Combined $^1$H and $^{31}$P NMR Studies of Cerebral Metabolism in Vivo

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NMR spectroscopic methods have recently been developed for measurement of several concentrated cerebral metabolites in vivo. At present, $^{31}$P spectra from the brain permit detection of ATP, PCr, Pi, and certain sugar and lipid phosphates. The resonant frequency of Pi also provides a measure of cerebral pH, and under some conditions ADP concentration can be calculated from information available in the $^{31}$P spectrum. The $^1$H spectrum of brain provides measurements of lactate, creatine, and several amino acids and choline-containing compounds. Both kinds of spectra can be obtained from the same subject. Our group at Yale used combined $^{31}$P and $^1$H methods to demonstrate that loss and recovery of phosphate energy stores and concomitant changes in cerebral amino acids during hypoglycemic coma in rodents could be observed in vivo. We then used the same methods to show that cerebral pH is normal while lactate is elevated in status epilepticus.

NMR spectroscopy performed in vivo provides an array of chemically specific measurements unavailable by any other non-invasive method. It is thought to be entirely free of deleterious biological effects; hence, its potential for use in humans is considerable.

Recent technical developments in nuclear magnetic resonance (NMR) spectroscopy make possible non-invasive, chemically specific measurements of some concentrated metabolites in the living, undisturbed brain. The method works by interaction of magnetic and radio frequency fields with magnetic nuclei in biological molecules. Nuclei such as $^{31}$P, $^1$H, and $^{13}$C precess about the axis of a strong static magnetic field at a frequency characteristic of the magnetic field strength, the particular nuclear species, and local perturbations of the static magnetic field by other constituents of particular molecules. The frequency of precession is different for the three $^{31}$Ps of adenosine triphosphate (ATP), the $^{31}$Ps in phosphocreatine (PCr) and inorganic phosphate (Pi), and the $^1$H nuclei bonded to the methyl carbon of lactate. These different resonant frequencies can be interrogated selectively with radio waves in such a manner that signals proportional to the concentrations of some metabolites are obtained. Various constraints dictate that only measurements of small, rapidly tumbling molecules present in millimolar concentrations are possible in vivo. An impressive array of compounds can already be measured, however, and the list will grow. At present, it includes ATP, PCr, Pi, and certain phosphates of sugars and lipids in the $^{31}$P spectrum, and lactate, glutamate, glutamine, aspartate, taurine, creatine, and some choline-containing compounds in the $^1$H spectrum. Other important quantities can be determined from information present in the spectra. Notable among these are intracellular (cytosolic) pH (pHi), which is given by the exact resonant frequency of Pi, and the concentration of adenosine diphosphate (ADP), which, though far below...
the directly detectable range, can be calculated from $^{31}$P spectra if the creatine kinase reaction is assumed to be at equilibrium. Any of these variables can be measured every few minutes by spectroscopic procedures which are thought to be entirely without hazard for biological tissue.

The thoughtful reader may wonder: Why was all this so long in coming? There are three answers: magnets, computers, and water. Magnets: These have to be big and of very high quality. Magnets big enough for laboratory animals and also capable of producing magnetic fields sufficiently stable and homogeneous for spectroscopy are less than ten years old. Magnets suitable for people are only now appearing. Computers: NMR spectroscopy has been computationally intensive since it came to depend on Fourier transform methods in the mid-1970s; in its academic incarnations, it is a principal beneficiary of the small computer revolution. Water: With two $^1$H nuclei per molecule and an abundance in biological tissues far higher than any other compound, water produces in $^1$H spectra a signal which is several orders of magnitude more intense than the strongest signals from other metabolites. The water signal is the basis of NMR imaging, but ways of suppressing it had to be found before $^1$H spectroscopy of other compounds could be useful in vivo. Spectroscopists working with samples in test tubes found such ways a few years ago. Our group at Yale adapted two of these methods for use with whole animals. That was an emphatically non-trivial achievement, the consequences of which will accumulate for years.

Developments in NMR spectroscopy of the living brain in the last five years are recounted in a comprehensive review in the 1986 Annual Reviews of Neuroscience [1]. Much of the work has been done by a group of spectroscopists and neurologists in the Yale laboratory of Robert Shulman. Two of our experiments illustrate the power of in vivo NMR methods especially well, because both $^1$H and $^{31}$P spectroscopic observations were made in the same animal.

Figure 1 contains $^1$H and $^{31}$P spectra obtained from the brain of a living rat before, during, and after insulin shock. The work was done principally by Kevin Behar, in collaboration with Jan den Hollander, Ognen Petroff, Hoby Hetherington, Robert Shulman, and James Prichard [2]. This was the first study to combine measurements from both nuclei in a single animal. The data illustrated have several important features.

First, the $^{31}$P spectra on the right document very clearly loss of the brain's phosphate energy stores during profound hypoglycemia and their partial restoration after administration of glucose. Since the electroencephalogram also did not recover fully, irreversible hypoglycemic brain damage may have occurred during the 35-minute period of electrocerebral silence. Another possibility is that full recovery would have taken longer than the period of observation in this experiment because of the need to resynthesize metabolic precursors of the high-energy phosphates lost from the brain during the insult [3]. The point emphasized here is that in vivo spectroscopy makes available the metabolic history of such an event in a single animal. Quantitative correlation of metabolic and electrical data obtained in vivo with morphologic and tissue extract data obtained later from the same brain can lead to more thorough and far more efficient analysis of experimental metabolic encephalopathies than has been possible previously.

Second, much of the phosphorus lost from PCR and ATP remained in the cells as Pi (spectrum G). Pi is hard to measure accurately by destructive methods; relatively few quantitative data concerning its behavior during metabolic stress are available from
FIG. 1. Metabolic effects of hypoglycemic shock on the brain of a rat under N₂O analgesia. The experiment was performed in a Bruker WH360 spectrometer. NMR signals were obtained from a surface coil double-tuned to ¹H and ³¹P and placed over the exposed calvarium. The course of the experiment is shown on the time axis between the two columns of spectra. Times of insulin (INS) and glucose (Glc) administration and spectrum acquisition are indicated. An additional dose of insulin (90 U/kg) was given at 75 minutes. The dashed box indicates the period during which the EEG was isoelectric; marked slowing dominated the EEG for several minutes before the isoelectric period and for the duration of the experiment after it. The "δ" on the abscissa is the symbol commonly used for "chemical shift." ¹H spectra (A–D) are the average of 64 transients acquired in 2.36 minutes. The large unlabeled resonance at 2 ppm is from N-acetyl aspartate; the labeled resonances are from the total creatine pool (Cr₉₀), aspartate (ASP), glutamine (GLN), and glutamate (GLU). The position of a lactate resonance is indicated (LAC), but at the concentrations lactate was present in this experiment, the signal from it is not clearly resolved from the lipid resonances in the same region. ³¹P spectra E and H are the average of 1,000 transients acquired in ten minutes; spectra F and G are from 400 transients acquired in four minutes. Resonances from Pi and PCr are labeled, as are resonances from the α, β, and γ phosphates of nucleoside triphosphates (NTP) and diphosphates (NDP). The "+" sign indicates that the NTP₉₀ and NDP₉₀ signals overlap, as do those from NTP₁₀ and NDP₁₀; in fact, nearly all of the signal in these three resonances is from ATP. Reprinted with permission from J Neurochem.
conventional experiments. With improvements in spectroscopic technique, Pi movements out of the volume of tissue being observed (or into a physical state which renders it invisible to NMR) can be measured. Any role that availability of Pi may have in resynthesis of PCr and ATP after depletion can be detected by such experiments.

Third, measurement of pH\textsubscript{i} is an inherent capability of \( ^{31}\text{P} \) spectroscopy. During hypoglycemia, an alkaline shift of pH\textsubscript{i} estimated by conventional techniques has been reported \cite{4}, and we observed one in rabbit brain in an earlier NMR experiment \cite{5}. There was only a very slight alkaline shift in the experiment illustrated by Fig. 1, and the data were quite stable. We do not yet know the reason for the difference. There is much still to be learned from determination of cerebral pH\textsubscript{i} by \( ^{31}\text{P} \) spectroscopy. It is the most direct pH\textsubscript{i}-sensing method available, and the only non-invasive one which can provide time-course data from a single subject. It is by no means artifact-free, but the artifacts it is susceptible to are in general different from those which beset other methods. There is great potential for confirmation or correction of observations made by the other methods and for collection of cerebral pH\textsubscript{i} data in situations which cannot be studied any other way.

Fourth, the frequency of the \( \beta \)-ATP resonance is related to \([\text{Mg}^{2+}]\) in a way which, in principle, allows the latter to be estimated \textit{in vivo} from \( ^{31}\text{P} \) spectra. Since the gap between principle and practice may be large when complicated technology is the link between them, we wished to validate the procedure by finding some experimental situation in which a change of \([\text{Mg}^{2+}]\) was likely to occur \textit{in vivo} and could be detected by a shift in the frequency of \( \beta \)-ATP resonance. The experiment of Fig. 1 had a good chance of meeting these requirements. A large fraction of the ATP in brain is complexed with Mg ions, which affect the frequency of the \( \beta \)-ATP resonance. Together with knowledge of the equilibrium constant of the \([\text{Mg}^{2+}]\)-ATP interaction, this measurement allows one to calculate \([\text{Mg}^{2+}]\). With a large decline in [ATP], one would expect a corresponding rise in \([\text{Mg}^{2+}]\). Ognen Petroff found that changes in the resonant frequency of \( \beta \)-ATP indeed indicated such a rise during the portion of the experiment when the \( \beta \)-ATP resonance was reduced but still detectable \cite{Petroff OAC, et al: in preparation}.

Finally, let us consider the \( ^{1}\text{H} \) spectra on the left side of Fig. 1. The first feature which deserves mention is the signal-to-noise ratio, which is much higher than in the \( ^{31}\text{P} \) spectra. In spectrum E, for instance, the peak-to-peak variation of the noise (the variations on each end of the spectrum, outside the range 5 to \(-20 \) parts per million) is much greater than that of the only discernible noise in spectrum A, which is between 0 and 0.5 parts per million. In addition, many more transients were averaged to make the \( ^{31}\text{P} \) spectra than to make the \( ^{1}\text{H} \) ones, so that the comparative signal-to-noise advantage of the latter is actually considerably better than it looks in Fig. 1. The reason for this comes from basic physics: \( ^{1}\text{H} \) gives a stronger signal per nucleus than any other magnetic nuclear species, including \( ^{31}\text{P} \). Adding to that the fact that \( ^{1}\text{H} \) is the most abundant nucleus in biological tissues, one quickly appreciates why \( ^{1}\text{H} \) spectroscopy has a rich present and an expansive future in biology and medicine. This experiment employed the first of the methods of water suppression developed by our group; the water resonance was selectively irradiated to remove it from the spectrum so that resonances of other compounds could be detected. The \( ^{1}\text{H} \) spectra in Fig. 1 show features of hypoglycemic coma which are well understood but could not previously be observed \textit{in vivo}. There are no changes in the larger resonances, but the smaller ones from glutamate and aspartate change in a most interesting way. The two resonances
from glutamate fell during the period of electocerebral silence and rose again after glucose was given. Two resonances from aspartate behaved in reciprocal fashion. These changes are precisely what is expected in hypoglycemic encephalopathy, as carbon skeletons from glutamate are fed into the tricarboxylic acid cycle via the aspartate aminotransferase reaction.

As a whole, this experiment is a leading example of the kind of validation which is necessary with any new technique: Does it show what it should when one knows what that is? Like several other kinds of validating experiments we have done, the answer in this experiment was resoundingly affirmative. Development of techniques for doing NMR spectroscopy in vivo has certainly had to overcome many difficulties, but unaccountably anomalous results have not been among them. With its reliability thus demonstrated, the ability to monitor such changes non-invasively in a single subject provides a rich new source of information about pathological chemistry in the brain, at present in animals, later in humans.

A second way in which our group used combined $^1$H-$^{31}$P spectroscopy was for study of bicuculline-induced status epilepticus in living rabbits. This time our efforts went beyond validation of NMR methods to exposure of a previously unreported aspect of pathological cerebral metabolism. The work was done mainly by Ongen Petroff and Takashi Ogino, in collaboration with Malcolm Avison, Jeffry Alger, Robert Shulman, and James Prichard [6]. This study was the first to use the second and more sophisticated of the water-suppression methods adapted for in vivo use by our group. Called the "1$^{331}$" sequence after the relative lengths of some of its pulses, it works by a different principle than the earlier method. Instead of exciting the water resonance and then suppressing it, the $^{1331}$ method selectively excites only the region of interest in the spectrum, in this case the region around the lactate resonance. The salient biological result of the study was demonstration of a pronounced and long-lasting dissociation between pH$i$ and lactate in the brain. Figure 2 illustrates the essential features of the phenomenon in two animals. Our measure of the "duty-cycle" of seizure discharge in the electroencephalogram showed that intense electrical disturbance lasted about 20 minutes. In both animals, pH$i$ initially moved about 0.3 pH units in the acidic direction, then gradually returned to normal. Lactate rose during the period of intense seizure discharge, but, in marked contrast to pH$i$, it remained elevated for the remainder of the experiment. For well over half an hour, the animals were in a state of non-acidotic cerebral lactatosis. Other animals which were given larger doses of bicuculline and had more prolonged seizures displayed no such dissociation; their brains remained acidotic until the end of the experiment. In animals which received smaller doses of bicuculline and had shorter seizures than the ones of Fig. 2, lactate rose but pH$i$ did not change, or at least any change which occurred did not reach our detection threshold of 0.05–0.1 pH unit measured in five-minute $^{31}$P spectra.

What is the pathological significance of the fact that the rabbit brain cleared excess $^1$H ions much more vigorously than excess lactate in these experiments? There are two general possibilities, each of which has several forms. Number 1: lactate may have been trapped in a metabolically inactive compartment—dead cells, for instance. Number 2: it may have remained elevated due to pathological regulation of intact metabolic processes. The distinction is important. In the first case, elevated lactate might be a marker for damaged tissue. In the second, analysis of the mechanism of persistent lactate elevation would lead to new understanding of metabolic control in the brain.
FIG. 2. Time course of EEG changes, cerebral pH, and cerebral lactate changes (lactate/NAA ratio) during bicuculline-induced status epilepticus and subsequent recovery in two rabbits under N₂O analgesia. One animal (solid circles) received 2.2 mg/kg of bicuculline, the other (open circles) 1.8 mg/kg. The experiments were performed in an Oxford Research Systems TMR 32/200 spectrometer, using a surface coil which could be mechanically switched between ¹H and ³¹P resonant frequencies. EEG seizure intensity is expressed as the percentage of a ten-second epoch occupied by spike-and-wave discharge (% seizure). pHi was obtained from the chemical shift of the Pi resonance in ³¹P spectra. The rise in cerebral lactate after bicuculline administration is expressed as the increase in intensity of the lactate resonance since the control period, relative to the intensity of the N-acetyl aspartate resonance, which did not change. The animals were killed by KCl injection at the times indicated. Reprinted with permission from Ann Neurol.

The matter can be investigated further by Yale-developed NMR methods which are still more advanced than the ones discussed so far. The most pertinent of these is ¹H observe-¹³C decouple spectroscopy, originally demonstrated by Laurel Sillerud, Jeffry Alger, and Robert Shulman in yeast [7], and adapted for use on the living brain by Douglas Rothman, Kevin Behar, and Hoby Hetherington, with the help of Robin Bendall, Jan den Hollander, Ognen Petroff, and Robert Shulman [8]. The ¹H observe-¹³C decouple experiment in status epilepticus would be this: Infuse ¹³C-glucose into a rabbit for a while; then stop the infusion and induce status epilepticus with bicuculline. Cerebral lactate will rise, as the experiments described have shown; since ¹-¹³C will be abundantly available, the ¹³C specific activity of the excess lactate will be high. Then wait. If the ¹³C specific activity of the elevated lactate pool falls as rapidly as that of blood, the entire lactate pool, though elevated, must be in its usual state of rapid exchange with pyruvate; there is no large amount of pathologically sequestered lactate. If the ¹³C specific activity of the lactate pool does not change as total lactate falls, much of the excess lactate must be trapped.
Quantitative evaluation of intermediate results can determine the size of trapped and exchanging lactate pools if both are present.

NMR spectroscopy of the living brain is still in a very early stage of development. It is unique among non-invasive methods in its capability to harvest a wide range of chemically specific information from the functioning brain as often as every few minutes. The examples discussed here reveal only part of this capability. Research in cerebral metabolism and the practice of clinical neurology are both likely to benefit from this technology as it matures.

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