JOURNAL OF MAGNETIC RESONANCE 51, 124–127 (1983)

A ^{31}P NMR External Reference for Intact Biological Systems

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Received July 20, 1982

A significant concern in the application of high-resolution ^{31}P nuclear magnetic resonance to intact biological systems is the development of a convenient external intensity (concentration) and chemical shift reference. Because one is often monitoring changing metabolite concentrations, derived from integrated resonance peak areas, it is essential to have a reference peak that will (i) monitor and identify nonmetabolic fluctuations in signal intensity (changes in probe tuning, spectrometer problems, etc.) and (ii) that will provide a correlation between integrated intensities and concentration. These considerations are especially critical when dealing with samples such as whole animals and perfused organs where metabolic differences between individual subjects can complicate attempts at quantitation of generalized metabolism.

For these purposes, a useful external reference needs to meet a number of criteria. (i) The reference should have a chemical shift near the phosphorus metabolite spectral region so that the reference peak can conveniently be observed simultaneously with the metabolite peaks but not so close as to overlap or cause baseline distortions of any of the metabolite peaks. (ii) The reference should not require proton decoupling to present a reasonably narrow line. This avoids unnecessary radiofrequency heating effects on conductive tissue samples. A narrow peak can also allow evaluation of field homogeneity stability during a long (many hour) experiment. (iii) The reference should have a minimal chemical shift change with temperature, over a small physiological range around 37°C, so that if needed it can also provide an external chemical shift reference at physiological temperatures that is not affected by minor temperature fluctuations. (iv) Finally, the reference compound should be easy to work with, readily available from a commercial supplier and reasonably priced.

Methylenediphosphonic acid, ($H_2O_3PCH_2PO_3H_2$) has been used frequently in ^{31}P NMR studies of intact biological systems (*1*). This compound requires ^{1}H decoupling to present a useful linewidth and has a chemical shift that is strongly pH dependent (*2*). The use of 85% H_3PO_4, generally used as a ^{31}P NMR external reference in a wide range of chemical systems (*3*), is unacceptable as it overlaps the phosphorus metabolite ^{31}P NMR spectral region.

We wish to suggest the use of hexachlorocyclotriphosphazene (HCCTP), I,

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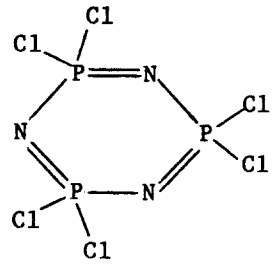

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as an external reference in ^{31}P studies of intact biological systems. As is shown in Fig. 1., a typical ^{31}P spectrum of perfused rabbit heart with HCCTP as an external reference, our proposed reference meets to a large extent the criteria discussed above. (i) The chemical shift of HCCTP is close to the phosphorus metabolite spectral region but not so close as to cause overlap problems. The chemical shift of HCCTP referenced to external 85% H_3PO_4 or phosphocreatine (PCr), the often used internal ^{31}P chemical shift reference in tissue studies (*6*), is given in Table 1. (ii) Because of the absence of proton coupling, the ^{31}P linewidth of HCCTP is relatively narrow, approximately 11 to 13 Hz at 145.8 MHz, and ^{1}H decoupling is not needed. (iii) A plot of the HCCTP chemical shift as a function of temperature, Fig. 2 shows a small linear variation about the normal physiological range. Between 8 and 50°C the chemical shift varies by only 0.34 ppm. Therefore, minor temperature fluctuations should not affect the use of HCCTP as an external chemical shift reference. (iv) HCCTP is commercially

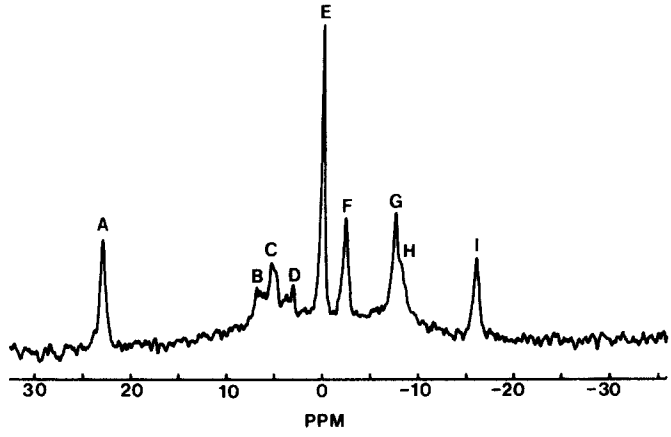

FIG. 1. ^{31}P NMR spectrum of Langendorff perfused, isovolumic rabbit heart (approximately 6 grams, 37°C) at 145.8 MHz. Spectrum represents four minutes of data averaging (240 scans, 45° pulses, 10,000-Hz spectral width, 1,024 total data points, zero filled to 2,048 points, 25-Hz exponential line broadening filter applied). No proton decoupling was employed. Intracellular phosphocreatine (PCr) is taken as 0.0 ppm and IUPAC convention (*4*) is used to define chemical shifts. Peak assignments are: (A) external hexachlorocyclotriphosphazene, 1 *M* in C_6H_6, contained in a Wilmad spherical microcell of approximately 35-μl volume and placed in the left ventrical; (B) sugar phosphates; (C) inorganic phosphate; (D) phosphoesters; (E) PCr, 0.0 ppm; (F) γ-phosphate of ATP and β-phosphate of ADP; (G) α-phosphate of ATP and ADP; (H) NAD; (I) β-phosphate of ATP.

TABLE 1

CHEMICAL SHIFT OF HEXACHLOROCYCLOTRIPHOSPHAZENE

δ^a (ppm)	External reference (0.0 ppm)	Reference orientation[b]
19.9	85% H_3PO_4	Microsphere
19.4	85% H_3PO_4	Cylinder perpendicular to B_0
20.8	85% H_3PO_4	Cylinder parallel to B_0
22.9	PCr	Rabbit heart

[a] All shifts determined at 37°C. Chemical shift defined by standard IUPAC convention (i.e., a more positive frequency shift is a more positive chemical shift) (*4*). HCCTP was bulk solution (100-m*M* in C_6D_6 in 10-mm NMR tube) except for final entry which was 1 *M* in C_6H_6 contained in a microsphere inside the left ventrical of perfused rabbit heart.

[b] Susceptibility shifts of the external reference depend on geometry of sample container and orientation to magnetic field, B_0 (*5*).

available (Aldrich Chemical Company, "phosphonitrilic chloride trimer"), and it may be purified by extraction and recrystallization (*7*). HCCTP is very soluble in common solvents (*8*) and remains stable in solution if protected from hydrolysis by storing in a sealed tube. We have found it easy to work with.

A potential problem for some applications is the relatively long T_1 exhibited by HCCTP which could lead to excessive saturation. If required, a suitable relaxation agent can be used to reduce T_1. For example, a 1 *M* HCCTP solution in C_6D_6 at

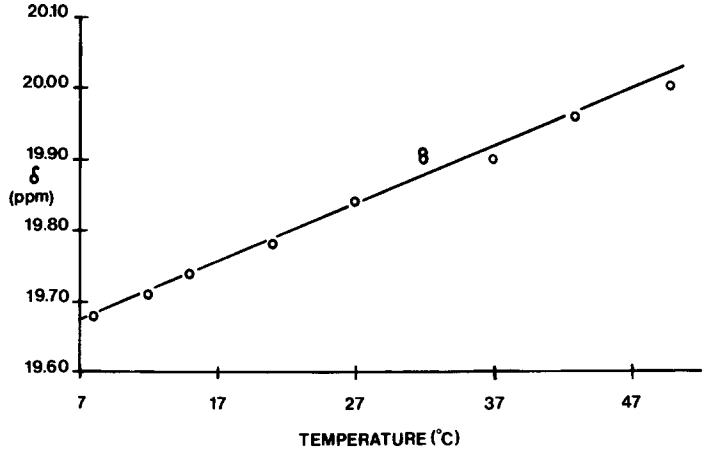

FIG. 2. Plot of the chemical shift of hexachlorocyclotriphosphazene, 100 m*M* in C_6D_6, referenced to 85% H_3PO_4 contained in a microsphere, as a function of temperature around the physiological range of 37°C. The external reference, 85% H_3PO_4, shows no change in chemical shift, within 0.1 ppm, over this temperature range (*3*).

37°C gives a T_1 of 16 to 17 seconds at 145.8 MHz. Addition of 62 m*M* chromium acetylacetonate reduces the T_1 value to 0.24 seconds, increases the linewidth by only 3 Hz and increases the chemical shift relative to an external microsphere of 85% H_3PO_4 by only 0.59 ppm. This approach can be used if needed to avoid any potential problems that might arise due to saturation of the HCCTP resonance signal.

In summary, we feel that hexachlorocyclotriphosphazene will make a convenient external ^{31}P NMR intensity and chemical shift reference for a variety of intact biological systems. Melting point capillaries can be cut to appropriate lengths, filled with a concentrated HCCTP/benzene solution and easily sealed with "Torr Seal," a low vapor pressure resin epoxy (Varian Associates). These capillaries can then be firmly attached to the back side of surface coils or with appropriate use of Teflon spacers, placed in the center of sample tubes containing a cell preparation. Perfused organs will usually require that the capillary be attached to the side of the sample container. Susceptibility shifts of HCCTP can be minimized by use of microsphere sample containers (spherical microcell sample bulb, Wilmad Glass Comp.) (*5*). A final note: for reproducible results, we find it necessary to keep solutions of HCCTP away from contact with metal, including stainless-steel syringe needles.

ACKNOWLEDGMENTS

We would like to thank Stephen M. Kinney of Monsanto Company for the generous gift of the HCCTP. We are also grateful to David Lipkin of Washington University for helpful discussions regarding the chemistry of hexachlorocyclotriphosphazene. Support for this work was provided by Washington University Intramural Funds, NSF Instrument Grant CHE 8100211, and NIH Grant 1 R01 GM30331-01 SSS. In addition, this project was also supported in part by BRSG S07 RR07054-17, -16, and -15, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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