Review

Response of Solid Tumors to Chemotherapy Monitored by in Vivo 31P Nuclear Magnetic Resonance Spectroscopy: A Review

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Abstract

In vivo 31P nuclear magnetic resonance (MR) spectroscopy has shown great promise as a tool for cancer research and the clinical management of solid tumors. It is now possible in some cases to integrate MR spectroscopy with routine MR imaging of the cancer patient, so that tissue identified as tumor on an MR image can be examined biochemically and monitored following treatment. Alterations have been observed in the phosphorus MR spectra of patient tumors after treatment, but the causes and consequences of these alterations are poorly understood. Here we review data obtained from experimental animal tumor models treated with chemotherapy in order to gain insight into the biological events reflected in MR spectroscopic changes, and to determine what information the spectra provide about the success or failure of therapeutic interventions. An attempt is made to relate these experimental findings to the cancer clinic and to analyze the contributions of MR spectroscopy to the understanding of tumor biology.

Introduction

NMR2 is a quantum mechanics phenomenon affecting certain atomic nuclei. When molecules are placed in a magnetic field, the magnetic field interacts with those atomic nuclei which possess unpaired protons or neutrons. These unpaired nucleons confer upon the atomic nucleus intrinsic magnetic properties which cause the nucleus to respond to magnetic fields, much as the needle of a compass responds to the earth’s magnetic field. Each nucleus within a molecule experiences a slightly different applied magnetic field depending upon how that nucleus is shielded by electron clouds on adjacent atoms. Each nucleus will resonate, or absorb and reemit radiofrequency, at a particular frequency determined by the local chemical environment of the nucleus within the molecule. Placing a molecule in a strong magnetic field and interrogating that molecule with a rf frequency, or chemical shift, information can be obtained with the same dependence to frequency dependence by Fourier transformation. The time-dependent loss of induced magnetic coherence is then translated from time domain to frequency domain (1). The time-dependent loss of induced magnetic coherence is then translated from time dependence to frequency dependence by Fourier transformation. Since each chemically equivalent nucleus resonates at the same rf frequency, or chemical shift, information can be obtained about bonding configurations and molecular structure. The Fourier-transformed spectrum can show the identity and concentration of metabolites in vivo and can sometimes reveal information about tissue localization of metabolites (for general background, see Refs. 1 to 6).

In vivo NMR techniques have shown great promise as a tool for cancer research and the clinical management of cancer. MRI can be used to create a proton density map, in which image features are determined by anatomical structures, and image brightness can be determined by the relative concentration and mobility of water protons or by particular pulse sequences designed to generate or highlight contrast. MRI produces high-resolution anatomical images, which are already the diagnostic method of choice for cranial malignancies and are becoming increasingly important in a range of other applications (for information, see Ref. 7). MRS can provide in vivo information about a number of biologically important nuclei such as 1H, 13C, 23Na, and 31P. Phosphorus-31 is a 100% naturally abundant nucleus present in measurable concentration in only a few metabolites, but these metabolites are the critical bioenergetic intermediates in all living tissues.

Phosphorus-31 MRS can detect phosphorylated compounds in vivo which are present at greater than roughly 100 μM concentration (1), including metabolites such as nucleoside triphosphates (NTP: ATP, UTP, and similar compounds), phosphocreatine, Pc, and a variety of phosphomonooester and phosphodiester compounds (8). While most cells and tissues are able to maintain relatively constant levels of these metabolites through homeostatic processes, tissues such as tumors, which can become hypoxic or ischemic, will often show altered levels of certain bioenergetic metabolites. As well as making in vivo measurements of steady-state levels of bioenergetic intermediates (Fig. 1), 31P MRS is capable of identifying and quantifying cell phospholipids or measuring tissue pH in vivo.

The only experimental requirement for collecting in vivo NMR spectra is that the tissue of interest be made to fit within the homogeneous magnetic field created by the NMR magnet without compromising the biological integrity of the tissue. Currently the highest field-strength magnet approved for human applications is 1.5 tesla (T), but experimental work can be done in animal systems at field strengths of 11 T or higher. Higher field strengths yield superior spectral resolution, so much of the work to date has involved animal experimental tumors in high field-strength magnets. The requirement that the tissue of interest fit within the homogeneous region of the magnet becomes increasingly stringent at higher magnetic fields, because the spatial volume over which the field is sufficiently homogenous becomes smaller at higher field strength. However, these limitations aside, NMR is currently the only technique by which in vivo bioenergetic information can be obtained in a noninvasive and nondestructive manner.

It is presently possible to collect information about the bioenergetic status of tissue in precisely localized regions of human tissue using 31P MRS (9). MR imaging can be integrated with MR spectroscopy in a single patient exam of roughly 1 h (10), and tissue identified as tumor on an MR image can be examined biochemically and monitored following treatment (11). Alterations have been observed in the 31P MR spectrum of tumors...
measured by in vivo NMR were positively correlated with the size of the well-perfused fraction in each tumor.

Tumor response to therapy has been monitored by $^{31}$P NMR in a number of animal tumors in vivo (5, 13, 15, 19, 22) and in human tumor xenografts in nude mice (14). In animal studies two qualitatively different patterns have emerged, as shown by an informal “meta-analysis” of the data in Table 1. Some therapeutic modalities, notably hyperthermia (15) and photodynamic therapy (23, 24), produce a tumor with little or no NTP and a very large P$_i$ resonance. This tumor decline has been hypothesized to result from severe tumor ischemia. However, for many types of tumors, response to chemotherapy or irradiation appears to involve the reversal of the trends seen in untreated tumor progression (19, 20, 25), leading to apparent increases in levels of high-energy phosphates. This pattern of response will be termed tumor activation.

Tumor Activation following Chemotherapy

Chemotherapeutic agents produce apparent tumor activation in 62% of the tumor-chemotherapy pairs reported to date (8 of 13; Table 1). No real patterns emerge as to particular drugs which produce activation, because several drugs (cyclophosphamide and Adriamycin) have been shown to be associated with both tumor activation and the opposite effect of tumor decline (cyclophosphamide: 20, 22; Adriamycin: 25). There is no evidence that tumor size plays a role in response to treatment, as very small tumors (≤0.5 cm$^3$) can respond to chemotherapy by showing activation (20, 25) or decline (25, 26). No solid evidence exists to support the hypothesis that human tumors respond to chemotherapy in any fundamentally different way from tumors of animal origin (Table 1). The fact that both activation and decline can be produced in tumors treated with chemotherapy, apparently depending upon the particular tumor-therapy pair, suggests that the NMR signal from a treated tumor is affected by the total tumor cell kill achieved with treatment, the rate of clearance of killed cells from the tumor, and the metabolic responses of the host and the surviving tumor cells (27).

Work with a drug-resistant subline of a drug-sensitive tumor (25) confirms the hypothesis that the NMR spectrum derived from treated tumors is affected by tumor cell kill achieved. Murine mammary adenocarcinoma 17/A, which is sensitive to Adriamycin, was selected for drug resistance by repeated Adriamycin treatment in vivo to obtain the Adriamycin-resistant subline 17/A/ADR (28). In vivo NMR spectra were obtained from both the sensitive and resistant sublines prior to and following i.v. administration of Adriamycin (12 mg/kg) (25). Significant differences were observed between pretreatment spectral parameters and parameters 1 day following treatment of the Adriamycin-sensitive tumors: (a) tumor pH increased to greater than 7.3; (b) the ratio of P$_i$ to NTP decreased to less than 1; and (c) the relative proportion of phosphorylcholine (PME) and glycerophosphorylcholine (PDE) decreased. Adriamycin-sensitive tumors also showed an increase in the ratio of phosphorylcholine to NTP following treatment, which was not significant for the population, although it was substantial in 25% of the tumors examined. In contrast to these several differences observed in the Adriamycin-sensitive tumors, no major changes resulted from treatment of the Adriamycin-resistant tumors. Therefore, the changes in the spectrum of the Adriamycin-sensitive tumors are associated with tumor cell kill. This conclusion is consistent with evidence that treatment of the Adriamycin-sensitive tumors produced a 19-day growth delay (1.6 log$_{10}$ cell kill), while the Adriamycin-resistant tumors

Animal Tumor Response to Chemotherapy Observed by NMR

During untreated growth of tumors the levels of energy-rich compounds (NTP and phosphocreatine) tend to decline relative to other spectral resonances, while the resonances due to P$_i$ and PMEs increase (5, 8, 12–20). These changes are frequently associated with an acidic shift in tumor tissue pH (5, 20). Because these changes resemble those observed in normal tissues made ischemic, it has been proposed that changes observed in growing tumors result from increasing hypoxia as the tumor outgrows its blood supply (15). Support for this hypothesis has come from $^{18}$O washout experiments (21), which demonstrate that the quantities of high-energy phosphates in RIF-1 tumors

fig. 1. In vivo (a) and in vitro (b) $^{31}$P NMR spectra derived from a s.c.-implanted 9L gliosarcoma at Day 12 after tumor implantation. The in vivo spectrum was taken using a Bruker AM 360 multinuclear spectrometer (8.5 T/8.9-cm bore) equipped with an Aspect 3000 computer. A home-built probe with a solenoidal radiofrequency coil doubly tuned to proton and phosphorus was used. The spectrum was acquired at 145.8 MHz using a 60° pulse ($\tau_{60}$) and a 3-s recycle time; the in vivo spectrum is the result of 200 scans. The tumor was then freeze clamped at liquid nitrogen temperature, extracted in perchloric acid, deionized in Chelex resin, and repeatedly lyophilized. The extract spectrum was acquired using the same collection parameters as above, except that 12,000 scans were summed. Peak assignments are as follows (19): PE and PC (1); P$_i$ (2); glycerophosphorylcholine (PDE) and glycerophosphorylcholine (PME). 3; phosphocreatine (4); Ï–NTP and nucleoside diphosphate (5); Ï–NTP and nucleoside diphosphate (6); unknown, possibly uridine diphosphate (7); and Ï–NTP (8).
experienced no growth delay (25). These results do not confirm an earlier report that the relative spectral contribution of phosphocreatine was elevated and PME and PDE depressed in a tumor cell line showing pleiotropic drug resistance (29).

Activation of a tumor following treatment is shown particularly clearly in the rat 9L gliosarcoma following treatment with BCNU (19). Tumor growth was associated with significant declines in the phosphocreatine and NTP resonances, consistent with an increase in the extent of tumor hypoxia during growth. Following treatment with BCNU, tumor levels of phosphocreatine and NTP increased with respect to age- and size-matched untreated controls, while the level of P1 decreased (19). Spectral changes following treatment with BCNU were dose dependent, with tumor activation occurring even at a dosage of BCNU (36 mg/kg) which caused massive tumor necrosis (19).

Spectral changes following treatment and then increased during the period of maximal DNA synthesis (33). The P1 decline in RIF-1 following cyclophosphamide is associated with a significant reduction of the P1 resonance with respect to other metabolites (20). The decrease in P1 is seen in perchloric acid extracts of treated tumors, so the decline of P1 in vivo cannot be explained as an increase in the fraction of P1 bound to macromolecules or sequestered in NMR-invisible compartments (20). Reduction of P1 is probably not the result of incorporation into DNA, as the P1 signal within the tumor declined during the period when DNA synthesis is reduced following treatment and then increased during the period of maximal DNA synthesis (33).

The P1 decline in RIF-1 following cyclophosphamide is associated with a tumor growth delay and is accompanied by an alkaline shift of tumor pH (20). A linear discriminant analysis combining tumor pH and the phosphocreatine:P1 ratio was able to successfully distinguish between treated and control RIF-1 tumors in 100% of cases, suggesting that, for some tumors, pH measured by NMR may be a sensitive indicator of therapeutic efficacy (20). Tumor pH has been reported to undergo an alkaline shift in murine mammary 17/A following Adriamycin (25), in the Glasgow osteogenic sarcoma treated with flavone acetic acid (26), and in RIF-1 following radiation (34). Conversely, an acidic shift has been reported following treatment of neuroectodermal tumors with high-dose chemotherapy (22), while no significant pH changes were observed following treatment of 9L with BCNU (19).

The estrogen-sensitive, NMU-induced rat mammary tumor (ES mammary, Table 1) also undergoes activation following treatment (32); although the treatment studied was not chemotherapy, the data may provide insight into the mechanism of

Table 1  
<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Species of origin</th>
<th>Treatment</th>
<th>Size at treatment (cm³)</th>
<th>Effect</th>
<th>Ref.</th>
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<tr>
<td>MOPC 104E</td>
<td>Mouse</td>
<td>CPA (200 mg/kg)</td>
<td>NA</td>
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<td>13</td>
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<tr>
<td></td>
<td>’BCNU (20 mg/kg)</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>13</td>
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<tr>
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<td>&gt;0.5</td>
<td>+</td>
<td>15</td>
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<td>’Radiation</td>
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<td>&gt;0.5</td>
<td>+</td>
<td>15</td>
</tr>
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<td>17/A</td>
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<td>Adria(12 mg/kg)</td>
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<tr>
<td>9L</td>
<td>Rat</td>
<td>BCNU (10-36 mg/kg)</td>
<td>&gt;1.0</td>
<td>+</td>
<td>19</td>
</tr>
<tr>
<td>RIF-1</td>
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<td>CPA (150-500 mg/kg)</td>
<td>0.2</td>
<td>+</td>
<td>20</td>
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<tr>
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<td>&gt;0.3</td>
<td>+</td>
<td>34</td>
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<td>“ES mammary”</td>
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<td>Ovariectomy</td>
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<td>+</td>
<td>32</td>
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<td>“Immunocytoma”</td>
<td>Rat</td>
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<tr>
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<td>100</td>
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<td>Mouse</td>
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<td>-</td>
<td>101</td>
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<tr>
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<td>&gt;1.0</td>
<td>-</td>
<td>102</td>
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<td>Mouse</td>
<td>PDT</td>
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<td>-</td>
<td>103</td>
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<tr>
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<td>Ovariect</td>
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<td>-</td>
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<tr>
<td>MCAIV</td>
<td>Mouse</td>
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<td>0.1-0.4</td>
<td>-</td>
<td>105</td>
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<tr>
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<td>Mouse</td>
<td>PDT</td>
<td>&gt;0.3</td>
<td>-</td>
<td>106</td>
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<tr>
<td>Glasgow OS</td>
<td>Mouse</td>
<td>FAA (100-200 mg/kg)</td>
<td>0.3-0.6</td>
<td>-</td>
<td>107</td>
</tr>
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* CPA, cyclophosphamide; NA, data not available; 5-FU, 5-fluorouracil; OS, osteogenic sarcoma; PDT, photodynamic therapy; FLC, Friend leukemia cells; TNF, tumor necrosis factor; MCA, mammary carcinoma.
Chemotherapy-induced tumor activation. Estrogen-sensitive mammary tumors were induced in situ by a course of NMU which caused 80% of animals to develop tumors. Untreated tumors showed a steady loss of high-energy phosphate and an increase in P, but after host ovariectomy the tumor phosphocreatine and β-NTP peaks rose and the P, peak declined. Spectral changes were detectable by NMR before any measurable regression of tumor volume, and control experiments were able to exclude the possibility that signal was detected from muscle adjacent to the rf coil during regression of the tumors. After sham operation of animals bearing estrogen-sensitive tumors or after ovariectomy of animals bearing estrogen-insensitive tumors, tumors continued to grow, and the high-energy phosphates were progressively lost. The paradoxical increase of high-energy phosphates in tumors during regression was postulated to be due to a decrease in demand for ATP, so that the cellular energy supply was again sufficient for the metabolic needs of the tumor (32). Other possibilities will be discussed in “Tumor Biology and NMR Spectroscopy.”

Tumor Decline following Chemotherapy

Chemotherapeutic agents did not produce tumor activation in 38% of the tumor-chemotherapy pairs shown in Table 1 (5 or 13). The opposite pattern of high-energy phosphate decline is observed in these tumors, often at an enhanced rate relative to the loss of high-energy phosphates in untreated tumors (22). The effect of extremely high doses of several different chemotherapeutic agents was examined in three different tumors of neuroectodermal origin (22). A human “neuroblastoma” (implanted in hamsters) given cyclophosphamide (300 mg/kg), a human “glioblastoma” (implanted in hamsters) given vincristine (2 mg/kg), and the rat glioma EA285 (implanted in CD Fischer rats) given vincristine (2 mg/kg) all showed tumor decline in the first 6 to 12 h following therapy. After i.v. injection of cyclophosphamide (300 mg/kg) to “neuroblastoma”-bearing hamsters, a complete loss of NTP and a marked increase in P, were observed in all animals within 10 h. In at least one tumor, P, and PME were the only peaks visible in the NMR spectrum. However, these results were obtained using frankly toxic doses of chemotherapy (50% lethal doses), so it is unclear what relationship these results bear to tumors treated with levels of chemotherapy attainable in the clinic. Sham-treated animals and animals treated with low-dose chemotherapy (cyclophosphamide: 30 mg/kg; vincristine: 0.5 mg/kg) did not show an enhanced rate of tumor decline compared to unmanipulated controls. These preliminary results were obtained using a delay between separate rf pulses of only 1 s (22), so it is probable that PME and P, were underestimated because of partial recovery of the signal between pulses (17). The more commonly used interpulse delay of 3 or more seconds (13, 15, 19, 20, 25, 34, 35) allows for more complete recovery of the NMR signal and a more accurate estimation of resonance intensity.

The Adriamycin-resistant murine mammary adenocarcinoma 17/A/ADR does not appear to show tumor decline in response to Adriamycin treatment, but neither does it show tumor activation (25). There were no significant differences between a treated population 1 day following treatment with Adriamycin (12 mg/kg) and a control population of age-matched untreated control tumors; NMR parameters were unaffected, and no tumor growth delay was produced. This is in contrast to the Adriamycin-sensitive line 17/A, which showed tumor activation and an alkaline shift in pH 1 day following Adriamycin (12 mg/kg) treatment and a growth delay which lasted 19 days (25). A similar unresponsiveness to treatment is shown by the estrogen-insensitive rat mammary tumors Rama 600 and 622 (32). In contrast to an estrogen-sensitive rat mammary tumor, which regressed following ovariectomy and showed clear tumor activation, the estrogen-insensitive tumor lines did not regress following ovariectomy. NMR spectral parameters of the estrogen-insensitive lines were apparently unaffected by ovariectomy; the β-NTP:P, ratio continued to fall after ovariectomy in the estrogen-insensitive tumors, whereas it had risen in the estrogen-sensitive tumors. This decline in tumor NTP occurred at the same rate as in a sham-operated group of animals with estrogen-sensitive tumors (32).

Tumor decline, or a relative decrease in the pool size of high-energy bioenergetic intermediates following tumor treatment, is clearly demonstrated by the Glasgow osteogenic sarcoma treated with FAA (100 to 200 mg/kg) (26). FAA produced a dose-dependent decrease in both NTP levels and tumor pH within 4 h of treatment. These results are striking because of their rapidity and magnitude with respect to a control, which underwent no significant changes over the 72-h course of the experiment. For the highest dose of FAA (200 mg/kg), tumor pH dropped 0.29 units relative to the control within 4 h, and NTP levels were undetectably low. Slight recovery was observed in NTP values by 72 h posttreatment, but levels were still low relative to the control. The partial recovery of the NTP:P, ratio at 48 and 72 h after treatment was postulated to reflect a repopulation of the tumor with viable cells (26).

Corollary measurements of tumor metabolic levels and blood flow rates demonstrate that NMR spectral changes are reflecting real changes in the physiology of the Glasgow osteogenic sarcoma (26). The ATP concentration determined by high-pressure liquid chromatography was 23-fold lower than control levels in the FAA (200 mg/kg)-treated tumors 4 h after treatment, and the rate of blood flow in treated tumors was more than 6-fold lower than the controls. Because both the magnitude and the duration of the pH and ATP reductions were correlated with therapeutic efficacy in a dose-dependent manner, the effects observed appear to be related to FAA activity. These effects may be mediated by an FAA-induced reduction of tumor blood flow, although FAA has been postulated to inhibit mitochondrial respiration (26) or to act indirectly as a biological response modifier similar to tumor necrosis factor (36).

Human Tumor Response to Chemotherapy Observed by NMR

Human tumor response to therapy has been monitored by 3P NMR spectroscopy in a number of patients in vivo. In interpreting these often preliminary studies, one must draw on insights gained from properly controlled animal studies; however, a note of caution is appropriate. Human studies often have limitations not encountered in animal studies: a variety of human tumors have been studied, but many tumor types have been analyzed only once; tissue heterogeneity within human tumors is often greater than within the smaller, less invasive, animal tumors; no correlations have yet been made between human tumor spectra and tumor histology; a great variety of treatments have been used, often in combination, so that it is impossible to ascribe effects to a particular agent; protocols of treatment and examination are often inadequately described; spectra may be processed and analyzed by a range of different techniques; and studies seldom follow individual tumors over time as their response to a treatment evolves.

Nonetheless, Table 2 presents findings selected from those papers in which a single tumor was followed during the response of the tumor to some form of therapy: papers which report data
for a series of untreated tumors without examining the same tumors after treatment are excluded from consideration. Similarly, papers which report spectra from treated tumors without reporting data from those tumors before treatment are also excluded. However, when multiple spectra are reported from a single tumor with some treatment given in the intervening time, each spectrum is treated as a separate datum [e.g., neuroblastoma 2 (37) is recorded 3 times in Table 2 because NMR spectra were reported from four different experiments on the same tumor].

Tumors responded to treatment by either progressing or regressing in 56% of all cases, while stable disease is reported (or no information is given) in the remaining 44% of cases (Table 2). If the latter category ("treatment efficacy = 0") is excluded from further analysis, then treatment was judged to be effective (efficacy was "+", as determined by some measure independent of the 31P NMR spectrum) in 80% of cases (8 of 10). In 75% of regressing tumors (6 of 8 cases), the tumor spectra underwent changes characteristic of tumor activation (levels of NTP or phosphocreatine increased, or Pi decreased, or no information is given) in the remaining 44% of cases (Table 2). If the latter category ("treatment efficacy = 0") is excluded, then treatment was judged to be effective in 80% of cases (8 of 10). In 75% of regressing tumors, the tumor spectra underwent changes characteristic of tumor activation (levels of NTP or phosphocreatine increased, or Pi decreased, or both). The remaining 25% of regressing tumors showed spectra characteristic of tumor decline following tumor treatment.

Of those tumors which progressed following therapy (2 of 10), both showed spectra characteristic of tumor decline. It is striking that no cases were seen in which an inefficacious treatment produced tumor activation. This is consistent with the observation that untreated experimental tumors characterizedly show spectra with decreasing contributions from high-energy bioenergetic intermediates during tumor progression (18–20). The acute effects of chemotherapy have been observed by collecting spectra from a neuroectodermal tumor in the thigh of a patient during infusion of Adriamycin and ifosfamide (38). Because good signal-to-noise was obtained in a control spectrum and five spectra were collected within 160 min of each other (without moving the surface coil), systematic errors were negligible. Linear regressions of resonance area ratios were made including the five spectra obtained during treatment, a pretreatment control spectrum, and a spectrum collected immediately after infusion of vincristine but before infusion of Adriamycin. Statistically significant increases in the phosphocreatine:Pi and phosphocreatine:β-NTP ratio were observed over the course of the roughly 3-h experiment. In addition, the tumor pH declined significantly from about 7.25 to about 7.20 (38). These observations corroborate observations of tumor activation following treatment of experimental tumors and argue strongly that the phenomenon of tumor activation is not an artifact. Tumor activation in the human neuroectodermal tumor occurred with such a rapid time course (38) that substantial changes in tumor volume are probably precluded.

The results in Table 2 show that human tumor spectra can undergo heterogeneous changes in steady-state levels of bioenergetic intermediates following therapy. Interpretation of patient tumor spectra is further complicated by limitations in current spectroscopy methods. For example, a spectrum derived from an intracranial lymphoma prior to treatment showed the low phosphocreatine:ATP ratio characteristic of an advanced tumor (9). The patient underwent radiation therapy (24.2 Gy) and showed both clinical improvement and a regression of the tumor mass in an MR image. Two wk later a second 31P spectrum collected from the area of the tumor showed a spectrum indistinguishable from normal brain tissue. The first spectrum was collected from a VOI of approximately 51 cm3, while the second spectrum was collected from a VOI about 61% larger. As the authors note, it is quite possible that normal brain tissue was included within the VOI centered over the tumor (9). Therefore, it is unclear whether the proportional increase in phosphocreatine within the VOI incorporating the tumor resulted from metabolic activation of the tumor or from changes in the proportion of normal brain contributing to the VOI. This is not due to the inadequate technique of the authors; it is simply not yet possible to center a small enough VOI over an intracranial tumor so that contribution from normal tissue can be excluded.

Monitoring the NMR signal of human tumors following chemotherapy has shown that other phenomena may be observed in addition to changes in tumor bioenergetics. Infants with neuroblastoma of the liver were observed to show elevated levels of phosphomonoesters (PME:β-NTP ratio), when compared to subjects with normal livers (37). The PME:β-NTP ratio declined during spontaneous regression of one neuro-
blastoma, while this ratio was elevated 7-fold in a progressing tumor relative to control liver. When the progressing tumor showed a clinical response to irradiation and chemotherapy, the PME:\(\beta\)-NTP ratio was observed to decline to a value typical of normal liver. The PME resonance was tentatively identified as PE and PC (37), which are also observed in neonatal brain, another rapidly growing neuroectodermally derived tissue (39). It is unknown to what extent elevated levels of PME are characteristic of neural tumors in particular, or tumors in general, or simply of all rapidly proliferating tissue (37). However, the PME signal appears to be proportionally large in several experimental rodent tumors including mammary 17/A (25), 9L gliosarcoma (19), RIF-1 fibrosarcoma (20); in rabbit VX-2 carcinoma (40); in a range of human tissues including neonatal brain (39, 41) and adult brain tumor (9); human tumors of bone, breast, muscle, and skin (38, 42–44); in normal and diseased pancreas (45); and in diseased liver (46). The frequently stated hypothesis that PE and PC are involved in phospholipid synthesis, and that elevated levels of PME are characteristic of rapidly proliferating tissue (47), has been rigorously tested in vitro (48) but not in vivo (see “Tumor Biology and NMR Spectroscopy”).

The in vivo NMR signal of human tumors can show striking differences from normal tissue in average pH. However, the magnitude and direction of these changes are variable. In 7 of 9 excised (perfused) hypernephromas and in 1 of 2 ex vivo Wilms tumors, a resonance at +4.2 ppm was identified as P, of pH 6.1 to 6.5. Following chemotherapy, this peak underwent an alkaline shift in 1 of 5 tumors (49). In 7 of 8 brain tumors and in 7 of 7 other tumors, tumor pH was more alkaline before treatment than normal brain: pH of normal brain was 7.03, while tumor pH varied from 7.01 to 7.33 (50). The pH values for 8 human brain tumors were not significantly different from the brain pH of a population of normal subjects (51), while the pH of 3 of 4 bone tumors was more alkaline than the surrounding muscle (42). The pH of an osteosarcoma underwent an alkaline shift during favorable response to chemotherapy, while the pH of a neuroectodermal tumor underwent an acidic shift during favorable response to chemotherapy and during acute infusion of a chemotherapeutic agent (38). These conflicting results are probably due to several different factors: larger tumors may achieve more acidic pH values than smaller, presumably better vascularized tumors (20); some tumors may maintain a higher glycolytic rate than others under hypoxic conditions, thereby producing more lactic acid (47); poorly vascularized tumors may be unable to clear lactate produced in glycolysis (18); and tumor pH may be a characteristic property of tumor lines rather than of individual lesions (52). These inherent sources of variability are compounded by the notorious difficulty of measuring pH in vivo with any accuracy (1). The latter problem is likely to be particularly acute when dealing with different-sized tumors of irregular geometry, so that pH values measured by NMR are probably suspect unless serial measurements are made on a single tumor without moving the rf coil (see Ref. 38). While it has been hypothesized that tumor alkalinity is associated with activation of the Na\(^+\)/H\(^+\) exchange and mitogenic stimulation of cell growth rate (50), further work is required to conclusively demonstrate that tumor pH changes are significant to the biology of the tumor.

Analysis of breast carcinomas and benign breast tumors by in vivo \(^{31}\)P NMR spectroscopy has shown that chemical shift differences between the \(\alpha\)-NTP and \(\beta\)-NTP resonances (\(\delta_{\alpha}\)) and between the \(\gamma\)-NTP and \(\beta\)-NTP resonances (\(\delta_{\gamma}\)) were larger by about 0.2 ppm in carcinomas (\(P < 0.003\)) (43). Differences in \(\delta_{\alpha}\) and \(\delta_{\gamma}\) are hypothesized to reflect primarily the fraction of NTP uncomplexed with magnesium. Calculations from the data show that the concentration of free magnesium ions in benign tumors was 3 times higher than in carcinomas. It is unknown whether this is due to higher levels of NTP present in the carcinoma cells or whether there is a difference in the total amount of free magnesium in the two tumor types (43).

Tumor Biology and NMR Spectroscopy

As with any costly new analytical technique introduced into the armamentarium of the biomedical scientist, it is appropriate to ask what this new technique has taught us that would not soon have become evident from some other, better established technique. In this section I will attempt to analyze the unique contributions of \(^{31}\)P NMR to the understanding of tumor biology and to relate these findings to the mainstream of tumor research.

Tumor Bioenergetics

Classic biochemical assays of enzyme activity utilize purified (or partially purified) preparations of enzyme to measure initial enzymatic rates at very high substrate levels, and therefore reflect enzyme kinetics at saturation (53). However, in the living cell, enzymes may be substrate limited or, alternatively, may form multienzyme complexes where substrate availability to a particular enzyme is enhanced (54). Furthermore, various metabolic pathways can compete for a single substrate in vivo, creating a complexity absent from the in vitro experiments of the biochemist. NMR spectroscopy has enormous potential as a means to attack the question of how the rates, reactions, and regulatory processes described by biochemists in vitro actually affect the physiology of tissue in vivo.

Tumor bioenergetics may be influenced by the various anaerobic and catabolic pathways used by tumor cells. Work with MCF-7 human breast cancer cells, perfused in vitro in an NMR spectrometer, has shown that about 60% of NTP production is dependent upon glycolysis, and that perfusion of cells in the presence of azide (an inhibitor of oxidative phosphorylation) has little effect on the \(^{31}\)P spectra of cells (55, 56). Similar results were obtained in perfused T47D human breast cancer cells; most NTP synthesis was via the glycolytic pathway despite the fact that cells were perfused with excess oxygen (53). These data confirm Warburg’s (57) hypothesis of enhanced aerobic glycolysis in tumors. It has been hypothesized that the high concentration of NDP found in T47D cells plays a role in maintaining high rates of aerobic glycolysis, since ADP is known to be a stimulator of the regulatory enzymes on the glycolytic pathway (53). Increased tumor glycolysis may also be due to tumor hypoxia and is consistent with NMR observations of depressed high-energy phosphates and acidification of tumor tissue.

Tumor bioenergetics and metabolism are likely to be strongly affected by tumor oxygenation. Several experimental tumors (RIF-1, KHT, and MLS) in vivo show a significant decrease in bioenergetic status (phosphocreatine plus \(\beta\)-NTP/P) with increasing tumor size or decreasing blood hemoglobin saturation (58). NMR spectral parameters measured on RIF-1 tumors were correlated with tumor perfusion, which had been estimated by analysis of the decay of \(^{15}\)O positron activity following uniform tissue activation in vivo by high-energy X-rays (21). The size of the well-perfused fraction of the tumor was positively correlated with the phosphocreatine:NTP resonance area ratio (\(P < 0.05\)) and negatively correlated with the P:\(\beta\)-NTP ratio (\(P < 0.05\)) (21). Doses of hydralazine (2 mg/kg), which produce
a drop in mouse arterial blood pressure, also produce a decrease in tumor pH and in the resonance intensity of phosphocreatine and NTP in the murine fibrosarcoma FSAI (59). The phosphocreatine:P\textsubscript{i} ratio of the FSAI tumor decreased with increasing tumor size (31) or when the host animals respired 10% O\textsubscript{2}, but increased when the animals were permitted to respire 100% O\textsubscript{2} (60). These observations clearly indicate a direct relationship between \textsuperscript{31}PNMR spectral parameters and tumor oxygenation.

The relationship between tumor size and the proportion of poorly oxygenated cells in a tumor (58) suggests that the progressive decline in NMR-visible high-energy phosphates during untreated tumor growth is due to an increasing proportion of hypoxic tumor cells (15). Intracapillary hemoglobin saturations below about 30%, corresponding to a blood Po\textsubscript{2} of 30 to 40 mm of Hg (depending on pH), result in areas of mouse tumor showing radiobiological hypoxia (a tissue Po\textsubscript{2} below about 3 mm of Hg) (61). This is consistent with biochemical evidence that ATP levels are lower in large human breast cancer xenografts than in small tumors (62). Tumor perfusion can become progressively poorer as tumors grow: small RIF-1 tumors (<0.5 cm\textsuperscript{3}) have an average blood flow rate (ml/100 g/min) more than twice as high as large RIF-1 tumors (>1.0 cm\textsuperscript{3}) (63). Hypoxic cells have been demonstrated in most animal tumors, and the hypoxic fraction can comprise, on average, 20% of tumor cells (64). The relationship between NMR spectral parameters and tumor hypoxia may also be influenced by intrinsic properties of the tumors, including the rate of cell respiration and the ability of the cells to survive under hypoxic stress (58).

Tumor hypoxia can have important implications for tumor biology and the success of tumor treatment. Hypoxic cells are known to be relatively more resistant to radiotherapy and certain types of chemotherapy, because these treatments depend upon the production of cytotoxic activated oxygen species (65). Increasing tumor hypoxia with increasing tumor volume may account for the frequent observation of a Gompertzian growth pattern in advanced tumors (66). The proportion of a tumor which is hypoxic may be related to the ability of that tumor to induce sufficient capillary angiogenesis (67). Tumor cells which are chronically hypoxic are less likely to encounter chemotherapeutic agents because hypoxic cells may be remote from the vasculature (65). Transient hypoxia can enhance the frequency of methotrexate resistance by inducing gene amplification in Chinese hamster ovary cells; this process has been implicated in the induction of multidrug resistance in advanced tumors (68, 69). The ability of NMR to estimate the hypoxic fraction of tumors in vivo and to follow the kinetics of changes in tumor oxygenation may have important consequences for patient treatment in the future.

If the decline in high-energy phosphates in progressing tumors is due to increasing tumor hypoxia, this suggests that tumor activation following chemotherapeutic treatment with BCNU could result from changes in subpopulations of cells within the tumor (19). In order to address this issue of tumor heterogeneity, histological comparisons were made of 9L tumor micromorphology in BCNU-treated tumors and sham-treated controls. Treated tumor sections had a significantly lower proportion of necrotic cells, a higher proportion of viable-appearing cells, and a 5-fold higher level of interstitial space than control tumor sections. These changes in micro-morphology could be sufficient to account for the spectral differences between treated and control tumors (19), but one is left to wonder what physiological mechanisms produced these morphologic differences?

Possible mechanisms which might produce the paradoxical phenomenon of metabolic activation following treatment remain to be investigated. These potential mechanisms include (19): (a) killing of some fraction of the treated tumor, producing an inflammatory response and recruiting macrophages into the tumor, so that the fraction of normal cells contributing to the tumor spectrum is increased; (b) cell killing within the tumor, reducing competition for oxygen or nutrients; (c) preferential killing of low-energy cells or recruitment of quiescent cells into a metabolically more active form, thereby enhancing the fraction of energized cells contributing to the spectrum; (d) cell killing decreasing intratumoral hydrostatic pressure and so improving blood flow to the tumor; and (e) chemotherapy directly affecting tumor vasculature, resulting in increased blood flow to the tumor or increased capillary permeability within the tumor.

Steady-state alterations in tumor cell metabolite levels following treatment are seen most clearly with perfused cells in vitro. Human HT29/5 colonic adenocarcinoma cells maintained in vitro in the NMR spectrometer without perfusion for 90 min display a spectrum with no high-energy phosphates (73), as is characteristic of tumor decline. However, when these cells are made aerobic, the spectrum changes over the course of a few minutes to one characteristic of tumor activation (73). HT 29 cells subjected to 1 h of anaerobic perfusion are depleted of phosphocreatine and relatively enriched with P\textsubscript{i}, but were able to maintain 60% of their NTP stores (74). When HT 29 cells were glucose starved, the NTP content decreased to 10% of the pretreatment control level within 1 to 2 h, yielding spectra typical of tumor decline. Refeeding with glucose in the perfusion medium led to a decrease in P\textsubscript{i}, an acidic shift of intracellular pH\textsubscript{in}, and an increase in NTP within 4 h (74). Therefore, a spectrum with depressed high-energy phosphates may be characteristic of metabolically inactive tumor cells rather than dead cells (73, 74). Steady-state metabolite levels also change when cultured RIF-1 cells in an in vitro perfusion system in the NMR spectrometer are treated with 4-hydroperoxy cyclophosphamide (the activated moiety of cyclophosphamide) at a con-
centration of 100 μM (75). Within 24 h of treatment, RIF-1 cells show a reduction in the phosphocreatine resonance accompanied by a substantial reduction in the P\(_i\) resonance (75), so that the spectrum of the treated cells shows a response which cannot be clearly classified as either tumor cell activation or decline.

In general, the effect of therapy on steady-state levels of tumor metabolites in vivo can be either tumor activation or tumor decline (Tables 1 and 2). Tumors treated in vivo can show short-term changes in steady-state levels of bioenergetic metabolites which may be mediated by positive or negative effectors; positive effectors result in increases in tumor steady-state metabolite levels, while negative effectors produce the opposite result (Table 3). Positive and negative effectors can be extrinsic to the tumor (referring to influences on tumor cells by the host) or intrinsic (referring to factors inherent to the physiology of the tumor cells). It is proposed that various tumor treatments produce characteristic alterations in steady-state metabolite levels depending upon their particular mechanism of action.

For example, hyperthermia and photodynamic therapy, which characteristically produce tumor decline (Table 1), may do so by inducing tumor ischemia (35). Tumor ischemia is therefore classified as a negative effector, mediated presumably by host endothelial cells, and therefore extrinsic to tumor cells (Table 3). Flavone acetic acid induces tumor decline in the Glasgow osteogenic sarcoma by a mechanism which may involve inhibition of mitochondrial respiration (26). This would fall into the category of loss of stoichiometry between glycolysis and the tricarboxylic acid cycle (Table 3), a negative effector intrinsic to the tumor cell. Alternatively, FAA has been proposed to act indirectly as a biological response modifier similar to tumor necrosis factor (36). Tumor necrosis factor produces tumor decline when administered to mouse tumors in vivo (76, 77), by an unknown mechanism.

Tumor activation is produced in estrogen-sensitive rat mammary tumors by host ovariectomy (32). Tumor activation may be the result of removal of a source of endocrine growth factors which are acting as positive extrinsic effectors (Table 3). It was hypothesized that when estrogens are removed and tumor cell growth ceases, demand for NTP falls and the cellular oxygen supply is again sufficient for the reduced metabolic needs of the tumor (32). However, removing a stimulus to growth may affect the per capita pool size of high-energy phosphates (78) as well as affecting turnover rate of these pools. The effect, if any, of cell cycle events on adenylate pool size and turnover rate is poorly understood. Growth factors and their effects on tumor cell cycle distribution are also poorly understood in the biology of tumors, so the effect of growth factors on tumor spectra has been categorized as potentially both positive and negative (Table 3).

### Tumor pH

In vivo studies of the Walker 256 carcinosarcoma (12) show a small (0.07 pH unit) but significant difference between tumor and muscle pH. However, when the tumor was rendered ischemic, a rather marked decrease of 0.78 pH units was produced (12). In the RIF-1 fibrosarcoma, a positive correlation was found between the well-perfused fraction of the tumor, as measured by \(^{13}\)O washout, and the tumor pH as measured by the chemical shift of the P\(_i\) peak (21). Tumor pH was also negatively correlated with the P\(_i\):NTP ratio. It was concluded that, as the well-perfused fraction of the tumor became smaller, relatively more cells became hypoxic or necrotic, resulting in decreased signal from phosphocreatine and NTP and an acidic shift of tumor pH (21).

Other work has shown that average RIF-1 tumor pH\(_{\text{NMR}}\) is positively correlated with the phosphocreatine:P\(_i\) ratio and undergoes an alkaline shift following treatment with cyclophosphamide (20). Tumor pH\(_{\text{NMR}}\) is not significantly higher in treated tumors than in controls until the third day after treatment, even though the phosphocreatine:P\(_i\) ratio is significantly elevated on the first day after treatment (20). The alkaline shift of pH\(_{\text{NMR}}\) is coincident with increased blood flow following treatment (79) and an increase in cell proliferation rate (80). In a number of different normal and malignant cells, an alkaline shift in intracellular pH is correlated with the initiation of cell proliferation (81). However, it is perhaps more likely that improved blood flow to the tumor, resulting in decreased lactate production and increased lactate clearance, is responsible for the alkaline shift following treatment (20).

Tumor pH may have an indirect but important effect on tumor oxygenation. Tumor pH has been shown to decrease during volumetric growth of the RIF-1, KHT, and MLS cell lines (58, 61). Hemoglobin-oxygen affinity is labile and is reduced by decreases in pH or increases in Po2 (the well-known Bohr effect; Ref. 82). Oxygen concentration measured in the BA1112 murine rhabdomyosarcoma varied between 0.5 and 34 mm of Hg, with regions of hemorrhage having a Po2 of about 16 mm of Hg (52). If we assume the Po2 of blood entering a hypoxic region of tumor to be about 20 mm of Hg, then hemoglobin-oxygen affinity will be sharply reduced by acidic pH. At a blood Po2 of 20 mm of Hg, hemoglobin saturation will be approximately 30% at pH 7.4 (the pH of normal arterial blood; Ref. 82), but only 20% at pH 7.2 (83). If the pH excursion of tumor blood is farther than 0.2 pH units from normal, this effect will be larger. If the oxygen partial pressure required for...

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<td>Catabolic processes involving nutrients other than glucose</td>
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<td>Accumulation of toxic products leading to cell impairment</td>
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Table 3: Potential effectors of per capita cellular energy metabolites of tumors in vivo

Positive effectors potentially result in increases in tumor cell levels of steady-state metabolites, while negative effectors produce the opposite effect. “Extrinsic” refers to influences on the tumor cells by the tumor host, while “intrinsic” refers to factors inherent in the metabolism of the tumor cells.
half-saturation of hemoglobin is 28 mm of Hg at pH 7.4, then half-saturation will occur at 49 mm of Hg at pH 6.9 (calculated from Ref. 82). This relationship will be further complicated by other factors such as tissue PO₂, temperature, and organic phosphate ligands such as diphosphoglycerate (83). Although it is impossible to predict the net result of interactions between all of these factors in a tumor, conditions within a tumor may result in oxygen dumping by hemoglobin before the perfusing blood reaches the hypoxic core of the tumor. This hypothesis must be critically examined to determine whether an alkaline shift of pH following tumor treatment may result in better hemoglobin oxygenation within the tumor.

**Phospholipid Metabolism**

Elevated levels of PME in phosphorus spectra have been hypothesized to be associated with phospholipid membrane synthesis and related to the rate of cell proliferation (37, 47). Human MDA-MB-231 breast cancer cells perfused in vitro with medium containing choline or ethanolamine show enhanced levels of PME and PDE observable by in vivo NMR (48). PMEs arise from the activity of choline and ethanolamine kinases, which are the first step in phospholipid biosynthesis, while PDEs are hypothesized to result from phospholipid catabolism. Cells in log phase growth have PME peaks with twice the intensity of those from cells at stationary phase, so increased levels of PME are associated with increased cell membrane synthesis (48).

However, the hypothesis that relative PME pool size is correlated with cell growth rate in vivo is somewhat difficult to resolve with experimental data from animal tumor models. The rat 9L gliosarcoma shows a significant increase in the PME:α-NTP ratio between 7 and 18 days postimplantation, during untreated tumor progression (19). During this same period the tumor doubling time for s.c. 9L increases from 48.2 to 109.4 h (84). Between Days 7 and 18 postimplantation, the adenylate ratio (β-NTP:γ-NTP) shows a significant decrease, suggesting that the metabolic state (19) and the growth rate of the tumor (84) are both declining. In the untreated tumor, increased levels of PME are therefore apparently associated with reduced cell growth rate rather than with increased cell proliferation. This would imply that membrane synthesis, or at least the accumulation of membrane precursors, is occurring at a time when production of new cells is decreasing.

Four days following treatment with BCNU, the PME:α-NTP ratio is significantly lower in treated tumors (19). The depletion of this ratio in treated cells occurs at a time when clonogenic cell survival following 10 mg/kg of BCNU is less than 1% (39). The growth fraction of treated 9L tumors decreases for the first 2 to 10 days post BCNU treatment (85). Therefore, decreased levels of PME in the treated tumors are associated with reduced cell growth rate. The mechanism by which both increased and decreased levels of PME can be associated with cells having decreased cell growth rates is unknown.

Spectra from RIF-1 fibrosarcoma, taken in the 3 days subsequent to a dosage of 150 mg/kg of cyclophosphamide, show a proportional decline in the PME peak relative to an untreated control (20). Cell proliferation in RIF-1 tumors, assessed by [³H]thymidine labeling index and primer-dependent DNA polymerase labeling assay, was inhibited for up to 3 days by cyclophosphamide (79, 80). Proliferative recovery of surviving cells began at Days 4 or 5 after chemotherapy (80), at a time when the PME peak began to undergo a proportional increase in the treated tumors (Fig. 1; Ref. 20). In the RIF-1 tumor, an enhancement of the PME resonance therefore appears to be associated with, and may predict, an increase in cell growth rate.

The Morris 7777 hepatoma shows greatly elevated levels of PME when examined in vivo after isolation from the rat host and perfusion with Krebs-Henseleit buffer (86). Associated with this elevation of PME is an unusual phosphorus metabolite identified as IcP, which is proposed to be derived from hydrolysis of phosphatidylinositol by phospholipase C (86). Products of phosphatidylinositide hydrolysis can act as second messengers in the response of tissues to hormones and growth factors. It has been proposed that observation of IcP (at a concentration estimated to be 70 μM) is suggestive of a metabolic defect in the phosphatidylinositide pathway, which may be associated with the loss of control of cellular proliferation rate in this fast-growing tumor (86). It is not known whether this metabolite is found in other rapidly proliferating tumors.

**Conclusion**

Data summarized in Tables 1 and 2 strongly suggest that the phenomenon of tumor activation following chemotherapy is not an artifact of inadequate spectroscopic methods, nor is it always a symptom of ineffective treatment. Metabolic activation of a treated tumor can occur in spite of substantial clonogenic cell kill and overwhelming host toxicity and is not simply a result of tumor volume changes. It is hypothesized that ³¹P NMR spectroscopy is sensitive to tumor hypoxia and that tumor activation results from some combination of factors which leaves the treated tumor less hypoxic than the untreated tumor.

The existence of hypoxic tumor cells, which are resistant to radiotherapy, is the major cause of failure of radiotherapy in the clinic (87). In vivo MRS appears to be sensitive to differences in tumor oxygenation brought about by anesthesia (88) and which may result in differences in radiation sensitivity (89). It is hypothesized that MR spectroscopy may be able to provide a noninvasive method of monitoring changes in tumor oxygenation produced by therapy. Therefore, it may eventually be possible to use these data to design and monitor individualized fractionated radiotherapy schedules for patients, in order to achieve maximal tumor radiotoxicity (75). Data from drug-sensitive and -resistant tumors suggest that NMR spectroscopy may also provide a sensitive, noninvasive way to assess the response of tumors to chemotherapy. It should be possible to monitor tumor response to therapy following a low, relatively nontoxic “test dose” of chemotherapy. This would allow patients with nonresponsive tumors to avoid the systemic toxicities associated with a full course of therapy and would aid in the selection of patients for clinical trials of new antitumor agents (25). NMR spectroscopy may also assist the clinician in developing novel combinations of chemotherapy and radiotherapy, designed so that changes in tumor oxygenation or perfusion that are induced by one modality can be exploited in another modality.

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Response of Solid Tumors to Chemotherapy Monitored by *in Vivo* $^{31}\text{P}$ Nuclear Magnetic Resonance Spectroscopy: A Review

R. Grant Steen


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