co.), (b) 166 mM KCl (Fisher Scientific), and (c) Dulbecco's modified essential medium (DMEM) with 10 mM HEPES buffer. The NMR spectra were acquired as described for the ethanol solutions in the preceding section, except that 100–300 FIDs were collected and the decoupler was off at all times except the first one-sixth of acquisition to avoid heating the sample.

^{19}F NMR of Trifluoroethanol/Water Solutions. ^{19}F NMR spectra were acquired on the same TFE/water solutions described above, with a 0.1 mM solution of sodium trifluoroacetate (Aldrich Chemical Co., Inc.) in 1:2 H_2O/D_2O in the coaxial reference tube. The spectra were acquired on a Bruker AM500 spectrometer using the decoupling channel of a 10-mm broad-band probe tuned to 470.56 MHz for fluorine. A one-pulse sequence was used with a 15-μs pulse (~30°), a 10-ppm sweep width, a 3.0-s recycle time, 8 scans, 16 K points, and no line broadening.

The Effect of Albumin on the Chemical Shift of DMMP. Bovine serum albumin (BSA fraction V; crystalline, >99%) was purchased from CalBiochem Corp. (La Jolla, CA). A coaxial tube was used as described above, with the inner reference tube containing 30 mM DMMP in 2:1 H_2O/D_2O. The outer tube contained 5 mM DMMP in water with varying amounts of BSA ranging from 0 to 316 mg/mL. The pH of all BSA solutions was decreased with HCl to between 5.18 and 5.22, and then the density of the solutions was measured (by weighing 1.00 mL of solution) to determine the final weight concentration of BSA. The pH of the solution without BSA was lowered to 5.25. The ^{31}P NMR spectra at 25 °C were acquired using a one-pulse sequence with a 5.3-μs pulse width, a recycle time of 2.6 s, and WALTZ-16 broad-band decoupling during acquisition; 1500 scans were accumulated, and a line broadening of 0.5 was applied.

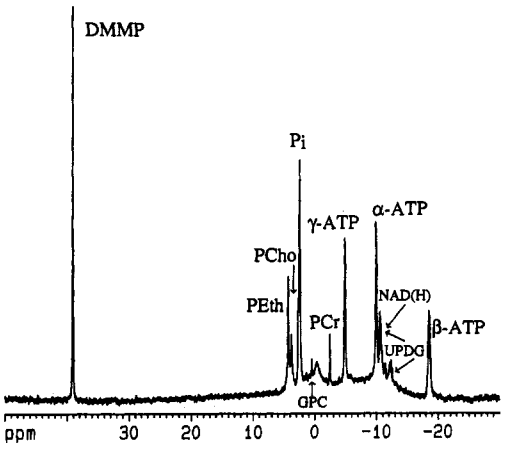

FIGURE 1: Proton-decoupled ^{31}P NMR spectrum of CHO cells attached to 650 mg of gelatin beads, after 351 hours of growth in a hollow-fiber bioreactor. The splitting of the DMMP peak is too small to be resolved here (cf. Figure 2). The spectrum was acquired with a 30-μs excitation pulse, a recycle time of 1 s, and 7200 transients. A 2-Hz exponential line broadening was applied prior to Fourier transformation.

Determination of Intracellular Protein Concentration. Intracellular protein concentration was measured on CHO, EAT, and RN1a cells as described (Gillies & Deamer, 1979). Briefly, cells were incubated for 60 min in DMEM containing 0.1 mCi/mL ^3H_2O and 0.01 mCi/mL ^{14}C-sorbitol. This medium was aspirated off, and the remaining cells were solubilized by washing in 1.0 mL of 0.1 N NaOH. Five hundred microliters of each sample was solubilized in Aquasol liquid scintillation counter cocktail (Dupont New England Nuclear, Boston, MA) and counted using double-label discrimination; counts were corrected for crossover to yield ^{14}C and ^3H counts. The ^{14}C counts were used to correct for extracellular volumes, and the remaining ^3H_2O counts yielded intracellular volumes. The remaining 500-μL aliquots were assayed in triplicate for protein content using the Lowry method with BSA fraction V (Sigma Chemical Co.) as a standard.

RESULTS AND DISCUSSION

DMMP in Mammalian Cell Cultures. A typical ^{31}P NMR spectrum of a hollow-fiber bioreactor cell culture is given in Figure 1. The intracellular DMMP peak in all six mammalian cell lines cultured in the bioreactor (CHO, C6 glioma, EAT, EPO, 9L glioma, and RN1a) was shifted by between 15 and 18 Hz (0.093 to 0.11 ppm) relative to the external peak, while the total integral of intra- and extracellular DMMP varied very little with increasing cell density (Figure 2). Since the intracellular T_1 value is equal to the apparent extracellular T_1 within experimental error (see below), the constant integral indicates that DMMP is neither sequestered nor excluded by

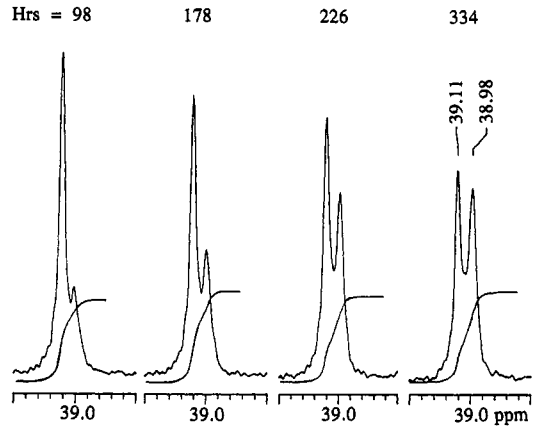

FIGURE 2: Extra- and intracellular ^{31}P NMR DMMP resonances as a function of the growth of an EAT cell hollow-fiber bioreactor culture. Note that the intracellular peak (lower frequency) grows with time relative to the extracellular peak as the cells occupy more of the bioreactor volume. Each unit increment on the frequency axis equals 0.1 ppm.

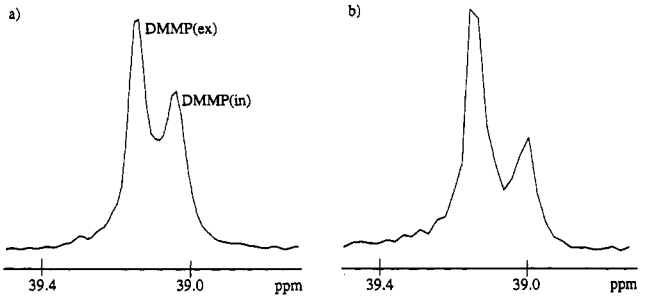

FIGURE 3: ^{31}P NMR DMMP resonances for a bioreactor culture of rat C6 glioma cells subjected to hyperosmotic shock by adding NaCl to the culture medium. The spectrum in (a) was acquired just prior to raising the extracellular Na^+ concentration, while that in (b) is a sum of 60 spectra acquired every 30 s from 30 s to 30 min following hyperosmotic shock (each spectrum consists of 16 acquisitions). The resulting cell shrinkage caused an increase in the shift of the intracellular peak from 16 Hz (0.099 ppm) to 22 Hz (0.136 ppm), as well as a decrease in peak height.

the cells. This is to be expected for a small, uncharged polar molecule and is consistent with results for erythrocytes that DMMP diffuses passively across the plasma membrane (Potts et al., 1989).

The experiments testing the toxicity of DMMP on CHO cells found that DMMP concentrations of up to 10 mM were nontoxic after 71 and 108 h (data not shown). DMMP concentrations of 2.5–5 mM are therefore safe for use in mammalian cell cultures.

The chemical shift of intracellular DMMP was measured as a function of cell density in bioreactor cultures of CHO cells. Measurements on 48 spectra during the growth of two CHO bioreactor cultures gave a mean separation between intra- and extracellular DMMP of 16.3 (±1.7) Hz, with no significant difference between the two experiments. No trend was found between the intracellular shift and the cell density in four different cell lines (CHO, C6 and 9L glioma, and EAT) in which the shift was monitored as a function of growth (Figure 2).

The effect of changes in cell volume on the intracellular shift was also examined. Hyperosmotically shocked C6 glioma cells showed an increase in the intracellular shift of DMMP (Figure 3), consistent with the trend reported in shrunken erythrocytes (Raftos et al., 1988). The cell shrinkage induced by raising the extracellular Na^+ concentration by 33% caused the amount of intracellular DMMP to decrease by 33%.

The intracellular T_1 value for DMMP was calculated in CHO cells to be 13.2 ± 0.9 s, while the *apparent* extracellular T_1 value was found to be 12.9 ± 1.4 s. The results from the inversion–recovery and progressive saturation experiments were equal within experimental error. The apparent extracellular T_1 value is an empirical measurement which includes the effects of the rapid flow of the medium through the bioreactor fibers and the much smaller Starling flow in the extracapillary spaces containing the cells. The T_1 relaxation time of DMMP in a bioreactor in the *absence of both cells and flow* was 14.9 ± 0.1 s. In order to determine if flow affects the apparent T_1 value of external DMMP under conditions normally used to acquire ^{31}P NMR spectra (as described in Materials and Methods), spectra were acquired on bioreactors without cells both with and without flow. The spectra, consisting of DMMP and P_i peaks, were identical (data not shown), indicating that the rate of flow has no effect on the apparent T_1 value of external DMMP when short recycle times are used.

The intracellular and apparent extracellular T_1 values can be used to correct the partially saturated DMMP peak integrals from the ^{31}P NMR spectra, allowing cell growth in the culture to be monitored in terms of the ratio of the intracellular to the extracellular compartment. Given the longitudinal relaxation times for all intracellular metabolites, the concentration of DMMP and the relative intra- and extracellular volumes can also be used to calculate the absolute quantities of the metabolites from each spectrum. A potential obstacle to calculating absolute quantities is the long excitation pulse often required for Ernst angle pulsing of 25-mm bioreactors using conventional probes. The longer a pulse, the smaller the frequency range that is uniformly excited. A square pulse produces an excitation envelope whose intensity distribution is defined by a sinc function. According to this function, at the edges of a spectrum 16 kHz wide the radio frequency power of a 30-μs pulse has been attenuated to 90% of the maximum power. The phosphorous resonances, contained in the central 10 kHz of the spectrum, experience no more than 5% loss of power. Therefore the experimental conditions used in this work result in a nearly uniform degree of radio frequency excitation.

Another possible source of error is in estimation of peak areas. These data can be fit with a Lorentzian function using Bruker software, and the residuals integrate to less than 5% of the total peak areas (Lien et al., 1992). In expressing the data as percent intracellular volume, the term for the area of the DMMP peak appears in both the numerator and the denominator. Accordingly, error propogation indicates that the errors inherent to estimating percent intracellular volume are 3.2% when intra- and extracellular peaks are of equal height and 5.3% when the intracellular peak is 25% of the total area. In addition, the Lorentzian fits indicate that the line widths of the intracellular DMMP are wider (15.8 ± 0.4 Hz) than those of the extracellular (11.6 ± 0.6), suggesting that the internal milieu is more heterogeneous.

The Mechanism for the Chemical Shift of Intracellular DMMP. There are two mechanisms by which solvent molecules can affect the resonance frequency of a solute molecule: (1) the bulk magnetic susceptibility of the solvent and (2) intermolecular solute–solvent and solute–solute interactions (Pople et al., 1959). In the current work, the latter mechanism involves hydrogen-bonding interactions and possibly ionic strength. The chemical shift of DMMP was not influenced by the presence of either KCl (166 mM), $CaCl_2$ (50 mM), or cell culture medium (162 mM), as the resonances

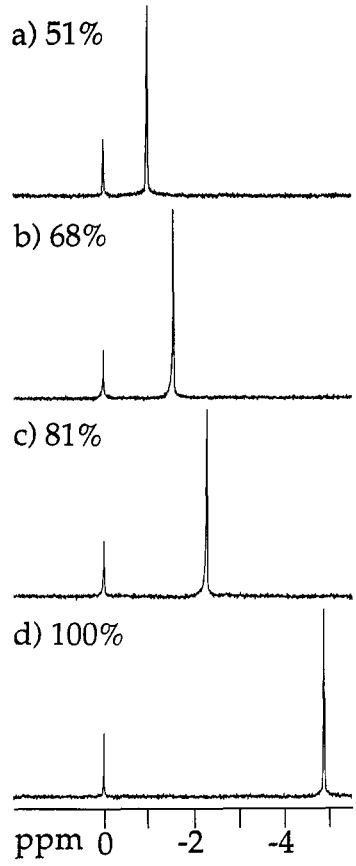

FIGURE 4: ^{31}P NMR spectra of DMMP in different mol % mixtures of water and 1,4-dioxane. The high-frequency peak at 0 ppm corresponds to an inner reference tube solution of DMMP. The outer reservoir of the NMR tube contains the dioxane solutions producing the low-frequency peaks. The shifts are plotted graphically in Figure 5.

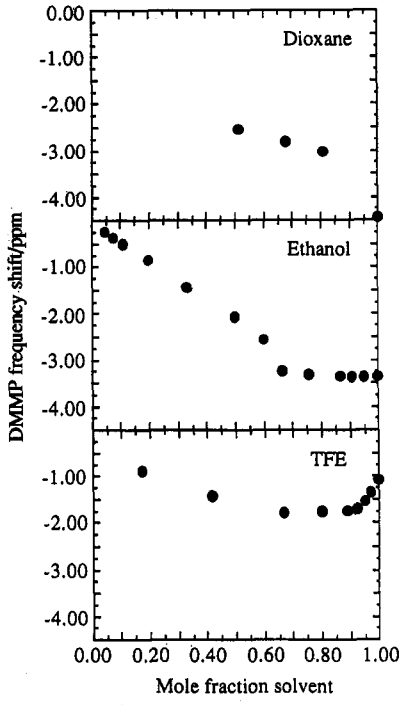

FIGURE 5: Dependence of the ^{31}P NMR chemical shift of DMMP on the mole fraction of three organic solvents in water. The shifts are relative to a coaxial tube containing DMMP.

were neither shifted nor broadened relative to pure water (line widths were consistently 3 to 4 Hz at half-height). Therefore ionic strength does not contribute to the intracellular shift of DMMP.

Kirk and Kuchel (1988) measured the chemical shift of DMMP in a number of pure solvents and found that it shifted to lower frequency in solvents which hydrogen bond less strongly than water to DMMP, and to higher frequency in sulfuric acid, which hydrogen bonds more strongly than water; susceptibility effects in these experiments were accounted for by measuring the chemical shifts relative to a spherical bulb reference. In an effort to provide additional evidence for the dependence of the chemical shift of DMMP on hydrogen bonding with the solvent, the shift in DMMP as a function of solvent concentration was compared in three solvents, 1,4-dioxane, ethanol, and 2,2,2-trifluoroethanol (TFE). ^{31}P NMR spectra for the dioxane/water mixtures are shown in Figure 4.

To assess the contribution of bulk diamagnetic susceptibility to the shifts in DMMP, the volume susceptibility, χ, in units of ppm was calculated (Chu et al., 1990):

$$\chi = 4\pi(10^6)\chi_M c \qquad (2)$$

where χ_M is the molar susceptibility of the solvent in units of $cm^3\ mol^{-1}$ and c is its concentration in mol cm^{-3}. If the NMR sample tubes are modeled as infinite cylinders (with respect to the NMR coil), the contribution of the susceptibility to the resonance frequency is one-third that given by eq 2 (Chu et al., 1990). Values for χ_M for dioxane, H_2O, and D_2O (−52.16, −12.97, and −12.76 × 10^6 $cm^3\ mol^{-1}$, respectively) were obtained from the literature (Weast, 1972), while χ_M for TFE was estimated from Pascal constants for the elements to be −44.6 × 10^6 $cm^3\ mol^{-1}$ (Pople et al., 1959) (The calculated value for TFE is believed to be an accurate estimate since the calculation from Pascal constants for *difluoroethanol* was only 0.18 × 10^6 $cm^3\ mol^{-1}$ less than the measured value (Weast, 1972).). The resulting contributions of susceptibility to the chemical shift were subtracted from the observed shifts; the residual shifts in DMMP due to hydrogen bonding are plotted in Figure 5. Note that the shifts in the TFE/H_2O mixtures decrease as the mixture approaches pure TFE. It is expected that the hydrogen bonding of water to the methyl fluorine atoms increases as the proportion of water increases. This is supported by ^{19}F NMR spectra of TFE/H_2O mixtures showing that increasing water content induces a steadily increasing shift in TFE to higher frequency (data not shown). Therefore the hydrogen-bonding capability of the hydroxyl group in pure (or nearly pure) TFE is greater than in hydrated TFE, causing the shift in DMMP to decrease.

Kirk and Kuchel (1988) proposed that lysozyme- and hemoglobin-induced shifts of DMMP to lower frequency occur by perturbing the hydrogen bonding between water and DMMP, and that this same mechanism was responsible for the intracellular shift of DMMP. In the current work we have observed shifts to lower frequency in the presence of a different protein, bovine serum albumin (BSA), which generates shifts about 4 times larger than those induced by either lysozyme or hemoglobin. The shift increased to 0.380 ppm (61.6 Hz) with a BSA concentration of 316 mg/mL. In suspension cultures of CHO, RN1a, and EAT cells, the intracellular protein concentrations were determined to be 155 (±5) mg/mL in both CHO and RN1a cells and 173 (±27) mg/mL in EAT cells.

In Figure 6 these values are plotted as a function of the intracellular chemical shift of DMMP, along with values for diamagnetic erythrocytes taken from Figure 1 of Raftos et al. (1988). Figure 6 shows that the dependence of the shift in cells is similar to that in pure albumin solutions and also that

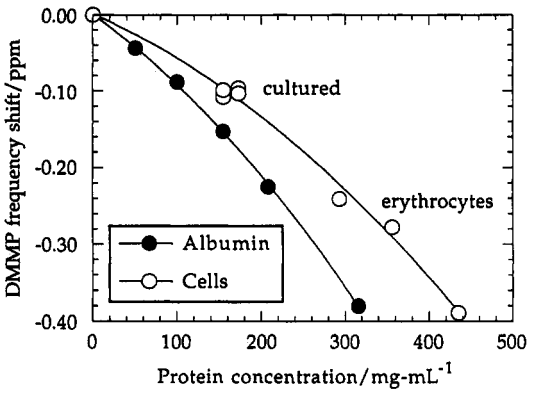

FIGURE 6: Dependence of the ^{31}P NMR chemical shift of DMMP on the concentration of protein. The filled symbols denote shifts due to solutions of bovine serum albumin at pH 5.2. The open symbols correspond to the intracellular shifts in four cell types: the upper four points correspond to cultures of CHO, RN1a, and EAT cells (two separate cultures of EAT cells), and the lower three points correspond to erythrocytes with carbonmonoxyhemoglobin, from Raftos et al. (1988). Both sets of points were fit to second-order polynomials.

the dependence of the intracellular shift on intracellular protein concentration is very similar in mammalian cell cultures and erythrocyte suspensions. We suspect that the difference in the curves for albumin and cells is related to the surface area/volume ratio of the proteins, since cell proteins have larger molecular weights than albumin and are sometimes partially buried in membranes. These data, together with the data in different solvent systems, indicate that the intracellular shift of DMMP is largely due to protein-induced reductions in the extent of hydrogen bonding of water to DMMP. This may be due to hydrogen bonding between DMMP and proteins, reducing the interactions of DMMP with water and thereby shifting to the left the equilibrium

$$\mathrm{DMMP} + \mathrm{H_2O} \Leftrightarrow \mathrm{DMMP{\cdot}H_2O}$$

The data also suggests that membranes do not contribute significantly to this effect, since the dependence of the shift is similar in cultured cells and erythrocytes, and the latter lack endomembrane systems.

SUMMARY

In this work we have extended the work of Kuchel and co-workers on the intracellular shift of DMMP in erythrocytes to mammalian cell cultures. Intracellular ^{31}P NMR shifts of the DMMP resonance in hollow-fiber bioreactor cultures of six cultured mammalian cell lines, at DMMP concentrations having no apparent effects on culture growth, can be resolved from the extracellular resonance. Consistent with shifts in erythrocytes, the intracellular shift in cultured cells increased when the volume of the cells was reduced, and the shift exhibited the same dependence on intracellular protein concentration as in erythrocytes. Evidence was presented showing that the resonance of DMMP in different solvent systems depends largely on hydrogen-bonding interactions between DMMP and the solvent.

Together with the observation that there is a similar dependence of the intracellular shift on intracellular protein concentration among different cell types, the above data support the hypothesis of Kuchel and co-workers that the intracellular shift is due to protein-induced reductions in the average hydrogen-bonding strength between water and DMMP. It has recently been shown that reduced hydrogen-bonding strength (i.e., solvation energy) could have a significant impact on the calculated thermodynamics of protein conformational transitions (Columb et al., 1992).

Longitudinal relaxation (T_1) values for intra- and extracellular DMMP were measured so that partially saturated DMMP peaks from the ^{31}P NMR spectra of mammalian cell cultures can be corrected to give the relative volumes of the intra- and extracellular compartments in the bioreactor. This information provides a relative measure of culture growth and can also be applied to quantify metabolites such as ATP during the growth of the culture.

ACKNOWLEDGMENT

The authors would like to thank G. M. Martinez for her technical help and Drs. Charles Springer and Philip Kuchel for their helpful discussions in the early stages of this project.

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