# Communication

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## Determination of Intracellular pH by $^{31}P$ Magnetic Resonance*

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### SUMMARY

**Observation of the $^{31}P$ signal from various intracellular phosphates can provide a convenient, nondestructive technique for determining intracellular conditions such as pH. This procedure has been explored with particular reference to the erythrocyte. Both the chemical shift of intracellular inorganic phosphate relative to that of serum phosphate and the positions of, and more especially the difference between, the chemical shifts of 2,3-diphosphoglycerate have been used to monitor intracellular pH of erythrocytes whose hemoglobin has been liganded with carbon monoxide.**

Eventually studies of the hemoglobin molecule in solution must be related to its function within the intact red cell. Organic phosphates, in particular 2,3-diphosphoglycerate, bind preferentially to unliganded deoxyhemoglobin, thereby reducing the oxygen affinity of hemoglobin (1, 2). The degree of hemoglobin oxygenation significantly affects pH through the alkaline Bohr effect and the intracellular pH in turn exerts a strong influence on the level of 2,3-diphosphoglycerate within the erythrocyte (3). Moreover, 2,3-diphosphoglycerate serves as a potent inhibitor of several red cell enzymes (4). A close association thus exists within the red cell between hemoglobin oxygenation, pH, and metabolism which is mediated in large measure by 2,3-diphosphoglycerate. In order to gain a better understanding of these interrelationships within the functioning erythrocyte we have undertaken $^{31}P$ magnetic resonance studies of hemolysates, red cell suspensions, and whole blood.

We have found $^{31}P$ NMR to provide a useful and direct means for determining the intraerythrocyte pH and the levels of several important red cell metabolites. Since $^{31}P$ is present at 100% natural abundance, exhibits a wide spectrum of chemical shifts, and is contained in many metabolites, it holds considerable promise as a probe in many biological systems.

### EXPERIMENTAL PROCEDURE

Fig. 1 shows the pH dependencies of the chemical shifts for several organic phosphates and emphasizes the large variation in this parameter as a function of the state of ionization. All spectra were obtained by pulsed Fourier transform techniques on a Varian Associates XL-100-15 spectrometer operating at 40.5 MHz. Fig. 2 shows a more detailed representation of the titration behavior of the 2- and 3-phosphate groups of 2,3-diphosphoglycerate. Assignment of the resonance at lower field to the 3-phosphate (which titrates with $pK_a = 6.3$) depends on the $^1H$-coupled spectrum in which this $^{31}P$ resonance appears as a triplet with $J = 5$ Hz which is due to coupling to a $CH_2$ group. The resonance at higher field (which titrates with $pK = 7.0$) was assigned to the 2-phosphate because its $^1H$-coupled spectrum gives a doublet with $J = 10$ Hz which is expected for attachment to a CH group. The relative chemical shift separation between the 2- and 3-phosphate $^{31}P$ nuclei depends on pH as shown in Fig. 2, and this separation may be used to measure pH directly in cases where diamagnetic or other bulk effects may complicate determinations based on absolute chemical shifts. Except in the region 6.4 to 6.6, the absolute chemical shift of the nuclei will distinguish between the 2-fold degeneracy inherent in the use of the difference parameter alone.

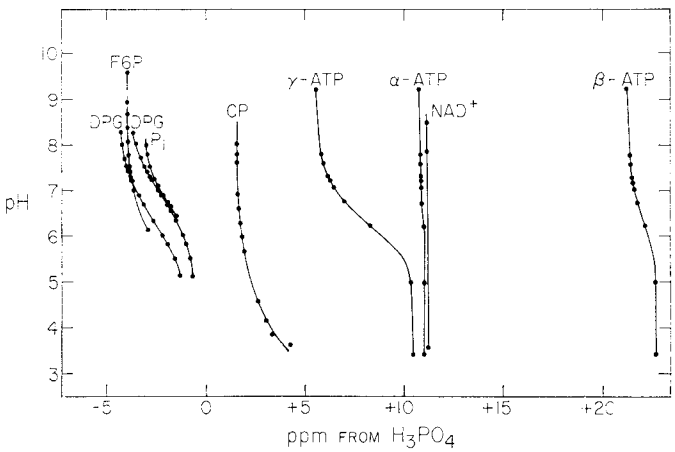


FIG. 1. The pH-dependent chemical shift behavior of a variety of biological organic phosphates. Chemical shifts are reported relative to external 1.0 M phosphoric acid. *F6P*, fructose 6-phosphate; *DPG*, 2,3-diphosphoglycerate; *CP*, carbamyl phosphate.

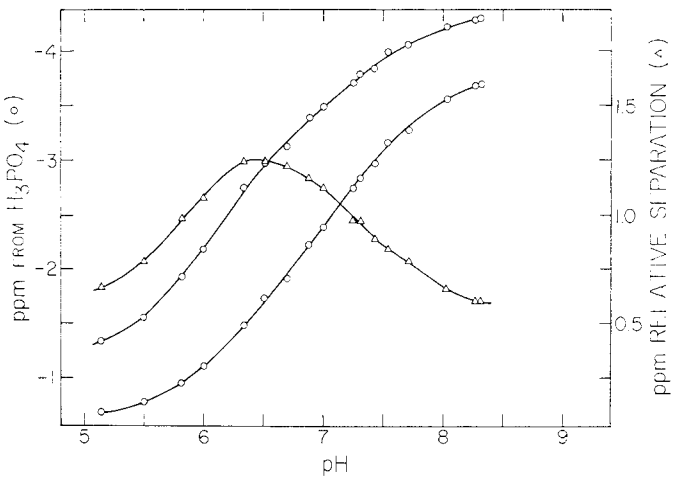


FIG. 2. The detailed pH-dependent chemical shift behavior of 2,3-diphosphoglycerate. Absolute shifts from external $H_3PO_4$ are indicated on the *left ordinate* while the relative separation of the $^{31}P$ resonances are shown on the *right*. The *upper* and *lower* titration curves correspond to phosphates at positions 3 and 2 of 2,3-diphosphoglycerate, respectively.

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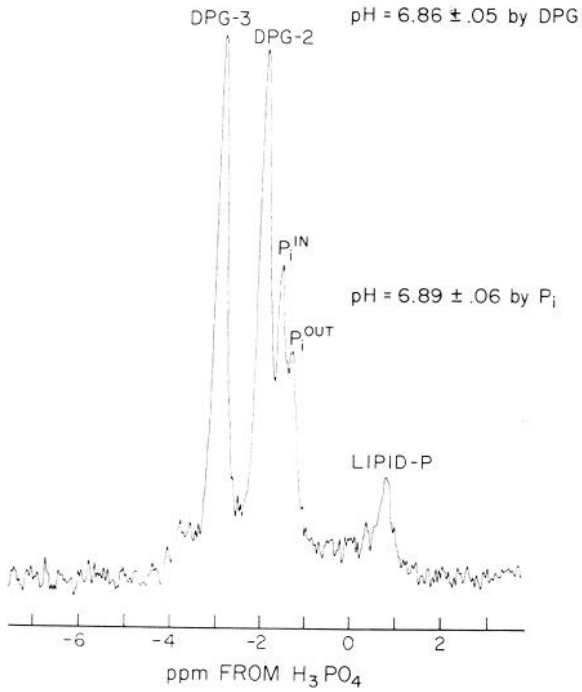


FIG. 3. Typical $^{31}P$ spectrum of carbon monoxide-treated whole rabbit blood obtained at 40.5 MHz by pulsed Fourier transform NMR. The anticoagulant used was acid-citrate-dextrose (ACD, Formula B). The whole blood pH was 6.70 ± 0.02 as determined on a Radiometer model 26 pH meter. The estimated intracellular pH values indicated are based on both the absolute chemical shifts and the relative separation of the 2,3-diphosphoglycerate (*DPG*) resonances, and the relative separation of the intra- and extracellular $P_i$ resonances. The phospholipid resonances of the red cell membrane are also indicated.

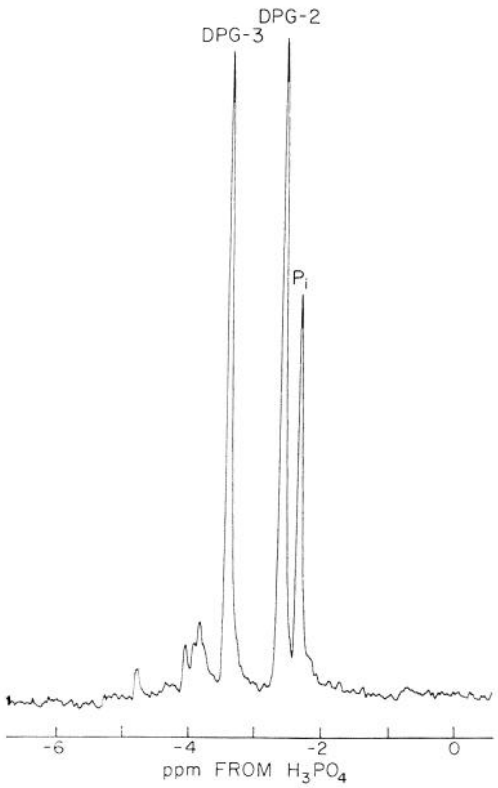


FIG. 4. A typical $^{31}P$ spectrum of a rabbit hemolysate obtained from sonicated cells. Sample pH = 7.52 ± 0.02. Data accumulation time was 5½ hours for this sample. Sufficient signal to noise ratios for determining 2,3-diphosphoglycerate (*DPG*) and $P_i$ chemical shifts are obtained after several minutes, however.

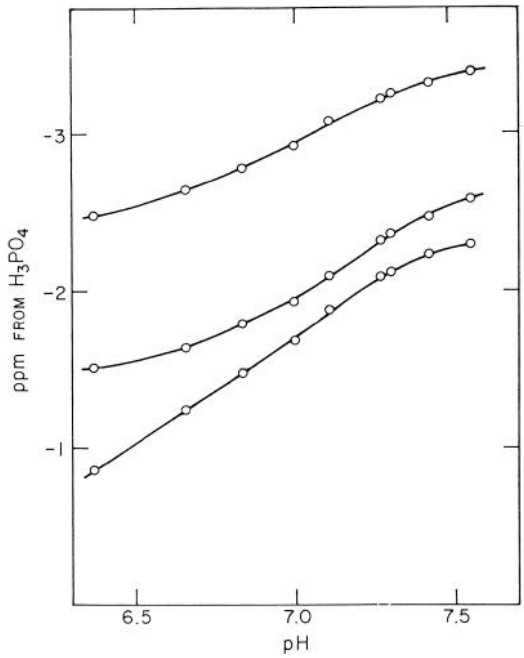


FIG. 5. A plot of chemical shift *versus* pH for carbon monoxide-treated rabbit hemolysates. The *upper* and *middle* titration curves correspond to phosphates at positions 3 and 2 of 2,3-diphosphoglycerate, respectively, and the *lower* curve corresponds to the titration behavior of $P_i$.

Typical physiological concentrations for 2,3-diphosphoglycerate in human red cells of 5 mM and in rabbit red cells of 7 mM make the $^{31}P$ resonances from 2,3-diphosphoglycerate easily observable by pulsed Fourier transform NMR in hemolysates, packed red cells, or whole blood. Fig. 3 shows a typical spectrum of carbon monoxide-treated whole rabbit blood. The sample was contained in a 10-mm tube which was not spun as this leads to packing of the erythrocytes around the sides of the tube which increases the magnetic field inhomogeneity within the sample and thereby artificially broadens the resonances.

Rabbit red cell hemolysates which were first treated with carbon monoxide so as to reduce interactions between 2,3-diphosphoglycerate and hemoglobin as much as possible by completely saturating the hemoglobin with ligand gave titration results similar to those of Fig. 2. Fig. 4 shows a typical spectrum of such a hemolysate and Fig. 5 summarizes the titration behavior of 2,3-diphosphoglycerate and inorganic phosphate in such a hemolysate.

These data, together with comparable titration data for the $^{31}P$ resonance of inorganic phosphate in the hemolysate, were used to estimate the intracellular pH of erythrocytes in whole blood which had been treated with carbon monoxide such as the sample shown in Fig. 3. The pH estimates indicated are based on both the differences in chemical shift for the two $^{31}P$ nuclei of the intracellular 2,3-diphosphoglycerate and for the $^{31}P$ nuclei of $P_i$ inside and outside the cell.

## DISCUSSION

Although providing a nondestructive and reasonably accurate and rapid way of measuring intraerythrocytic pH, this method must be used with caution as the interpretation of the observed resonances can be complicated by the interaction between 2,3-diphosphoglycerate and deoxyhemoglobin. On binding to deoxyhemoglobin, the 2,3-diphosphoglycerate resonances experience a large downfield shift which probably results largely from

an increase in degree of ionization for the bound 2,3-diphosphoglycerate, as the magnitude of the observed downfield shift closely parallels that observed on ionization of 2,3-diphosphoglycerate in solution. Other factors, such as aromatic ring currents or bond anisotropies, for example, may also influence chemical shift. (The 2,3-diphosphoglycerate exchanges rapidly relative to $\sqrt{2}/2\pi\Delta$ so that the observed signal represents the weighted average for the 2,3-diphosphoglycerate in its two environments—solution and bound to hemoglobin.[1]) Accordingly, one must calibrate the experiment by first titrating a hemolysate which has the same relative oxygen tension as the blood sample of interest if one is to obtain accurate data. Nevertheless this chemical shift data may be acquired in a relatively short time (only several minutes per point are required) and the technique is therefore comparable in ease to other methods of measuring intraerythrocytic pH, *i.e.* electrode determination of the pH in hemolysates obtained from packed red cells which have been lysed by repeated freezing and thawing of the cells (5) or by sonication. In this regard we have found no change in intraerythrocytic pH in packed red cells or their hemolysates with respect to whole blood samples. This technique offers a further advantage in that no disruption of the cell membrane is necessary; measurements can be made of intact, functioning cells. Thus one can directly study the response of intracellular pH to various external stimuli. Further, the total concentrations of 2,3-diphosphoglycerate and $P_i$ can be determined by integration of their respective $^{31}P$ resonances in spectra requiring data accumulation times of an hour or less.

While most other cellular systems do not possess significant concentrations of 2,3-diphosphoglycerate, they often do contain other phosphates such as $P_i$, ATP, and inositol hexaphosphate with concentrations approaching 1 mM. Use of analogous $^{31}P$ techniques may therefore provide a basis for intracellular pH measurements in a variety of biological systems. Once again appropriate controls in solution must be carried out so that one can unambiguously dissect the influence on chemical shift of pH changes from other effects such as interaction with intracellular macromolecules or cations, for example. Moreover, phosphates incorporated into synthetically prepared vesicles may provide a probe for measurements of internal pH for the study of Donnan equilibrium or other properties of model membranes.

[1] R. B. Moon and J. H. Richards, unpublished observations.

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