

Measurements of *in Vivo* ³¹P Nuclear Magnetic Resonance Spectra in Neuroectodermal Tumors for the Evaluation of the Effects of Chemotherapy¹

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ABSTRACT

The effects of chemotherapy on living tumor tissue in hamsters and rats were investigated by measuring the ³¹P nuclear magnetic resonance spectra using topical magnetic resonance. Human neuroblastoma, human glioblastoma, and rat glioma tumor cells were inoculated s.c. in the lumbar region of the animals. After the diameter of the tumors increased to 1.5 cm, *in vivo* ³¹P nuclear magnetic resonance spectra were measured selectively in the tumors with a TMR-32 spectrometer. Adenosine triphosphate, inorganic phosphate (P_i), phosphodiester, and phosphomonoester peaks were observed. The phosphocreatine peak was hardly detectable, adenosine triphosphate and phosphomonoester peaks were high, and tissue pH, calculated from the chemical shift of P_i, declined. Regardless of the tumor origin or the histological type, the spectral pattern of each neuroectodermal tumor was found to be essentially the same. After i.v. injection of a large dose of a chemotherapeutic agent, adenosine triphosphate peaks decreased and P_i increased gradually, resulting in a dominant P_i peak pattern after 6 to 12 hours. However, during the same period, there were no observable changes in the spectra of normal organs. These findings indicated that the drugs have a selective and direct action on the energy metabolism of tumor cells. With lower drug doses, no remarkable changes were seen in the spectrum. Measurement of *in vivo* ³¹P nuclear magnetic resonance spectra is valuable not only to investigate the energy metabolism in tumor tissue but also to evaluate the effects of chemotherapy.

INTRODUCTION

The effects of chemotherapy on tumor tissue have been investigated extensively by histological, biochemical, and radioisotopic methods. The cell culture model and the tumor-bearing animal model are used to examine the effects of various therapies. The former model is commonly used to examine the direct effect on tumor cells, but it is not suitable to clearly determine the effect on the tumor mass *in situ*. The latter method, on the other hand, has an advantage in that it is possible to observe the effect of the therapy on the tumor as a whole. However, this method requires many animals and much time because the

effects of the therapy could be evaluated only by measuring the change of tumor size serially and/or by calculating the survival rate of the animals. It is desirable to develop a more convenient and reliable method for examining the effect of therapy on tumor-bearing animals.

Recently, it has become possible to measure energy metabolism in a living animal sequentially using *in vivo* ³¹P NMR³ (1, 3-5, 7-17). We have indicated that this method is very useful not only for the examination of *in vivo* energy metabolism (13, 15) but also for the evaluation of the effects of therapy on many diseases *in situ* (14). Therefore, we applied this method in the assessment of the effects of chemotherapy on implanted tumors by measuring the acute reaction of living tumors to antitumor agents.

MATERIALS AND METHODS

Three experimental groups of neuroectodermal tumors were inoculated s.c. in the lumbar region of the animals. The first group, consisting of 26 newborn hamsters pre- and posttreated with antilymphocyte serum, received a suspension of 10⁶⁻⁷ cultured human neuroblastoma cells (18). The second group, consisting of 21 similarly treated hamsters, was given the same quantity of a suspension of cultured human glioblastoma cells (2). The third group, consisting of 23 CD Fisher rats, received the same quantity of cultured rat glioma (EA285) cells (20). Animals were used for the experiment when the tumors grew to over 1.5 cm in diameter. Measurements of *in vivo* ³¹P NMR spectra in the tumors were made using a TMR-32 spectrometer (Oxford Research Systems, Oxford, United Kingdom). After anesthetizing with an i.p. injection of sodium pentobarbital (35 mg/kg), animals were placed in a supine position in the superconducting magnet (1.8 teslas) so that the tumor site was situated in the center of the effective homogeneous magnetic focusing field, which measured 1.5 cm in diameter. A 4-turn, solenoid-shaped radio-frequency coil (1.5 cm in diameter) was applied over the tumor site to obtain a resonant signal of ³¹P at 32.5 MHz from the tumor. The parameters were as follows. The homogeneity in the measured volume was about 0.2 ppm, pulse width was 13 μs (40° pulse), and scans were repeatedly made 250 or 500 times at 1-s intervals. Data points numbered 4096, spectrum width was 4000 Hz, and acquisition time was 0.51 ms (15). A 5-Hz exponential noise filter and a 200-Hz convolution difference were applied to the free induction decay signal prior to Fourier transformation. Following this control study for each group, antitumor agents such as cyclophosphamide, vincristine, and methotrexate were injected i.v., and their effects on the ³¹P NMR spectra of the tumor were monitored sequentially for 48 h after the administration.

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³ The abbreviations used are: NMR, nuclear magnetic resonance; PCR, phosphocreatine; PME, phosphomonoester.

These treated groups were further subdivided into 2 subgroups according to the dose of the drugs; high-dose groups, which received about 80% of the 50% lethal dose, and low-dose groups, which received about one-eighth to one-tenth that of the high-dose ones. Observations were limited to a short period in order to simplify and facilitate comprehension of the data. As a sham-operated group, 4 animals in each group received an i.v. injection of 0.9% NaCl solution in the same volume as the solution of the chemotherapeutic drug. Tissue pH was calculated from the chemical shift (σ ppm) of P_i in each spectrum using the equation

$$pH = pK' + \log \frac{\sigma - \sigma_a}{\sigma_b - \sigma}$$

where pK' is 6.75, σ_a is -3.15 ppm and σ_b is -5.65 ppm, assuming that the chemical shift of PCR is 0 ppm (16). In cases showing extremely small PCR peaks, 0 ppm was assumed from the α -ATP peak which was located at 7.5 ppm. Moreover, in cases showing no ATP peaks, a peak of PME's was used temporarily to determine the chemical shift of P_i .

RESULTS

The typical spectra of an untreated human neuroblastoma, a human glioblastoma, and a rat glioma are shown in Charts 1a, 2a, 3a, 4a, and 5a. Several peaks are shown in the spectra. They are, in the order of high resonant frequency: β -phosphate of ATP; α -phosphates of ATP and ADP including $NAD^+/NADH$, γ -phosphate of ATP, and β -phosphate of ADP; PCR; phosphodiester; P_i ; and PME. Regardless of the tumor origin or the histological type, the spectral pattern in the 3 types of neuroectodermal tumors was essentially the same. The PCR peak could hardly be detected while PME and ATP peaks were obvious. Tissue pH at this stage, calculated from the chemical shift of P_i , was 7.15 ± 0.05 (S.D.; $n = 52$). Anesthesia had no effect on the spectral pattern in any group. As the tumor growth advanced,

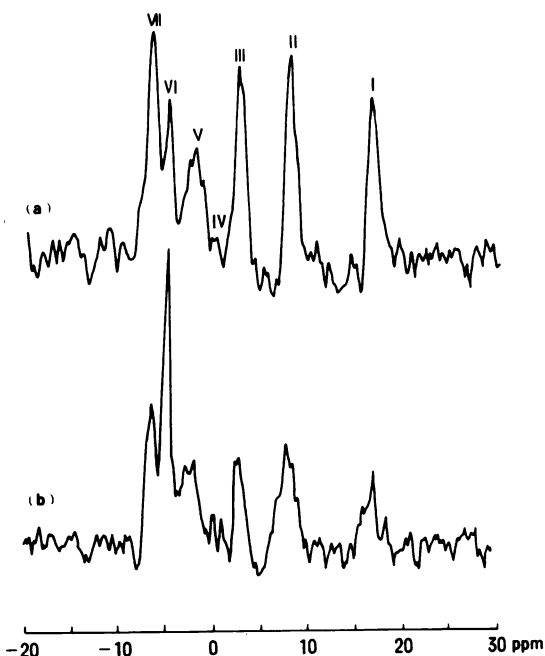


Chart 1. ³¹P NMR spectra of the rat glioma in 2 different stages of tumor growth. The assignments of ³¹P NMR signal peaks are: I, β -ATP; II, α -ATP and α -ADP with $NAD^+/NADH$; III, γ -ATP and β -ADP; IV, PCR; V, phosphodiester; VI, P_i ; VII, PME's. As the stage of tumor growth advanced from the active stage (a) to an advanced stage 4 weeks later (b), ATP peaks decreased with a concomitant increase of P_i .

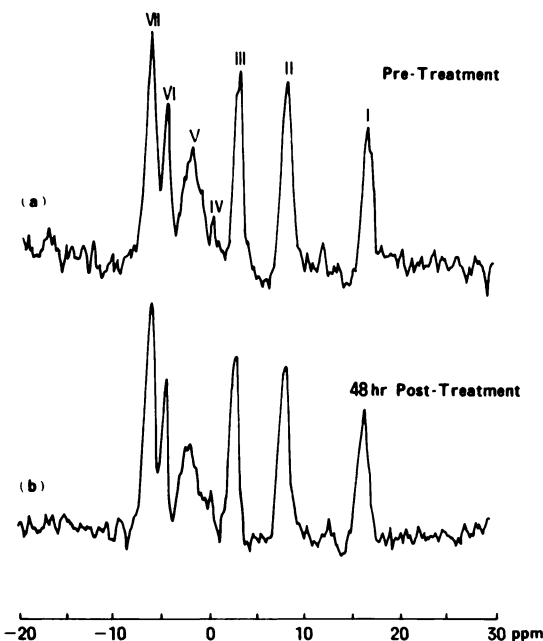


Chart 2. ³¹P NMR spectra in the human neuroblastoma pre- and postadministration of the low dose of cyclophosphamide. a, pretreatment; b, 48 h after i.v. injection of cyclophosphamide at a dose of 30 mg/kg body weight. There were no changes in the spectrum.

ATP peaks decreased with a concomitant increase of P_i , finally resulting in a P_i -dominant pattern (Chart 1). It takes about 2 weeks for the neuroblastoma and glioblastoma and about 4 weeks for the rat glioma to progress to such an advanced stage after NMR measurement. Tissue pH at this advanced stage was 7.07 ± 0.08 ($n = 20$).

In the sham-operated group, there were no changes in the spectrum after i.v. injection of 0.9% NaCl solution. There were also no changes in the treated group with low doses (Chart 2), except for one case of neuroblastoma showing a temporary decrease of ATP after the administration of cyclophosphamide. On the contrary, in treated groups with high doses, almost all animals showed the same drastic changes in tumor spectrum within a short period. For example, after the i.v. injection of cyclophosphamide to 5 neuroblastoma-bearing hamsters at a dose of 300 mg/kg body weight, a complete loss of ATP and a marked increase of P_i were observed in all 5 animals within 10 h (Chart 3). At this time, however, no histological changes were detected in the tumor tissue. These changes in the spectrum lasted for at least 6 days in surviving cases. Histological examination, performed after this 6-day period, showed extensive necrosis in the tumor tissue. Similar changes in spectra were also seen with other antitumor agents. Administration i.v. of vincristine at a high dose of 2 mg/kg caused a decrease of ATP and an increase of P_i in all 4 neuroblastomas within 6 to 12 h. Even in rat glioma and human glioblastoma, antitumor agents at a high dose had nearly identical effects (Charts 4 and 5). There were no concomitant changes in the spectrum of normal organs such as muscle and brain for 2 days after the experiment (Chart 6). Therefore, it was supposed that spectrum changes were specific to the tumor tissue. The results of cases which showed such ³¹P NMR spectral changes are shown in Table 1. In the group showing changes in the spectrum, tissue pH was $7.09 \pm$

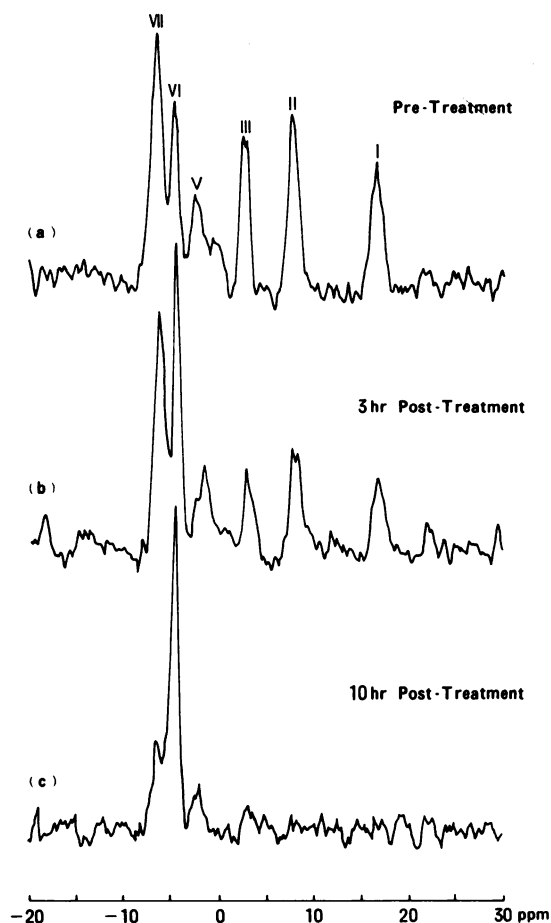


Chart 3. Changes in the ^{31}P NMR spectrum in the human neuroblastoma caused by the administration of a high dose of cyclophosphamide. a, pretreatment; b, 3 h after the i.v. injection of cyclophosphamide at a dose of 300 mg/kg body weight; ATP peaks decreased and the P_i peak increased; c, 10 h after i.v. injection; ATP peaks disappeared, and the P_i peak increased markedly.

0.10 ($n = 21$), which was more acidic than the pretreatment level.

DISCUSSION

Measurement of the *in vivo* ^{31}P NMR spectrum has been made for various organs and tissues with the use of a surface coil method and a topical magnetic resonance method (1, 3, 4, 8, 13, 14, 17). Several reports concerning the *in vivo* energy metabolism of tumor tissue have been made using Walker sarcoma, Dunn osteosarcoma, mammary carcinoma, and astrocytoma (5, 9-12, 16). Comparison of these previous reports with ours has shown that the spectrum of the neuroectodermal tumors resembled malignant tumors in other organs. This suggests that the energy metabolism of the tumors is essentially the same regardless of the origin of the tumor or of the histological type. A large PME peak and an extremely small PCR peak are characteristic findings of the ^{31}P NMR spectrum in tumors. The chemical shift of P_i in tumor tissue is slightly smaller than in normal tissue, indicating a decrease of tumor tissue pH. Our present report also suggested that ^{31}P NMR spectrum measurement could be used possibly as a sensitive monitor for the effect of antitumor agents. From the results obtained, the loss of high-energy phos-

phate and the consequent predominant P_i peak were characteristic changes in the spectrum caused by the administration of antitumor agents in high doses. These changes lasted at least 6 days, indicating the presence of damage in tumor cells. Histological examination showed no changes in the tumor tissue at an early period after the ^{31}P NMR spectrum changed. However, extensive necrosis was seen in the tumor tissue 6 days after the treatment upon an additional examination. Therefore, it was supposed that the tumor cell kill caused by the high-dose administration of antitumor agents was detected early with the ^{31}P NMR spectral measurement. The mechanism of the effects of these drugs on the ^{31}P NMR spectrum could not be explained from the basis of antitumor action on cell kinetics because it took at least 2 to 3 days for a complete cycle of cell division. As detectable by this method, these drugs acted directly on the energy metabolism of tumor cells without any apparent effect on normal tissues. The acute effects of chemotherapy on ATP and NAD has been suggested previously by the ordinary biochemical analysis *in vitro* of tumor tissue (6). Our *in vivo* data supported these results. Ng *et al.* (16) reported different results in ^{31}P NMR spectral changes using MOPC 104E myeloma implanted in mice, *i.e.*, a striking increase of PCR with a concomitant decrease of P_i and ATP within the first 2 days after the administration of 1,3-bis(2-chloroethyl)-1-nitrosourea. In another paper, they also re-

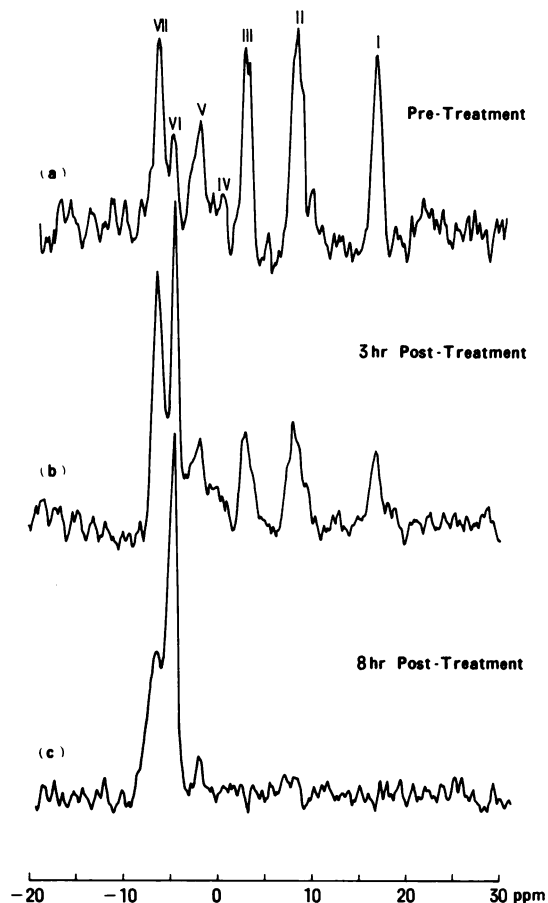


Chart 4. Changes in the ^{31}P NMR spectrum in the human glioblastoma caused by the administration of a high dose of vincristine. a, pretreatment; b, 3 h after i.v. injection of vincristine at a dose of 2 mg/kg body weight; ATP peaks decreased and the P_i peak increased; c, 8 h after the injection; the spectrum demonstrated a predominant P_i pattern.

ported similar but partial changes in the spectrum of mammary 16/C adenocarcinoma after the high-dose administration of Adriamycin (5). However, we could not find such changes in the spectrum in any case. This discrepancy may be due to the dose of the drugs administered or the susceptibility of the tumor tissue to each drug. They also reported that a P_i-dominant pattern was shown after other effective therapy such as hyperthermia (5, 16). Therefore, this predominant P_i pattern is one of the most characteristic findings of the response of a tumor to effective therapy including chemotherapy. Thus, *in vivo* ³¹P NMR spectral measurement could be used as a sensitive monitor of tumor response

to antitumor agents at a high dose.

The above-mentioned response was limited to the acute reaction of tumor mass to single-shot, high-dose chemotherapy, and this reaction was not produced by low doses. Practically, to prove the usefulness of this method as a good monitor of tumor therapy, it is necessary to further observe spectrum changes during longer periods after repeated administration of each drug with lower doses. However, careful attention should be paid in drawing conclusions from such an experiment, because similar spectral changes may occur spontaneously according to tumor growth as shown in Chart 1. This is a very serious problem in malignant tumor models such as Walker sarcoma, Dunn osteosarcoma, and Lewis lung cancer due to rapid growth in a short period. However, in our experimental model, this problem is out of consideration because it takes 4 to 6 weeks for the tumor to grow to a size where spontaneous changes occur. Although the low-dose administration of an antitumor agent with a single shot did not affect the spectrum, a preliminary examination in 2 cases demonstrated that the spectrum changes did occur after the repeated injection of low-dose drugs just as in high-dose administration (Chart 7). Therefore, this finding indicates that the

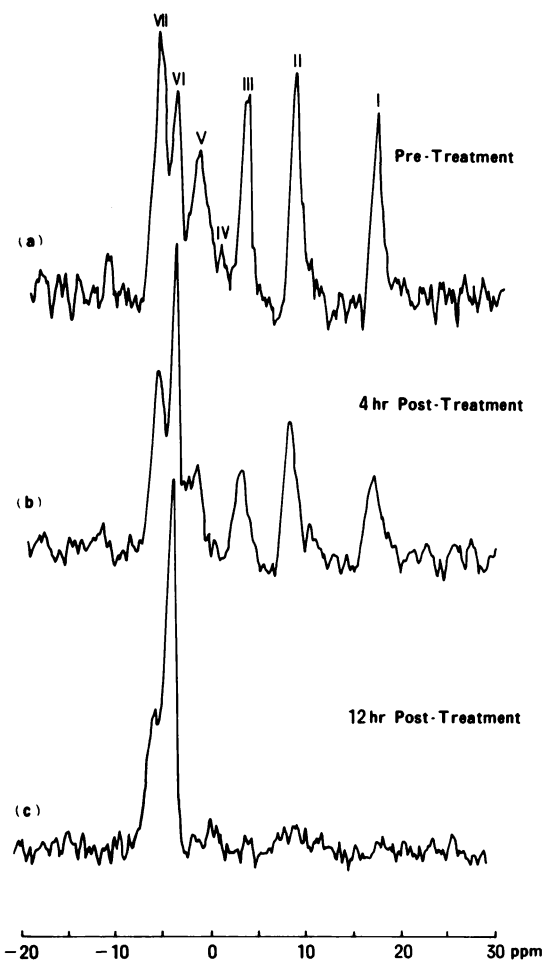


Chart 5. Changes in the ³¹P NMR spectrum in the rat glioma caused by the administration of a high dose of vincristine. a, pretreatment; b, 4 h after the i.v. injection of vincristine at a dose of 2 mg/kg; ATP peaks decreased, and the P_i peak increased slightly; c, 12 h after the injection; a P_i-dominant pattern was shown in Charts 4 and 5.

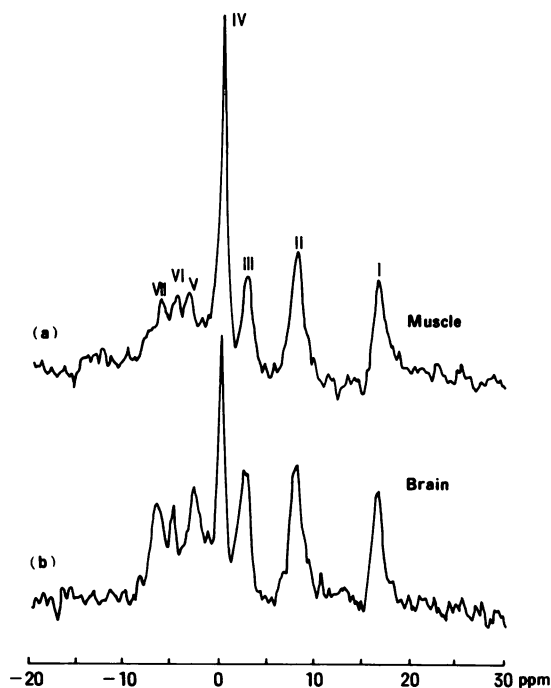


Chart 6. ³¹P NMR spectra of the lumbar muscle and the brain in a rat treated with vincristine. There were no concomitant changes of spectra in these normal organs after high-dose administration of antitumor drugs. This result was followed up for 7 days.

Table 1
Summary of cases showing changes in the ³¹P NMR spectrum within 2 days after chemotherapy

The spectrum was judged to be changed when it showed a P_i-dominant pattern.

	Cyclophosphamide		Vincristine		Methotrexate		Sham operation (0.9% NaCl solution)
	30 mg/kg	300 mg/kg	0.5 mg/kg	2 mg/kg	15 mg/kg	100 mg/kg	
Human neuroblastoma	1 (3) ^a	5 (5)	0 (3)	4 (4)	0 (4)	3 (3)	0 (4)
Human glioblastoma	0 (2)	3 (3)	0 (3)	4 (4)	0 (3)	2 (2)	0 (4)
Rat glioma	0 (3)	3 (3)	0 (3)	3 1 ^b (4)	0 (3)	2 1 ^b (3)	0 (4)

^a Numbers in parentheses, number of cases examined in each group.

^b No. of case showing partial and temporary ATP and P_i changes in ³¹P-NMR spectrum.

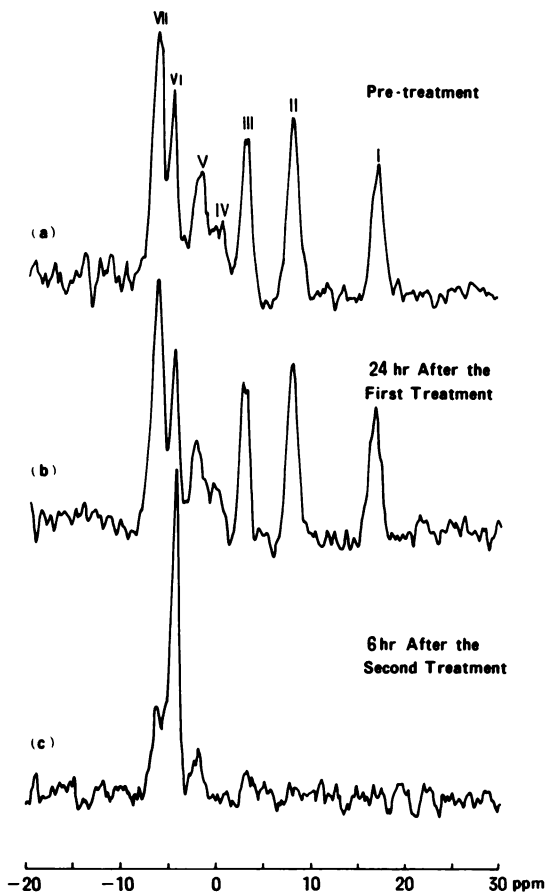


Chart 7. Changes in the ³¹P NMR spectrum in the neuroblastoma with the repeated administration of cyclophosphamide at a lower dose. a, pretreatment; b, 24 h after the i.v. injection of cyclophosphamide at a dose of 30 mg/kg; there were no changes in the spectrum at this time. c, 6 h after the additional injection of the same dose of cyclophosphamide at 24 h after the first injection; ATP peaks disappeared, and the spectrum showed a predominant P_i peak pattern.

measurement of ³¹P NMR spectrum for evaluation of the effects of antitumor agents can be useful in determining the best administration method and the minimal dose of the drug required for effective therapy.

Recently, it has become possible to take *in vivo* ³¹P NMR spectra clinically with a magnetic resonance imaging instrument with a high-field superconducting magnet. The results of the present report will favor and encourage such trials.

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