Hassell, unpublished observation, 1985), and dermatan sulfate binds to lipoproteins at physiological ionic strength[33].

Fibroblast-like cells generally have a limited life span of about 40 doublings[5]. The presence of a limit suggests that changes occur as a culture ages. Such aging becomes particularly important when experiments are performed using clones derived from single cells. As cell numbers in a developing clone increase, the number of potential doublings remaining in the lifespan decreases. At very high numbers, the cells are approaching senescence and are probably not functioning at normal levels of activity. If results from studies of clones are to be used to understand normal cell biology or the pathogenesis of connective tissue disorders, it would be advantageous to use cells early in their lifespan, and this necessitates using lower cell numbers per assay. Russell et al. performed a study of collagen synthesis by clones of human dermal fibroblasts and noted considerable variation among control groups[19]; we would suggest that this was due to differences in the population density or the age of these clones. Russell et al. advised that the limited proliferative potential of normal cells may make it impossible to design cloning experiments in which culture age is not a confounding factor. However, the data reported in the present study, demonstrating the feasibility of examining cellular synthetic activities using very small numbers of sparsely seeded cells of young age, support the use of single-cell clones to study normal cell functions as well as disorder. Recent work in our laboratory and elsewhere supports this finding[20-22,24].

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Detection of pyrimidine 5′-nucleotidase deficiency using ^{1}H- or ^{31}P-nuclear magnetic resonance

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Summary. We describe here a further Japanese family with pyrimidine 5′-nucleotidase (P5′N) deficiency diagnosed using a nuclear magnetic resonance (NMR) spectrum, in Kumamoto prefecture where two families having the disease have been reported before. The specific spectra in ^{1}H-NMR of P5′N deficient erythrocytes were due to three methyl protons of CDP-choline at 3.22 ppm and to H-2, H-8 and ribose-1′ of pyrimidine nucleotide phosphate(s) in the lower fields (at 5.82 and 8.00 ppm). The other specificities in ^{31}P-NMR spectra were due to CDP-choline, CDP-ethanolamine and UDP-glucose. Those spectra were not detected in other types of hemolytic anemia.

Key words. Pyrimidine 5′-nucleotidase (P5′N); nuclear magnetic resonance.

Nuclear magnetic resonance (NMR) studies of whole cell biology, especially the non-invasive study of whole cell biochemistry, has been pioneered by investigations of energy metabolism with ^{31}P-NMR spectrometry[1,2]. Furthermore, ^{13}C-NMR instrument has demonstrated its potential power for the investigation of ^{13}C-enriched metabolites in cell suspensions[2,3]. It is well known that ^{1}H-NMR can detect a wide range of metabolic compounds simultaneously and at higher sensitivity than ^{13}C- or ^{31}P-NMR. Thus, ^{1}H-NMR has been used in recent years to reveal many intracellular events[4-7].

Pyrimidine 5′-nucleotidase deficiency associated with hereditary hemolytic anemia was first described by Valentine et al.[8], and about 35 cases[9] of the deficiency have been reported from other laboratories[10-19]. The enzyme deficiency was first detected by a somewhat laborious assay in which the Fiske-Subarow method was used to measure the quantity of inorganic phosphate released from CMP or UMP during a 2-h incubation. Torrance et al. developed a new method for the assay of this enzyme using ^{14}C-CMP as substrate[20]. However, the method can be useful only for diagnosis and not for metabolic analysis of P5N deficient

erythrocytes. We have been using ^{1}H-NMR[21] and ^{31}P-NMR instruments for clinical hematology[22,23], and the results reported here demonstrate that NMR instruments are a powerful tool for the detection of abnormal metabolites in P5'N deficient erythrocytes.

Materials and methods. The proband was a 27-year-old male (No. 7 in fig. 1). He was admitted to our hospital for further examination of his hemolytic anemia. At 16 years of age he was found to be anemic with slight jaundice. He noticed his reddish urine at 20 years of age. Two weeks before entry he came to the clinic with complaints of general fatigue and edema, and hemolytic anemia was diagnosed. His hemoglobin was 12.2 g/dl with a hematocrit value of 34.3% and 11.8% reticulocytes. Erythrocyte count was 332 × 10^4/mm^3 with distinct basophilic stippling. Leukocyte and platelet counts were 5100 and 156,000/μl, respectively. The leukocyte differential count was normal. Serum bilirubin was 4.0 mg/dl with only 0.5 mg direct reacting, and haptoglobin was less than 10.0 mg/dl. LDH isozyme analysis showed LDH_1 40.8%, LDH_2 42.8%, LDH_3 12.7%, LDH_4 1.9% and LDH_5 1.8%.

Ham acid hemolysis, Coombs, Heinz body, osmotic fragility and sugar-water tests and hemoglobin electrophoresis were all normal. History revealed no known exposure to toxic agents and no family history of blood dyscrasia. Test for urine lead was negative and that for hemosiderin was positive. Bone marrow aspiration revealed erythroid hyperplasia.

Venous blood from the patient with P5N deficiency and from healthy donors was drawn into heparin-containing tubes and centrifuged 1000 rpm for 10 min. Serum, white blood cells and platelets were removed. Packed cells thus obtained were used for ^{31}P-NMR measurements. Spectra were obtained under the same conditions and using identical numbers of cells. Metabolic phosphates of erythrocytes were extracted, after lysis by distilled water and deproteinization with perchloric acid, and then neutralization with potassium chloride, followed by centrifugation.

NMR measurement. ^{31}P-NMR and ^{1}H-NMR spectra were obtained with a JNM-FX 200 NMR spectrometer (JEOL, Tokyo) operated at 80.76 MHz and 199.5 MHz, respectively, in the Fourier transform mode. ^{31}P-NMR spectra were collected by applying 1000 (intact cells) and 4000 (extracts) 45 °C pulses at 2-s intervals. Chemical shifts are referred to 85% (w/v) phosphoric acid as an external reference. Samples were contained in 15-mm diameter observation tubes. ^{1}H-NMR spectra were collected by applying 1000 60 °C pulses at 2-s intervals. Extract samples were deuterized and contained in 5-mm diameter observation tubes. Chemical shifts are referred to DSS as an internal reference.

High performance liquid chromatography. A column of Hitachi gel 3013-N was used. Elution was performed at 70 °C by applying a linear gradient from 0.06 M NH_4Cl, 0.01 M each of KH_2PO_4 and K_2HPO_4 in 6% (v/v) CH_3CN to 0.3 M NH_4Cl, 0.05 M each of KH_2PO_4 in 6% (v/v) CH_3CN. The amount of each nucleotide was determined by cutting out each peak from the chromatogram chart, weighing and comparing with the weight of the corresponding peak from the corresponding chromatogram of a standard mixture.

Pyrimidine 5'-nucleotidase acitivity. Pyrimidine 5'-nucleotidase activity of erythrocytes from the patient and his family was measured by the method of Swallow et al.[24].

Results. Figure 1 demonstrates the pedigree of the family of the patient with pyrimidine 5'-nucleotidase deficiency. Subjects 1 and 4 are cousins. Figure 2 shows the typical 80.76 MHz ^{31}P-NMR spectra of fresh normal red cells (A), intact red cells (B) from a patient with P5'N deficiency reported before[13] and those from the new patient with hemolytic anemia (C). Comparison of the spectra A, B and C distinctly shows that the intensity of signals for α-, β- and δ-phosphorus of nucleotide triphosphates is approximately 2-fold greater in cells of B and C. The difference in intensity directly reflects the difference in the level of nucleotides between A, B and C. The spectrum (C) is similar to that (B) of other cases with pyrimidine 5'-nucleotidase deficiency. Thus, pyrimidine 5'-nucleotidase deficiency was diagnosed prior to the enzyme assay. In fact, the enzyme activities in the patient were 2.45 μmole Pi per gHb per h for CMP and 0.16 for UMP, but 13.73 − 3.57 for CMP and 12.84 + 3.12 for UMP in the control (table). Signal assignments were performed by comparing the chemical shift of each signal with those of an authentic compound added to the deproteinized sample, and high pressure liquid chromatography. Thus, the abnormal signals at −10.3 ppm(a), 11.0 ppm(b) and −11.9 ppm(c) were assigned to CDP-ethanolamine-phosphorous, CDP-choline-phosphorous and UDPG-phosphorous, respectively. The peaks a, b and c in figure 2B remained unhydrolyzed for 8 days at 4 °C. Any difference was detected in the intensity for 2,3-diphospho-glycerate signals (4.3 and 3.5 ppm) between the deficient and normal cells (4.0 and 3.2 ppm).

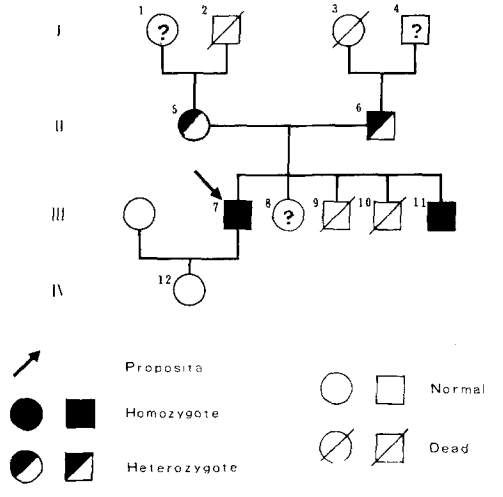

Figure 1. Pedigree of the family of the new patients with pyrimidine 5'-nucleotidase deficiency. Subjects 1 and 4 are cousins.

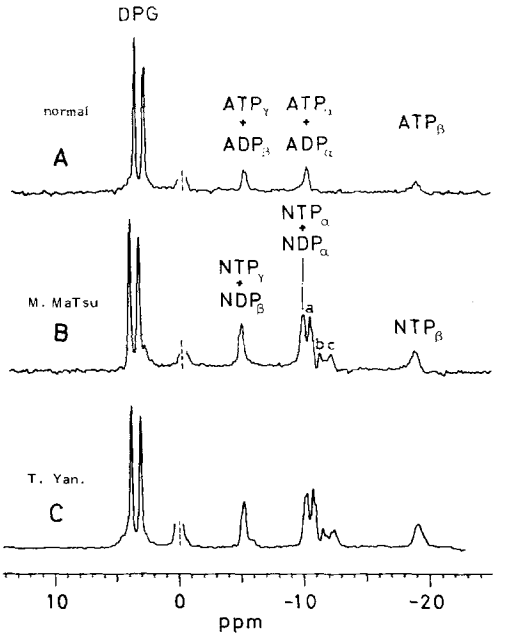

Figure 2. ^{31}P-NMR spectra of packed red cells from a normal control A, from P5N-deficiency B previously reported[13,23,25], and of the new patients (Nos. 7, 11 in fig. 1) from P5N-deficiency C. The dotted vertical line shows the chemical shift position for 85% H_3PO_4 as an external reference. NTP, nucleoside triphosphates; NDP, nucleoside diphosphates; DPG, 2,3-diphosphoglycerate.

Pyrimidine 5'-nucleotidase activity (μmol pi/g·Hb/H)

Substrates	Patient	Control*
CMP	2.45	13.73 ± 3.57
UMP	0.16	12.84 ± 3.12

*The results are given from 7 individual subjects as mean ± SD.

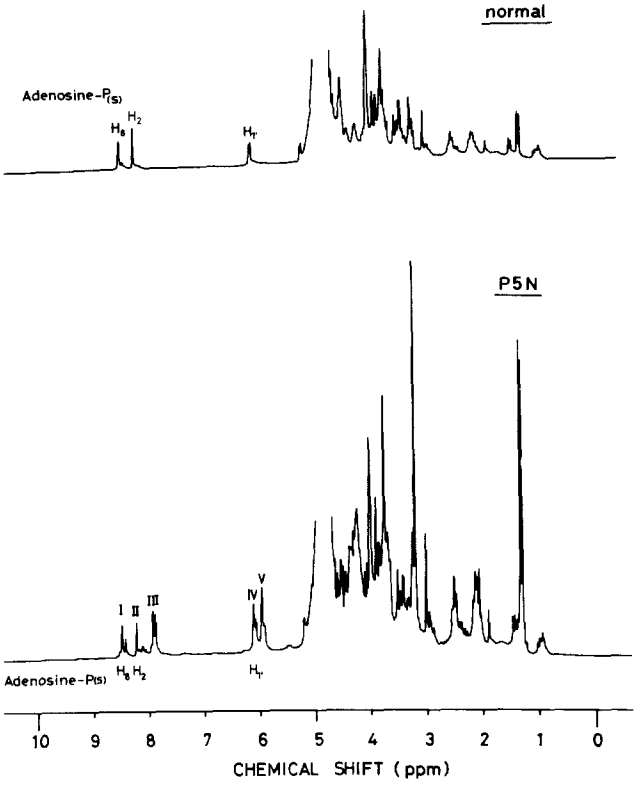

Figure 3. ^{1}H-NMR spectra of neutralized perchloric acid extracts from normal and P5N-deficient cells from previous patient and from the new family in figure 2. Resonances I and II result from H-8 and H-2 of adenosine phosphate(s), respectively; III, from H-6 of uridine and cytidine phosphate(s), IV, from H-5 of cytidine phosphate(s) and from H-1, of adenosine phosphate(s); V, from H-5 and H-1, of uridine phosphate(s) and from H-1, of cytidine phosphate(s).

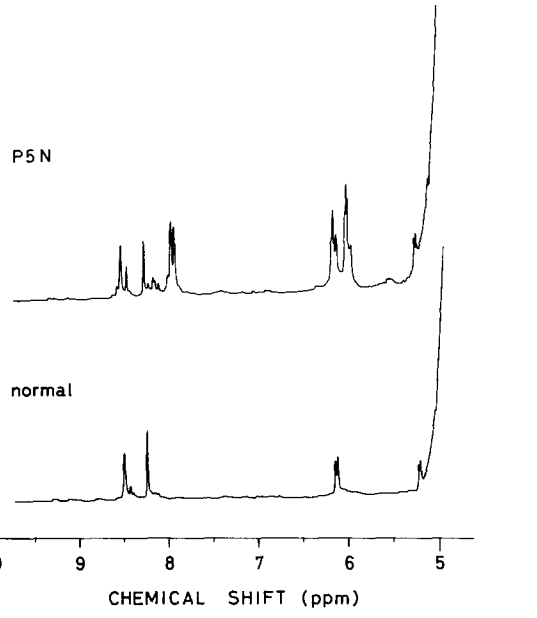

Figure 4. An expanded ^{1}H-NMR spectrum at a region from 5 to 9 ppm from the spectrum in figure 3.

Figure 3 shows a comparison of ^{1}H-NMR spectra of neutralized perchloric acid extracts from normal and P5N-deficient cells from patients M. Matsu and her brother (homozygous), spectrum B in figure 2. The eminent resonance line at 3.22 ppm appears to reflect methyl protons (9 protons per molecule) of CDP-choline. The resonance line at 3.76 ppm reflects methylene protons of glutathione (oxidized form). A group of signals from 4.18 to 4.36 ppm in the spectrum for P5N-deficient cells may reflect mainly ribose protons of nucleotides. Striking differences are observed in the lower field region. Namely, the spectrum for the extract from normal cells exhibited only resonance lines for H-2, H-8 and H-1′ protons of adenosine phosphate(s), whereas in the extract from P5N-deficient cells additional resonance lines for pyrimidine nucleotide(s) were seen. By comparison with chemical shift positions and spin couplings in model solutions, peaks I, II, III, IV and V were assigned as follows. Peaks I and II are due to H-8 and H-2 of adenosine phosphate(s), respectively; peak III, H-6 of uridine and cytidine phosphate(s); peak IV, H-5 of cytidine phosphate(s) and H-1′ of adenosine phosphate(s); peak V, H-5 and H-1′ of uridine phosphate(s) and H-1′ of cytidine phosphate(s).

Figure 4 shows an expanded ^{1}H-NMR spectra of neutralized perchloric acid extracts from a normal subject and from new patients T. Yan and his brother (No. 11 in fig. 1, spectrum C in fig. 2) with P5N-deficiency. These results of the ^{1}H-NMR study were also demonstrated in red cells from a previous family with P5N-deficiency; these results cannot be observed in heterozygotes carrying P5N-deficiency (Nos. 5 and 6 in fig. 1) and in other type of hemolytic anemia, such as spherocytic hereditary hemolytic anemia, paroxysmal nocturnal hematurias and autoimmune hemolytic anemia (not shown).

An additional advantage is that for ^{1}H-NMR less than 1 ml of packed red cells is needed. The ^{1}H-NMR spectrum is a powerful and convenient instrument for the diagnosis and metabolic study of P5N deficiency.

Discussion. We have tried the NMR instrument for medical applications[21,22] and have emphasized the potential usefulness of the ^{31}P-NMR method as a simple rapid diagnosis of this enzyme deficiency[23–25].

The ^{31}P-NMR and ^{1}H-NMR spectra of the new patient were nearly identical to those in other cases of P5N deficient erythrocytes[13,23]. Thus, this patient was strongly suspected, with basophilic stippling as an additional finding, to have P5N deficiency, and in fact P5N activities were one-tenth of normal (table). Clinical signs and symptoms and hematological findings for the new patient described here were almost the same as those published previously[9–11,13,19,26–28].

Biochemical or metabolic studies on P5N deficient erythrocytes have been performed previously[29–35], and especially the existence of UDPG in the erythrocyte was already reported in 1981[25]. After that, Swanson et al. showed the same results[36]. The peaks a, b and c in figure 2B, the same as in figure 2C, remained for 8 days at 4 °C without hydrolysis, which demonstrates that the enzymes for UDPG and CDP-choline or CDP-ethanolamine are not working in these erythrocytes. Thus, the basophilic stippling of P5N deficient erythrocytes may be due to these compounds. Swanson et al.[37] identified cytidine diphosphodiesters in erythrocytes from cases of P5N deficiency with ^{1}H-NMR spectra. However, the resonance lines due to pyrimidine nucleotide phosphate(s), compared with normal erythrocytes, have not been reported. Thus, the present results have not only added further information on the consequence of an extensive disturbance of pyrimidine nucleotide metabolism in the P5N deficient erythrocyte, but have also shown the potential usefulness of ^{1}H-NMR and ^{31}P-NMR spectroscopy for diagnosis of P5N deficiency.

Abbreviations used are:
P5'N = pyrimidine 5'-nucleotidase.
^{1}H-NMR = proton unclear magnetic resonance.
^{31}P-NMR = phosphorous nuclear magnetic resonance.
CDP = cytidine diphosphate.
UDPG = uridine diphosphate glucose.

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Contact and cellulolysis in *Clostridium thermocellum* via extensile surface organelles

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Summary. The ultrastructural distribution of the cellulosome (a cellulose-binding, multi-cellulase protein complex) of the thermophilic anaerobe, *Clostridium thermocellum*, was investigated. The cellulosome is compacted into protuberant cell surface structures, which, upon interaction with cellulose, form extended contact corridors wherein cellulolysis apparently occurs.
Key words. Cellulase; cellulosome; *Clostridium thermocellum;* exocellular organelle.

Considerable interest has recently been focused on the cellulase system in *Clostridium thermocellum*[1–3]. The cellulolytic apparatus in this bacterium comprises a multiple enzyme system consisting of both exo- and endo-β-glucanases which act synergistically in order to hydrolyze the complicated paracrystalline structure of cellulose. We have recently shown that the major enzymes responsible for cellulose degradation in this organism are arranged into a distinct multisubunit complex which we have called the 'cellulosome'[4–7]. The cellulosome appears both in an extracellular form and in a cell-associated form[4,8]. The latter is considered to comprise a discrete cell surface organelle, which is also responsible for the adherence of the bacterium to its insoluble substrate.

By using histochemical and immunocytochemical means, we demonstrate in the present report the ultrastructural localization of the cellulosome on the cell surface of *C. thermocellum*. In addition, we were able to trace its fate upon binding of the bacterial cell to cellulose.

Materials and methods. Cells of *C. thermocellum* YS were grown anaerobically in serum bottles containing salts, yeast extract and supplemented with either cellobiose (0,8 % w/v) or cellulose (0.8% w/v) as described previously[4]. Cells were harvested during the early stages of growth (usually between 8 and 15 h). Pelleted samples were resuspended gently in 0.7 ml of 0.9 % NaCl (saline) and 0.3 ml Karnovsky's fixative was added[9]. After a period of 20 min, the fixed cells were washed three times with saline, resuspended in 0.7 ml saline and treated either with cationized ferritin (CF; BioYeda, Israel) or by the immunochemical procedure outlined below. CF-treatment entailed the addition to the desired cell suspension of a saline solution (0.3 ml) containing 0.5 mg CF. The cells were incubated for 1 h at room temperature, centrifuged, washed twice (gently) with saline, and fixed with Karnovsky's fixative. Immunocytochemical labeling, using biotinylated anti-cellulosome (S1-specific) antibodies[4], was accomplished according to our previously described method[10,11]. The biotinylated antibody [30 µg in 200 µl 1% (w/v)