Determination of erythrocyte pyrimidine 5′-nucleotidase activity by ^{31}P nuclear magnetic resonance: Comparison of normal controls and multiple sclerosis patients[1]

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Summary. A technique to assay erythrocyte pyrimidine 5′-nucleotidase activity in situ using ^{31}P nuclear magnetic resonance spectroscopy is presented. The assay is chemically specific, simple and applicable to untreated lysates. A comparison of enzyme levels in normal controls and in multiple sclerosis patients employing the assay yielded no significant differences between both groups. Difficulties encountered in the quantitative analysis of the assay using ^{1}H-NMR spectroscopy are briefly discussed.
Key words. Pyrimidine 5′-nucleotidase; ^{31}P NMR; multiple sclerosis; erythrocyte.

Reduced pyrimidine 5′-nucleotidase (EC 3.1.3.5.; P5N) activities have been reported in cases of low-level lead exposure[2], hereditary P5N deficiency[3,4] and in a variety of myeloproliferative and lymphoproliferative disorders[5]. The erythrocyte enzyme has been purified[6] and the kinetic parameters which describe the activity of the semi-purified enzyme have been determined[7]. Structural analogues of cytidine monophosphate (CMP), cytidine and cytosine, act as inhibitors of the enzyme[8,9].
The enzyme is present in the myelin sheath and the activity is increased in spinal cord myelin from dysmyelinating 'shiverer' mice[9].

While present assay methods employ radioactive substrates and subsequent extraction and counting of labelled products[10–12] the original method measured the amount of inorganic phosphate released[13]. The latter technique did not prove chemically specific[8] and the former are, by nature, time consuming, require expensive radioactive substrates and are not amenable to studies under in situ conditions.
^{31}P NMR spectroscopy has previously been used to determine NAD glycohydrolase in human erythrocytes[14] and in a study of hereditary P5N deficiency to measure directly the intracellular pH and to characterise some phosphate metabolites[15]. ^{31}P NMR

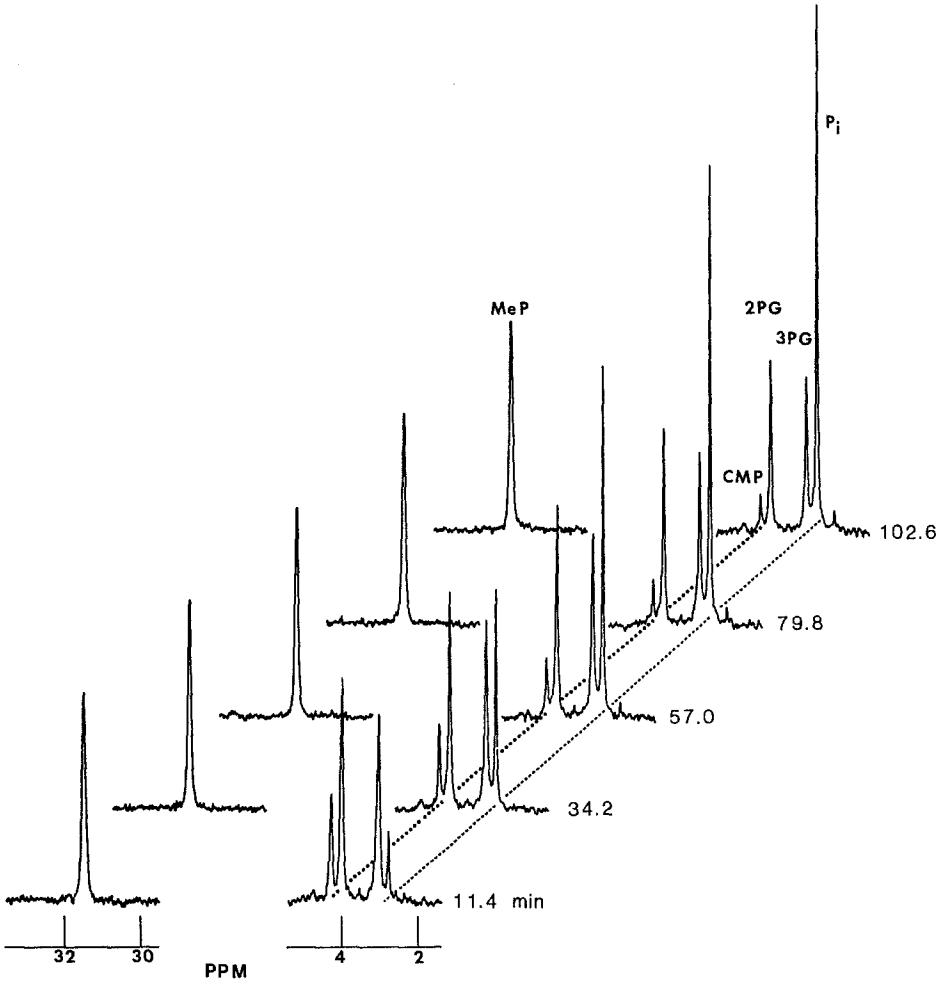

Figure 1. A time-series of ^{31}P NMR spectra of red blood cell lysate (Hc = 0.80) to which cytidine monophosphate (5 mM) was added at zero time. Temperature was 37 °C and 256 transients were averaged per spectrum. Assignments are: MeP, methylphosphonate; CMP, cytidine monophosphate (substrate); 2PG and 3PG 2,3-bisphosphoglycerate; and P_i, inorganic phosphate (product).

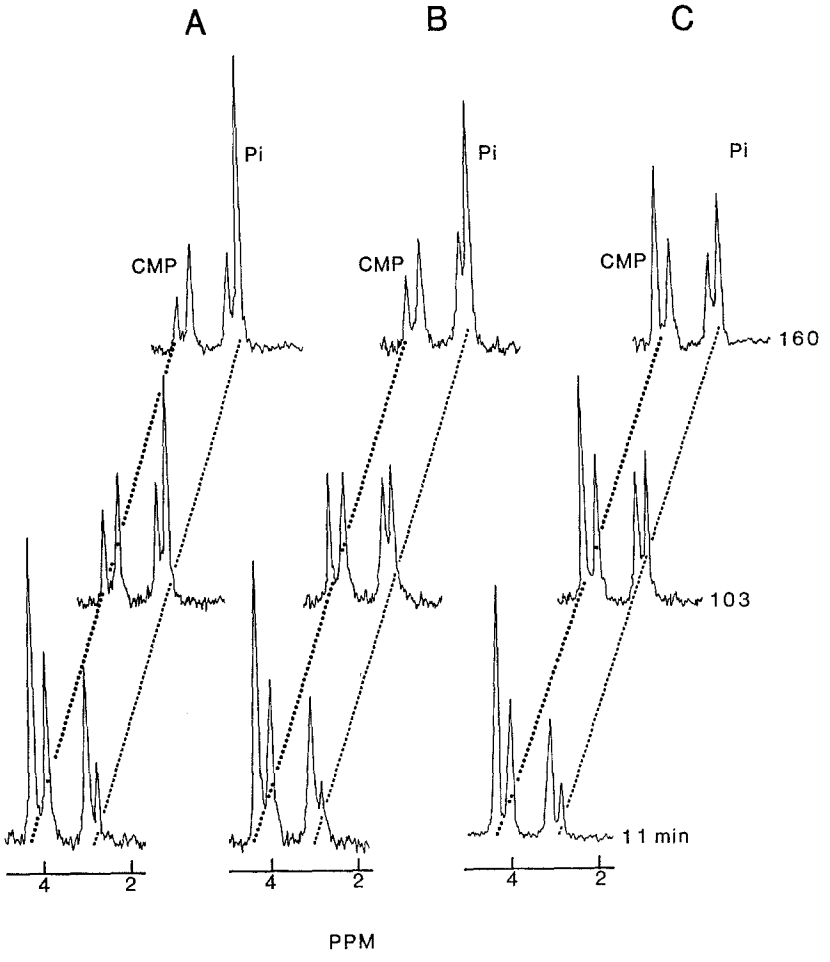

Figure 2. Comparison of ^{31}P-NMR spectra of hemolysates to which CMP (5 mM) was added, in the presence of no inhibitor (A), cytosine (1.25 mM) (B), or cytidine (5 mM) (C). Spectral Assignments are as for figure 1.

spectroscopy offers major advantages for the determination of erythrocyte P5N activities; the technique employed here is a modification of the proton NMR method of Beilharz[16]. The assay is chemically specific, rapid, simple, and applicable to untreated lysates.

The aim of the study was to develop a simple, direct method for assaying P5N activity in situ in order to effect a comparison of P5N levels in normal control and multiple sclerosis patients.

Materials and methods. Blood was collected from normal controls (n = 12, age range 22–43 years, 7 males, 5 females) and patients diagnosed as having multiple sclerosis (n = 16, age range = 25–54, 8 males, 8 females) by venipuncture into heparinized tubes. Red cells were washed once with isotonic saline (0.154 M NaCl, 4 °C, 5 vol) and three times with Krebs bicarbonate buffer[17] (pH 7.4, 4 °C, 5 vols). A final wash was carried out in Krebs buffer constituted in ^2H_2O (99.96% ^2H, Institute for Nuclear Science and Engineering, Lucas Heights, N.S.W., Australia) to provide a lock signal for the spectrometer. Cell suspensions were adjusted to approximately 80% haematocrit and gently oxygenated (O_2/CO_2, 19/1)[18]. For ^{31}P NMR measurements aliquots (2.75 ml) were dispensed into NMR tubes (10 mm o.d., Wilmad, Buena, NJ, USA) and nicotinamide (50 µl, 0.5 M, pH 7.4), glucose (50 µl, 0.25 M, pH 7.4) and Krebs buffer (50 µl, ^2H_2O, pH 7.4), were added. Cytosine (50 µl, 0.075 M) or cytidine (50 µl, 0.3 M) replaced this aliquot of buffer in the inhition studies. Nicotinamide, which was added to the lysates to inhibit NAD glycohydrolase[14], did not affect P5N activity. Lysates were prepared by repeated freezing (−195 °C) and thawing of the samples. CMP (0.15 M, 100 µl) was added and spectra were acquired over the next 3 h. All fine chemicals were the purest grades from Sigma, St. Louis, MO, USA. Other chemicals such as salts were analytical reagent grade.

^{31}P chemical shifts are quoted with respect to the methylphosphonate resonance at 31.40 ppm[18] of a 1% solution contained in a coaxially located capillary. ^{1}H chemical shifts were referenced with respect to the methyl resonance of tetramethyl silane at 0.000 ppm in a 0.2% ^2H_2O solution contained in a capillary. The final concentration of CMP in the sample was calculated taking into account the haematocrit, any additions and the available aqueous volume. A standard curve was constructed by addition of CMP (0–100 µl, 0.15 M) to lysates. In each measurement the volume was adjusted to 3.0 ml with Krebs buffer. The integral of the CMP resonance relative to the integral of the reference was determined and compared with the standard curve.

Free induction decays were collected using a Bruker WM-400 and a Varian XL-400 spectrometer operating in the pulsed Fourier transform mode with quadrature detection. The instrumental parameters were as follows: probe temperature 37 °C, operating frequency 162 MHz, spectral width 4.0 kHz; number of transients 256, memory locations 16384. A pulse angle of 70° was used in ^{31}P spectral acquisition in accordance with the optimum parameters described by Becker et al[19]. Exponential filtering of 3 Hz was applied prior to Fourier transformation.

Results and discussion. Figure 1 shows ^{31}P NMR spectra acquired at various times after addition of CMP. The resonances corresponding to the 5'phosphorus nucleus of CMP (4.30 ppm; substrate) and the inorganic phosphate (2.83 ppm; product) are

clearly resolvable. The other resonances arise from the methylphosphonate (MeP) and from 2,3-bisphosphoglycerate (DPG), which is converted to inorganic phosphate and ATP in glycolytic reactions.

While both the decline in substrate and increase in product resonance can be measured, conservation of peak area (substrate + product = constant) is not observed, because the inorganic phosphate is consumed and released in a variety of endogenous reactions (e.g., ATPase activity, production of glucose 6-phosphate for glycolysis and the pentose phosphate pathway, phosphorolytic reactions, etc.). Hence, an accurate estimate of P5N activity may only be obtained by observing the decline in CMP resonances. The P5N activities (μmol/L of packed cells/min) obtained for red cells of patients and normal controls were 36 ± 2.9 and 32 ± 2.7, respectively. A t-test for unequal sample sizes[20] showed that the two sample populations were not significantly different at the level of $p < 0.005$. As expected, cytosine and cytidine each inhibited the P5N, as can be seen by comparing figure 2A with figures 2B and 2C.

The quantitative analysis of the assay using ^{1}H-NMR spectroscopy did not yield good results for the following reasons. The ^{1}H spectrum exhibits greater complexity and is less resolved than the ^{31}P spectrum. This arises from the J-coupling phase modulation in spin-echo spectra[21], the larger number of proton nuclei present and the inherent small chemical-shift difference between substrate and product resonances. In the aromatic region, the C5 and C6 protons of the pyrimidine ring appear at 5.890 ppm and 7.828 ppm, respectively. Product resonances appear at slightly lower frequencies and are partially obscured by nicotinamide and hemoglobin-histidyl spectral lines. This complicates considerably the computer integration of the peaks and subsequently the determination of P5N activity.

The ^{31}P NMR method would be suitable for use in lower field, less expensive, spectrometers. Further, it considerably simplifies and expedites in situ assay of P5N and hence the diagnosis of P5N deficient subjects.

Although anomalies in P5N activity have been reported in other myelin associated disorders, there is no apparent change in the red blood cells of multiple sclerosis patients.

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Chronic hydrogen peroxide intake and peroxide metabolizing enzyme activities in some tissues of mice and rats

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Summary. Chronic daily intake of 0.5% H_2O_2 in drinking water decreased Se-dependent glutathione peroxidase (Se-GSHPx) activity in rat skeletal muscle, kidney and liver. Non-Se GSHPx activity decreased in kidney. Deprivation of drinking water decreased Se-GSHPx activity in kidney and non-Se GSHPx activity in kidney and liver. H_2O_2 intake decreased activity of catalase in rat skeletal muscle. H_2O_2 intake or water deprivation caused no changes in these enzyme activities in mice.

Key words. Hydrogen peroxide intake; glutathione peroxidase; catalase; lipid peroxides; antioxidants.

Certain chemicals e.g. CCl_4, ethanol[2] or phenobarbital[3] may cause the formation of lipid peroxides and other organic hydroperoxides in biological tissues. It is observed that H_2O_2[4] or cumene hydroperoxide[5] can initiate lipid peroxidation in vitro. Glutathione peroxidase (GSHPx) and catalase are the two main enzymes responsible for the enzymatic decomposition of H_2O_2. The GSHPx activity can be divided by different substrates into two activities[6]: H_2O_2 is the substrate for the selenoenzyme, Se-dependent GSHPx only, and cumene hydroperoxide is the substrate for both the Se-dependent and the Se-independent (non-Se) GSHPx activities. The non-Se activity arises from a group of glutathione S-transferases[7]. The detoxification of H_2O_2 is not affected by the antioxidant vitamin E[8].

In rat liver, lipid peroxidation increases as a result of an excess of dietary iron. This increase is potentiated by a deficiency of Se and/or vitamin E[9]. The increase in lipid peroxidation is reflected by a shift in the two activities of GSHPx; the Se-dependent GSHPx activity decreases with the increase of iron concentration. The decrease is additionally enhanced by the simultaneous deficiency of Se or vitamin E. This effect is partially compensated by an increase of the non-Se activity. Lee et al.[9] also observed the increase of liver catalase activity as a response to the increase of dietary iron, which increase also was potentiated by the deficiency of Se and/or vitamin E. The purpose of this study was to find out whether oral H_2O_2 intake could provoke an oxidative stress in some tissues of mice and rats as reflected by changes in the levels of catalase and glutathione peroxidases. Knowledge of possible enzymatic adaptations would be useful in studies on the role of scavenger systems in tissue injuries.