02/0017/31

นิพนธ์ต้นฉบับ

In vitro study of ^{31}P nuclear magnetic resonance in human red blood cells*

การศึกษาในหลอดทดลองเกี่ยวกับ ฟอสฟอรัส -31 นิวเคลียร์ แมกเนติก รีโซแนนส์ ในเม็ดเลือดแดงคน

Sompongse Chinayon[1] *Wanida Jinsart*[2] *Somchai Eiam-ong*[3]
Rampai Suvanpha[3] *Pongsak Pansin*[3]

บทคัดย่อ : **สมพงษ์ จินายน, วนิดา จีนศาสตร์, สมชาย เอี่ยมอ่อง, รำไพ สุวรรณภา และ พงษ์ศักดิ์ พันธุ์สิน. 2531. การศึกษาในหลอดทดลองเกี่ยวกับฟอสฟอรัส -31 นิวเคลียร์ แมกเนติก รีโซแนนส์ ในเม็ดเลือดแดงคน. วารสารวิจัยวิทยาศาสตร์การแพทย์ 2(1) : 1-8**

ศึกษาสารฟอสฟอรัส เมตาบอไลท์ในเม็ดเลือดแดงคน โดยใช้เครื่องมือนิวเคลียร์ แมกเนติก รีโซแนนส์ ชนิด JEOL FX 90Q ที่ความถี่ 36.2 เมกะเฮิรทซ์ ได้ตรวจพบสัญญาณฟอสฟอรัส -31 ของสาร 2, 3- ไดฟอสฟอท์กลีเซอเรท และฟอสฟอรัสอนินทรีย์ในเม็ดเลือดแดงแตกตัวและเม็ดเลือดแดงคงรูป ไม่พบสัญญาณฟอสฟอรัสอนินทรีย์ในเม็ดเลือดแดงคงรูปเมื่อวัดในเวลาเพียง 4 ชม. (2,500 pulses) ตำแหน่งสารประกอบฟอสฟอรัสที่พบในสเปคตรัม แสดงเป็นค่า พี พี เอม และเปลี่ยนแปลงได้ตามภาวะความเป็นกรดด่างในเซลล์ หรือชีววัตถุ

Abstract : **Sompongse Chinayon, Wanida Jinsart, Somchai Eiam-ong, Rampai Suvanpha and Pongsak Pansin. 1988. In vitro study of ^{31}P nuclear magnetic resonance in humen red blood cells. Thai J Hlth Resch 2(1) : 1-8**

Phosphorus metabolites in human red blood cells were studied by a nuclear magnetic resonance spectrometer, JEOL FX 90Q, operating at 36.2 MHz. ^{31}P resonance signals of 2, 3- diphosphoglycerate (DPG) and inorganic orthophosphate (Pi) were recorded in hemolysates and intact erythrocytes. However, the Pi peak was absent in the intact red blood cells, detecting for 4 hours (2,500 pulses). The chemical shift values (in ppm) of these biological phosphate compounds appearing in the spectrum were pH dependent.

---

* *This work was a part of the research project supported by the Rachadapiseksompoj-China Medical Board Fund (CMB 76357), Faculty of Medicine, year 1987.*

รายงานนี้เป็นส่วนหนึ่งของโครงการวิจัยที่ได้รับทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์ ปี พ.ศ. 2530.

[1] *Department of Medical Technology, Faculty of Medicine, Chulalongkorn University, Bangkok 10500*
ภาควิชาเทคนิคการแพทย์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กทม. 10500

[2] *The Scientific and Technological Research Equipment Center, Chulalongkorn University, Bangkok 10500.*
ศูนย์เครื่องมือวิจัยวิทยาศาสตร์และเทคโนโลยี จุฬาลงกรณ์มหาวิทยาลัย กทม. 10500

[3] *Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10500.*
ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กทม. 10500

Introduction

^{31}P nuclear magnetic resonance (NMR) spectroscopy has been introduced to monitor cellular phosphorus metabolism in research in clinical biochemistry. This technique is easy and nondestructive to identify or to quantify phosphate metabolites in cells, tissues, and biological fluids in vitro. ^{31}P nucleus is present at 100% natural abundance. The chemical shift values, in parts per million (ppm), are referenced to the usual standard of 85% orthophosphoric acid. ^{31}P resonance of phosphate compounds exhibits a wide spectrum of chemical shifts (40 ppm). Although the NMR spectrometer is a sophisticated and expensive instrument requiring an experienced operator, the advantages are the relative accuracy and sensitivity of analytical performane characteristics (Gadian and Radda, 1981 ; Brown *et al.*, 1984). ^{31}P resonances were taken in the studies of energy metabolic processes in cells regarding organophosphorus compounds, for example, adenosine triphosphate and creatine phosphate. As well, resonance peaks of inorganic orthophosphate (Pi), 2, 3-diphosphoglycerate (2, 3-DPG) and monoesterified sugar phosphates were observed in pieces of muscles and erythrocytes of experimental animals and man (Yoshizaki, 1972; Moon and Richards, 1973; Henderson *et al.*, 1974; Hoult *et al.*, 1974; Burt *et al.*, 1976). Moreover, the pH dependent chemical shifts of some phosphate metabolites are the basis for determination of the intracellular pH in physiological and pathological conditions (Moon and Richards, 1973; Burt *et al.*, 1976; Yoshizaki *et al.*, 1979; Seo *et al.*, 1983; Monti *et al.*, 1987). The NMR spectroscopy is a high technology and an entirely new approach for elucidation of intracellular phenomena in vitro without performing any pretreatments (such as extraction, separation and purification). ^{31}P NMR spectroscopy was applied to identify phosphorus metabolites in the whole bloods, packed red blood cells and hemolysates, as well as to measure intracellular pH (Moon and Richards, 1973; Henderson *et al.*, 1974; Fabry and San George, 1983; Labotka, 1984; Monti *et al.*, 1987). The efficacy of NMR spectrometer to detect the nuclear resonance signals is also depended on the intensity of magnetic field, as indicating by the high resolution of NMR spectrometry operating, the better separation of resonances obtained (Monti *et al.*, 1987). JEOL FX 90Q NMR spectrometer comprising of an electromagnet (2.16 Tesla) is presently available to study ^{31}P resonance at Chulalongkorn University. The aim of this work is to observe ^{31}P NMR spectrum of phosphorus compounds in human red blood cells by using the relatively low resolution magnetic field spectrometer. The pilot experiments were assigned to study the appropriate experimental conditions of spectrometer and sample preparation procedures. The information obtained will be further applied in monitoring cellular phosphate metabolism and intracellular pH measurement in some diseases.

Materials and Method

1. Preparation of erythrocytes

The procedures are modified techniques of Monti *et al.* (1987). Freshly drawn venous heparinized blood samples were obtained from normal, fasted, adult volunteers. To prepare packed red blood cells, the blood samples in closed tubes were centrifuged, without delay, at 1000 rpm, and at 4°C for 10 minutes. The plasma and buffy coat were gently aspirated. The remaining red blood cells at a packed cell volume of about 60-80% were kept in capped tubes and stored in crushed ice.

Venous hemolysates were obtained by two repeated freeze-thawings of packed erythrocytes in sealed tubes. The pH of the lysates was adjusted in different values (pH 6.959-7.595) with 1.0 N HCl or NaOH and measured with a Blood gas analyzer. Samples of the packed erythrocytes or hemolysates were studied for ^{31}P NMR spectra of phosphate metabolites as following : 1.7 ml aliquot of samples was placed in a NMR tubes, 10 mm (outer diameter), containing 0.3 ml of deuterium oxide (D_2O) which was needed for field frequency locking of NMR spectrometer. After insertion of a tight-fitted teflon plug, a mixture of 85% H_3PO_4 and D_2O (15% v/v) in a sealed 2 mm diameter reference capillary was held at the center of the NMR tube through a central pore of the plug. The tube was then capped and placed in the NMR spectrometer for measurements within 60 minutes. (Monti *et al.*, 1987)

2. *^{31}P NMR measurement*

^{31}P NMR experiments were performed at 23°C with a JEOL FX 90Q spectrometer operating at 36.2 MHz (by The Scientific and Technological Research Equipment Center, Chulalongkorn University, Bangkok). Spectra were recorded in the pulsed Fourier-Transform mode, with a broad-band noise decoupling procedures. Experimental conditions for intact red blood cells were : Sweep width 1,501 Hz, pulse width 12 μsec (45°), repeatition time 5.72 sec, number of scans 2,500. Similar conditions were operated for recording ^{31}P resonance in hemolysate except the number of scans using were 10,000.

Assignment of ^{31}P signals was made by comparing them with the chemical shifts of 85% H_3PO_4 which served as an external reference (Henderson *et al.*, 1974). All chemical shifts were shown in parts per million (ppm), as downfield displacements from the resonant frequency of orthophosphoric acid (0 ppm) contained in the inner concentric tube. Resonance peak positions of phosphate metabolites were read out by the computer of the instrument.

3. *Chemical reagents*

Deuterium oxide (D_2O) containing more than 99.8% of D(code 31240) was purchased from Fluka company, Switzerland. The 2, 3-diphospho-D glyceric acid, pentacyclohexylammonium salt (2, 3-DPG) is a product of Sigma chemical company, U.S.A. A 24.9 mM/L solution was recorded for ^{31}P resonance to demonstrate the typical peaks of 2, 3-DPG in the spectrum.

Results

^{31}P NMR spectroscopy of standard 2, 3-DPG solution, phosphate metabolites in the intact erythrocytes and hemolysates are depicted as the followings:

Figure 1 shows 36.2 MHz, ^{31}P NMR spectrum of a standard solution of 2, 3-DPG at a concentration of 24.9 mM/L. The two phosphate resonances at 3.536 and 3.030 ppm arise from the 3- and 2-positions respectively, of 2, 3- diphosphoglycerate. 85% H_3PO_4 (sealed capillary) served as the external reference (0 ppm).

The ^{31}P spectrum of packed erythrocytes measured for 23 hours is demonstrated in Figure 2. The broad signals of 3-phosphorus peak and 2-phosphorus peak of 2, 3- DPG are observed at positions of 3.07 and 2.2 ppm. The resonance of intracellular inorganic orthophosphate (Pi) is present at 1.75 ppm. The Pi resonance is absent in the spectrum of packed red blood cells recorded for only 4 hours as shown in Figure 3. Moreover, the average chemical shifts of ^{31}P resonance for intact erythrocytes from 4 healthy volunteers were 3-P group of DPG, 3.101 ppm: 2-P group of DPG, 2.315 ppm. The main resonances of phosphate metabolites in erythrocytic hemolysate, Figure 4, are similar to those identified in intact red blood cells. However, the peaks of ^{31}P NMR in hemolysate are sharper and the signal intensities (peak area) are more prominent. Additionally, there are also group of minor resonances centered at 3.556 and 3.455 ppm, arising from orthophosphate monoesters of sugars. Since the chemical shifts of some biological phosphate compounds are pH dependent, in table 1, the pH induced changes in chemical shifts of 3- and 2- phosphate groups of 2, 3-DPG and of Pi in 5 venous hemolysates are shown. The time course observation of ^{31}P NMR spectra of a venous hemolysate was sequentially taken (2.37, 3.48 and 15 hours). The peak amplitude of Pi resonance in the sample was increased in association with concomitant decrease in signal intensities of 2, 3-DPG, as a function of time (Figure 5). There were no changes in chemical shift values at different recording times, as demonstrating the 3-P and 2-P groups of DPG and the Pi at 2.37 hrs was equal to 3.15, 2.32 and 1.82 ppm; and at 15 hrs was equal to 3.15, 2.35 and 1.82 ppm, respectively.

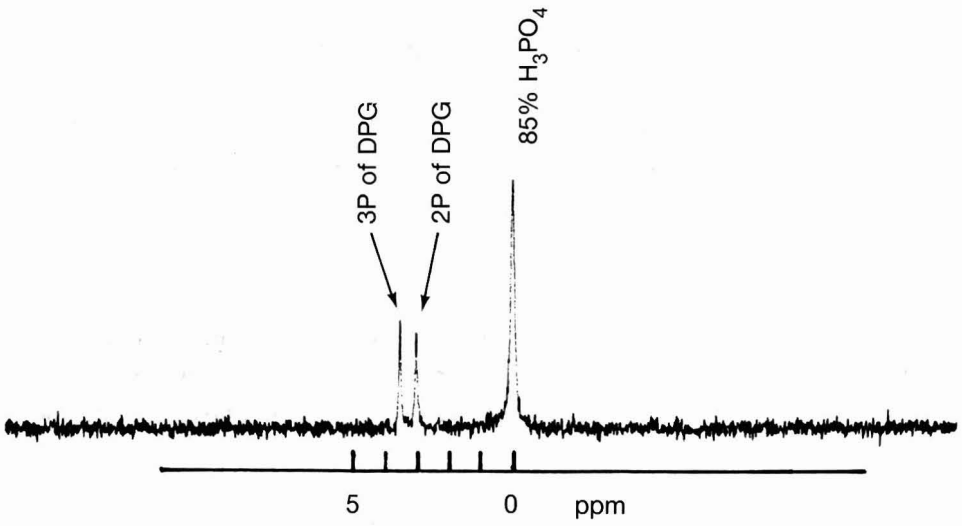


Figure 1. 36.2 MHz ^{31}P - NMR spectrum of 24.9 mM/L 2, 3 diphosphoglyceric acid (2, 3-DPG). NMR spectrum was taken in 10-mm sample tube at 23°C with 11300 pulses. Chemical shift is given in ppm form external, reference 85% H_3PO_4. (H_3PO_4 = 0 ppm, 3-P of DPG = 3.536 ppm, 2P - of DPG reference = 3.030 ppm)

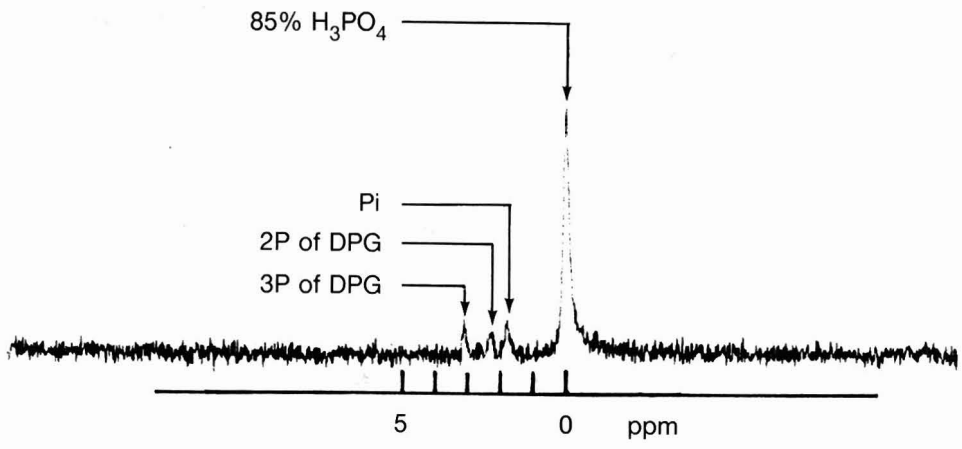


Figure 2. ^{31}P NMR spectrum of packed human red blood cells. Data accumulation time was 23 hours (14600 pulses) at 23°C. The major resonance bands are indicated by appropriate abbreviations : 2, 3 DPG, 2, 3 diphosphoglyceric acid (3-P, 3.07 ppm; 2-P, 2.2 ppm); Pi, inorganic orthophosphate (1.75 ppm). H_3PO_4 (85%, sealed capillary) served as the external reference (0 ppm).

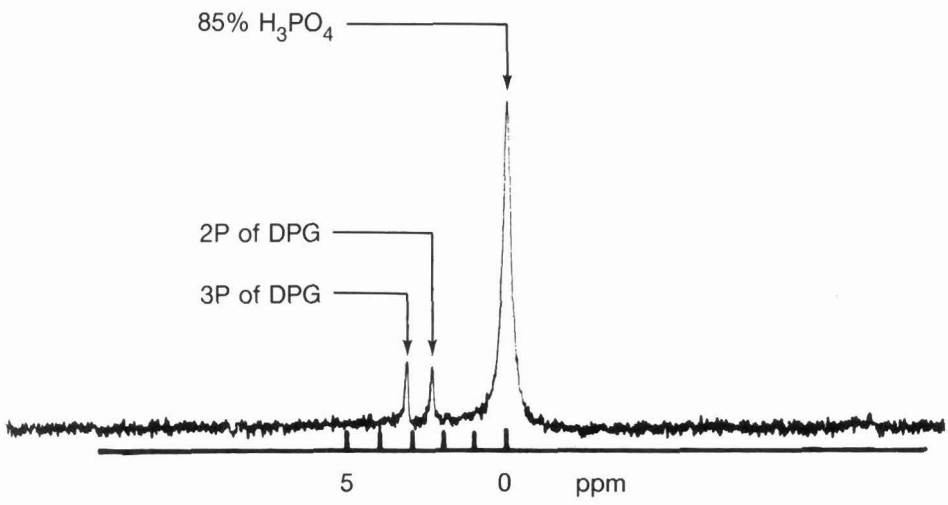


Figure 3. ^{31}P NMR spectrum obtained from packed human red blood cells recorded 4 hours (2500 pulses) for NMR analysis. Note the absence of Pi peak. The other resonances of spectrum are indicated in Figure 2. (3-P of DPG = 3.071 ppm, 2-P of DPG = 2.293 ppm).

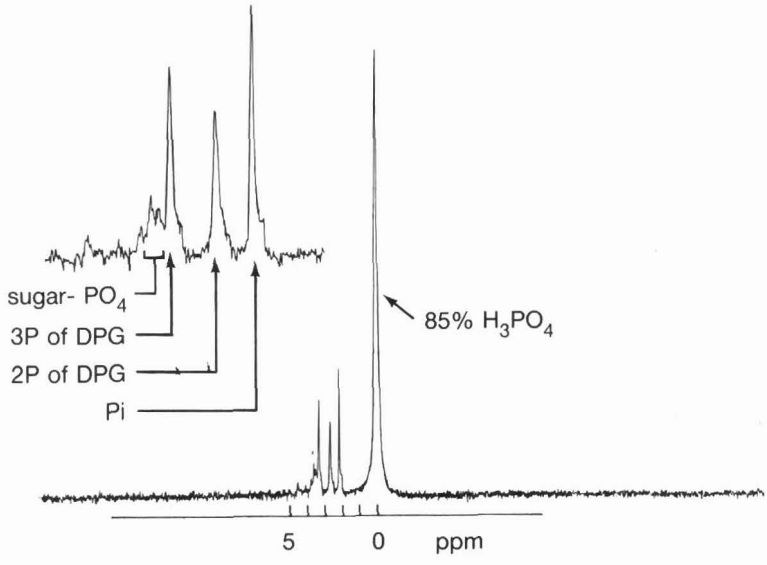


Figure 4. ^{31}P NMR specturm of venous hemolysate (pH 7.595) observing for 15 hours at 23°C (10000 pulses) for NMR analysis. The top spectrum is an expended scale read out of the lower spectrum. The main resonances are DPG (3-P group = 3.283 ppm, 2-P gruoup = 2.646 ppm) and Pi (2.111 ppm). The minor resonances (3.556, 3.455 ppm) positioned on the left of 3-P group of DPG are hexose or triose monoesterified phosphates.

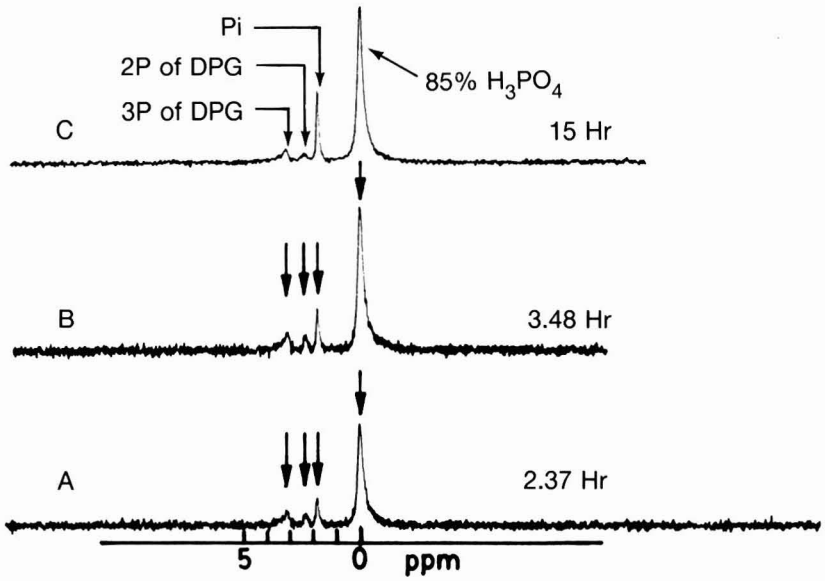


Figure 5. Changes in erythrocyte content of 2, 3-diphosphoglycerate and Pi, as determined by ^{31}P NMR analysis of venous hemolysate as a function of accumulation time. Accumulation of ^{31}P NMR data for spectrum A was begun within 3 hours after the blood was drawn and continued for 2.37 hours (1,500 pulses), 3.48 hours for spectrum B (2,200 pulses), 15 hours for spectrum C (10,000 pulses), respectively.

Table 1. *The ^{31}P chemical shifts of biological phosphorus compounds in erythrocytic hemolysates, measured as a function of pH.*

hemolysate	pH units	resonant frequency (chemical shift in ppm)		
		3-P of DPG	2-P of DPG	inorganic orthophosphate
1	6.959	2.788	2.340	1.252
2	7.093	3.010	2.342	1.656
3	7.286	3.142	2.455	1.778
4	7.439	3.172	2.465	1.980
5	7.595	3.283	2.646	2.111

Discussion

In our study ^{31}P resonance in the red blood cells, by the JEOL FX-90Q NMR spectroscopy (36.2 MHz), it is not able to record the three resonance bands of the polyphosphate chains of ATP. Henderson *et al.* (1974) used a Bruker HFX-5 operating at 36.43 MHz taken ^{31}P resonance of whole human blood after storing at 4° C with acid-citrate-dextrose for 24 hours. They had demonstrated γ, α and β resonances of ATP at chemical shifts of 5.9, 10.7 and 20.2 ppm respectively, to the right of external reference (85% H_3PO_4, 0 ppm). This controversy may, in part, cause by partial hydrolysis of

some organic phosphorus compounds during hemolysis or pretreatment (heparin added) and storage (Monti *et al.*, 1987). Moreover, the capability of the NMR spectroscopy operating at low frequency of magnetic field in another possibility (Moon and Richards, 1973, Monti *et al.* 1987) for the absent resonance of energy phosphate compounds, ATP. In this study, we have attempted to apply ^{31}P - NMR spectroscopy to the intact red blood cells and hemolysates in vitro and have demonstrated that some phosphorus metabolites present in the samples can be identified as previously reported (Moon and Richards, 1973; Henderson *et al.*, 1974; Monti *et al.*, 1987). The spectra contained resonances of monoesterified phosphates, 2, 3-DPG and inorganic orthophosphate, as shown in Figure 2 and 4. The absence of Pi resonance in the intact red cells in Figure 3 may be due to the short data accumulation time (2500 pulses). On the other hand, the physical state of phosphorylated compounds in the samples may effect the signal intensity of resonance (Gadian and Radda, 1981; Fabry and San George, 1983). The less immobilized molecules of Pi in the intact erythrocytes (than their behaviour in the hemolysates) may the cause of undetectable Pi peak. Otherwise, the resonances of 2, 3-DPG signals observed in packed cells were broader than those found in hemolysates as previously reported (Moon and Richards, 1973; Fabry and San George, 1983).

As shown in Figure 5, the signal intensities of Pi in venous hemolysate are increased with data accumulation time. The chemical shift values of 3- and 2- phosphate groups of 2, 3- diphosphoglycerate (DPG) and of Pi do not change at different pulses used. However, the peak areas of 2, 3-DPG are decreased with data accumulation time of 15 hours (10,000 pulses) of Fourier transform ^{31}P NMR analysis. These observations are in general agreement with those seen in aged red blood cells incubated in vitro at 25°C (Henderson *et al.*, 1974), of which show 2, 3-DPG decreasing and Pi increasing with time.

The titration behaviour of the 3-phosphate group of 2, 3-DPG and inorganic orthophosphate (Pi) in hemolysates of pH ranging from 6.959-7.595 (Table 1) can be a basis for measuring intracellular acidity in red blood cells as suggested by others (Moon and Richards, 1973; Monti *et al.*, 1987). In this study only 2, 3-DPG resonances were taken in intact, functioning, red blood cells, by NMR analysis at 36.2 MHz. Therefore, the pH induced chemical shift values of 3-phosphate group of DPG may be applied for direct determination of intracellular pH in the living cells of blood samples. Furthermore, if the standard 2, 3- diphosphoglycerate solution of known concentration is measured by NMR analysis under the identical instrumental conditions to the samples, the amount of 2, 3-DPG in the intact red blood cells or hemolysate can be quantitated.

REFERENCES

Brown CE, Battocletti JH, and Johnson LF. 1984. Nuclear magnetic resonance (NMR) in clinical pathology: Current trends. Clin Chem 30 : 606-618.

Burt CT, Glonek T, and Barany M. 1976. Analysis of phosphate metabolites, the intracellular pH, and the state of adenosine triphosphate in intact muscle by phosphorus nuclear magnetic resonance. J Biol Chem 251 : 2584-2591.

Fabry ME, and San George RC. 1983. Effect of magnetic susceptibility on nuclear magnetic resonance signals arising from red cells : A warning. Biochemistry 22 : 4119-4125.

Gadian DG, and Radda GK. 1981. NMR studies of tissue metabolism. Ann Rev Biochem 50 : 69-83.

Henderson TO, Costello AJR, and Omachi A. 1974. Phosphate metabolism in intact human erythrocytes : Determination by phosphorus - 31 nuclear magnetic resonance spectroscopy. Proc Nat Acad Sci 71 : 2487-2490.

Hoult DI, Busby SJW, Gadian DG, Radda GK, Richards RE, and Seeley PJ. 1974. Observation of tissue metabolites using ^{31}P nuclear magnetic resonance. Nature 252 : 285-287.

Labotka RJ. 1984. Measurement of intracellular pH and deoxyhemoglobin in deoxygenated erythrocytes by phosphorus -31 nuclear magnetic resonance. Biochemistry 23 : 5549-5555.

Monti J-P, Gallice P, Baas M, Murisasco A, and Crevat A. 1987. Modification of intra-erythrocytic homeostasis in uremic patients, as studied with ^{31}P nuclear magnetic resonance. Clin Chem 33 : 76-80.

Moon RB, and Richards JH. 1973. Determination of intracellular pH by ^{31}P magnetic resonance. J Biol Chem 248 : 7276-7278.

Seo Y, Murakami M, Watari H, Imai Y, Yoshizaki K, Nishikawa H, and Morimota T. 1983. Intracellular pH determination by a ^{31}P -NMR technique. The second dissociation constant of phosphoric acid in a biological system. J Biochem (Tokyo) 94 : 729-734.

Yoshizaki K. 1972. Phosphorus nuclear magnetic resonance studies of phosphorus metabolites in frog muscle. J Biochem (Tokyo) 84 : 11-18.

Yoshizaki K, Nishikawa H, Yamada S, Morimoto T, and Watari H. 1979. Intracellular pH measurement in frog muscle by means of ^{31}P - nuclear magnetic resonance. Jpn J Physiol 29 : 211-225.