

## ADJUNCTIVE MEDICAL KNOWLEDGE

### Multinuclear NMR Studies of Naturally Occurring Nuclei

C. Tyler Burt and Jason A. Koutcher

*Massachusetts General Hospital, and Dana Farber Cancer Institute, Boston, Massachusetts*

**The ability to obtain nuclear magnetic resonance spectra from spatially localized regions of living animals and patients has led to the possibility of measuring biochemical processes in vivo. Localization is generally achieved through the use of surface coils. Using this technique, intracellular pH, and concentrations of high-energy phosphates and "abnormal" marker compounds have been measured in animal organs (both in vitro and in vivo) and in human brain and muscle (in vivo). The majority of studies have used the P-31 nucleus, but carbon (C-13) and hydrogen (H-1) have also been studied. However, both C-13 and H-1 experiments have technical difficulties. Carbon-13 has a low natural abundance, and H-1-containing metabolites may have their signals obscured by the large water peak. The phosphorus studies have been largely preclinical, but diagnostic possibilities are appearing from the many research problems now under investigation.**

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In the first article, we described the basic phenomena of nuclear magnetic resonance (NMR) (1). The impetus for such an article was an appreciation and understanding of the background of the various biomedical applications of NMR that have begun to appear (2-20).

In this second article, the emphasis will be on methods and results obtained from high-resolution in vivo NMR. In particular, we will discuss the techniques of obtaining and interpreting spectra, and will develop an understanding of the biochemical and physiological phenomena. The third article will feature those methods that give mainly anatomical detail. This is a natural division, since biomedical research groups using NMR seem to divide themselves into either those that study high-resolution spectra (yielding chemical information) from relatively large but spatially localized volumes (2-10) or those that generate x-ray-like representations of anatomic features based primarily thus far on a single nucleus—the H-1 in H<sub>2</sub>O or the —CH<sub>2</sub>— groups of fat (11-20). Few chemical data other than relaxation times are obtainable in the latter type of study, although excellent anatomic detail can be obtained.

The reasons for this dichotomy in research will become apparent from the two articles. One is that spectral information is discussed in terms quite different from anatomic imaging and somewhat foreign to common imaging thought processes. Imaging is discussed and is limited primarily to anatomic terms, whereas high-resolution spectral data are more akin to a chemical map (i.e., the presence or absence of ATP or inorganic phosphate peaks). A second reason is the inherent insensitivity of operations with nuclei other than the proton, which has required almost all biomedical imaging to be limited to H-1 NMR. In contrast, biological and physiological applications of NMR have concentrated on C-13 and P-31 nuclei, and have been very productive in charting metabolic processes in normal and pathological states. The problem of sensitivity may be overcome to some extent with higher-field superconducting magnets, in which case the two avenues of research can begin to move closer together. Indeed, some attempts to bridge the gap have lately begun to appear (21-24).

#### SPATIAL SELECTION AND SURFACE COILS

The clinical applications of NMR, whether to obtain images or spectra, depend on the introduction of methods of spatial selection, which are fundamental to NMR imaging.

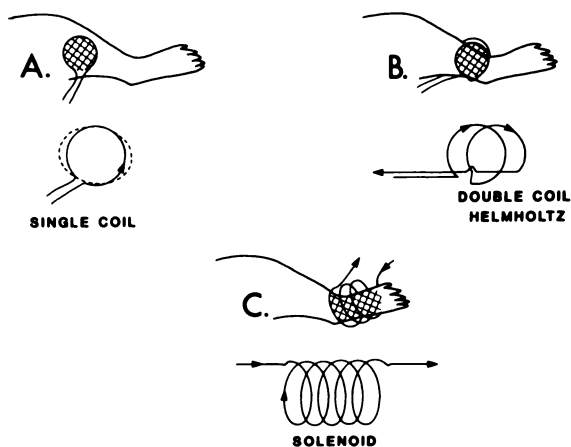
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For reprints contact: C. Tyler Burt, PhD, Research Bldg, Rm. 501, Mass. General Hospital, Boston, MA 02114.

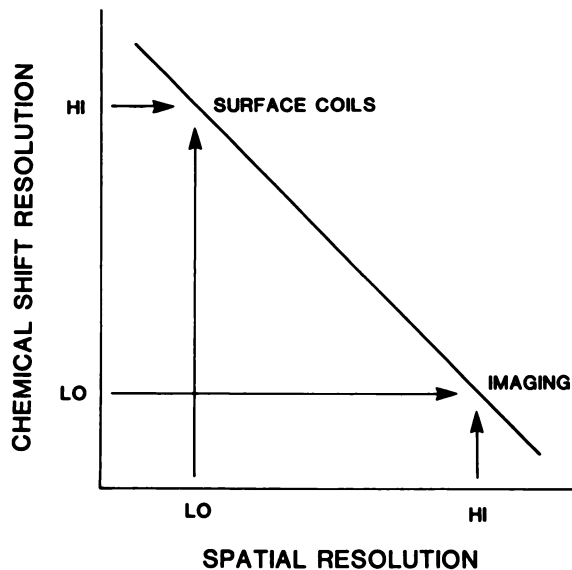
Current flowing in a loop of wire will generate a magnetic field. By winding electrical coils in various configurations, one can add to or subtract from the main field ( $H_0$ ) in localized areas, thereby shaping the geometry of the net magnetic field. The use of small auxiliary magnets (shim coils) is necessary to obtain the field homogeneity (one part in  $10^9$ ) required for high-resolution NMR (25). These coils are also used extensively in the generation of magnetic gradients for anatomic imaging (26-27) and will be discussed in the next paper.

As shown in the block diagram of an NMR system in the first paper, the magnet itself, together with the  $H_1$  coil, automatically serves as a primitive spatial selector, in that only the sample in the  $H_0$  field and the  $H_1$  coil will be able to generate NMR data. This limited the initial spectral studies to a sample that would fit into an NMR tube, which in turn had to fit into the magnet. From the mid 1970s to the present, there have been spectral studies on isolated cells (28-34), excised tissues (35-38), and isolated perfused organs from small animals (39-44).

Up to a few years ago, this effective sample size for high-field instruments was less than 25 mm, and therefore the possibility of gathering spectra was limited to the foregoing sample types. But now superconducting magnets of relatively high uniformity have been constructed into which whole animals can be placed. There are two general methods of obtaining *in vivo* high-resolution NMR spectra from a spatially localized volume: either shape the magnetic field using shim coils, or arrange the transmitter-receiver coils so that only a specific area will be sampled (2,3,45). The first method is called topical magnetic resonance (3) while the second is the surface-coil approach (2). Figure 1 shows some of the types of coil that have been used in the collection of lo-



**FIG. 1.** Three kinds of surface coils used in *in vivo* NMR. (A) Single coil, which samples roughly hemisphere on either side of it, is placed externally on sample to be studied, so that only half of sample volume is used. (B) Double or Helmholtz coil design. Sample area lies in gap between coils. (C) Solenoid design has very uniform field within coil. It can be used where sample is to be fitted into it.



**FIG. 2.** Schematic of current strategies of use of NMR in biomedical application. It symbolizes that in general one must trade off chemical-shift information for spatial resolution.

calized spectra. Note that the coils can be combined with field shaping to achieve, in principle, even higher spatial selection, although this option is not commonly used.

As Fig. 1 shows, each coil can be designed so that a particular body volume can be studied (46). The most common design is the single- or multiple-turn circular coil that roughly samples a hemisphere or "ice-cream scoop" of tissue. Large sample sizes are required because compounds containing nuclei like P-31 or C-13, and even H-1, are generally present in millimolar or lower concentrations.

The preceding introduction is summarized in Fig. 2. Conventional high-resolution studies lie at the upper left-hand corner and generate chemical-shift spectra, i.e., chemical information. Surface-coil investigations move along the spatial resolution axis, but some of the chemical-shift resolution is lost. Figure 3 shows a comparison of an extract of a muscle, a high-resolution spectrum of an intact muscle, and a surface-coil study of a muscle. The loss of spectral quality as one proceeds from a chemical situation (i.e., tissue extract) to an individual organ—and eventually to a spatially localized region of the body (*in vivo*)—is quite evident.

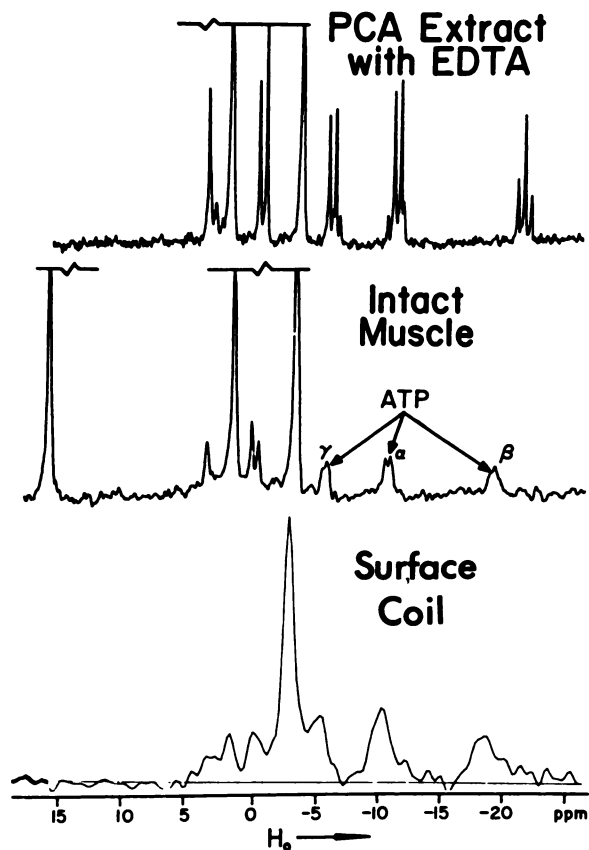
At the other extreme are pure NMR imaging studies that contain no chemical-shift information but excellent anatomical features (Fig. 2, lower right). It is hard to predict whether anyone can design an instrument capable of performing any mode of analysis along the locus shown in Fig. 2, so for the foreseeable future (5-10 yr) NMR instruments will either perform the kind of analysis outlined in this chapter (chemical shift) or will do H-1 spatial imaging.

#### SENSITIVITY

Sensitivity in a regional spectrum depends on several

factors. In general the signal-to-noise ratio (S/N) of a particular peak depends on (a) the concentration of the chemical species present, (b) its gyromagnetic ratio, (c) the sample volume, and (d) the magnetic field strength of the instrument being used. The first consideration is that the signal obtained is proportional to the concentration of a species present. It is the chemical species that contains all of the physiological significance and for most molecules varies at the millimolar level. Notable biological exceptions are water, fats, and glycogen particles. To detect millimolar concentrations is a severe requirement for an insensitive technique like NMR. The nucleus' gyromagnetic ratio affects sensitivity by way of Eq. 4 of Article 1 (1). The differences in sensitivity will vary in a nonlinear manner due to the exponential nature of Eq. 4.

The third factor is the sample size, and this is governed by the diameter of the coil for single-turn coils or the distance between the gap in solenoid and Helmholtz coils.



**FIG. 3.** Example showing application of Fig. 2. Top figure is from extract of frog muscle to which chelator EDTA has been added. This substantially sharpens lines. For classification of peaks see Fig. 5. Middle figure is from intact frog muscle that has been placed in NMR tube and run in high-resolution machine. Although the peaks are somewhat broadened, muscle filling sample space allows good resolution. Bottom figure is from rabbit muscle sampled in vivo with Helmholtz coil. Broadening of peaks is obvious. They have been artificially broadened by about 10 Hz to help signal-to-noise ratio, but lines are still considerably broader than compared to the first two.

Coil design plays a role through the factors involved in removing noise self-generated in them. An exposition on small-gap coil design by Hoult shows that increases of S/N roughly following the square of the magnetic field strength might not be unexpected (47). These factors are shown in Fig. 4.

The final factor is the magnetic field strength. As mentioned in the first article, higher field strength can dramatically increase S/N. In general, the S/N varies as a higher-than-unity power of the field strength, as was pointed out in the first paper.

The ultimate factor is time. Any NMR-active element can be measured if one averages the signal long enough. However, biological samples often change or degenerate with time, so it is best to optimize the other factors that affect S/N and minimize the time required to collect a spectrum. This tends to be the *sine qua non* in clinical situations.

#### EFFECT OF FIELD INHOMOGENEITY

There is one final problem that must be confronted with in vivo surface-coil studies and is highlighted in Fig. 3. The ability to recognize two peaks only a few hertz apart—the so-called spectral resolution factor—can be a problem in surface-coil or topical NMR studies. Part of that problem arises because the magnetic field strengths used in whole-body studies are still relatively low ( $\omega \leq 60$  MHz for proton NMR). We noted in the previous paper that the separation between peaks (measured in hertz) caused by chemical shifts differences will increase as the magnetic field increases.

The primary problem, though, is that there exist boundary areas in the various spatial localization techniques that show magnetic-field inhomogeneities with field-shaping techniques, or variability of the tip angle ( $\alpha$ ) with surface coils (see Article 1). This also represents a condition of inhomogeneity. If one returns to Fig. 11 of the first article, it would be as if some of the tops in their boxes were spinning (giving signals) under uniform conditions but other little boxes were signaling at slightly different frequencies. This means that what might be a single sharp line centered at one frequency would become a broad one made up of contributions from a population of peaks clustered around the main frequency. This results in the widening of the peaks seen in the in vivo spectral experiment.

Moreover, there is an additional theoretical limit to the sharpness of NMR peaks that can be obtained, and this is related to the inherent relaxation time in the transverse direction ( $T_2$ ). The theoretical half-width of a peak ( $\nu_{1/2}$ ) is:

$$\nu_{1/2} = \frac{1}{\pi T_2} \quad (1)$$

For most species of interest, this fundamental limit is

less than a few hertz, and occurs even in the most homogeneous of fields. However, the kind of inhomogeneity introduced by surface coils or field profiling seems to add additional broadening on the order of 5–10 Hz or more, which is sufficient to destroy much of the fine structure in an NMR spectrum.

NMR OF PARTICULAR NUCLEI

Table 1 in Article 1 showed a sampling of nuclei available for observation. Of these, only three naturally occurring ones appear to be capable of yielding information about specific biologic compounds. These are hydrogen (H-1), carbon (C-13) and phosphorus (P-31). Other nuclei like sodium (Na-23) can be imaged in a manner similar to protons (49), and eventually P-31 may yield x-ray-like images of the distribution of a single metabolite such as phosphocreatine (PCr).

Some new compounds, such as those containing fluorine (F-19) are proceeding towards exploitation (50–52), and nuclei of species enriched in C-13 have been added to biologic samples, and their metabolic fate followed with NMR (53). A fuller description of these will be presented in the last paper.

Among the nuclei under consideration, there are three categories of biomedical information to be gained: measurement of the concentration of labile species; the measurement of phenomena based on the chemical exchange rate, such as measuring pH from the phosphate chemical shift; and the measurement of compounds that serve as markers of development or disease.

Figures 5, 6, and 7 show some typical resonance positions for compounds using signals from P-31, C-13, or H-1. Of these, phosphorus has been the primary natural-abundance nucleus investigated.

The most immediate benefit from phosphorus NMR is the study of bioenergetics. This suggests that the analysis of the three phosphorus atoms in ATP, the one in phosphocreatine, and inorganic phosphate would be the focus of most surface-coil studies and of perfused organs and cells as well.

ATP has been shown to be the primary energy source of animals. Its fall during ischemic bouts has been shown to be related to tissue damage and eventual death. In muscle and brain, ATP levels are buffered by the phosphocreatine kinase reaction (Fig. 8). Certainly from a research viewpoint, a significant question would be whether there exists an ATP concentration beyond which tissue damage is irreversible.

What NMR offers is the promise of a method to measure noninvasively the ATP levels in a clinical setting. The noninvasive possibilities, coupled with the lack of ionizing radiation, mean that the procedure can be repeated as often as required.

**Examples.** The bioenergetics of several different organ systems have now been studied by NMR. For the study of cells one can consult any of the previously cited papers on the subject. We will now summarize some of the NMR research that has been done on each organ. This summary is not meant to be all-inclusive, since the field has expanded so rapidly that it is impossible to summarize all the P-31 biological data in a single paper.

**Skeletal muscle.** Isolated muscle has been studied intensively, both because excellent isolated preparations exist and also because skeletal muscle has been the paradigm for bioenergetic studies throughout this century (54–56). The previous premier method of investigation has been the analysis of tissue extracts. NMR has allowed sequential analysis of the compounds *in vivo*.

The NMR results on intact tissue have tended to confirm previous chemical findings (6,36,38). However, NMR does show new perspective in that cytosolic free ADP is not present above 0.5 mM, and hence its appearance above this level during chemical analysis of extracts is an artifact. Moreover, cytosolic free inorganic phosphate is very low in resting muscle, generally 1–2 mM or less. This is not a minor contribution, in that it shows exactly what conditions should be used to approximate *in vivo* conditions.

In the clinical domain, the use of surface coils has been adopted in human forearms or legs. Spectra from subjects or patients have been obtained using either ischemic or aerobic exercise against no load or a graded load in the magnet (7,8,57). Normal limbs under ischemic exercise show a decrease of phosphocreatine and pH, coupled with constant ATP levels. There is some rise in sugar phosphates as glycolysis becomes a larger source of ATP generation, with resulting acidification. By comparison, a patient with McArdle's syndrome shows a more rapid than normal drop of PCr and an actual alkalization of the intracellular milieu (7). This is because the critical enzyme phosphorylase needed to break down glycogen is missing and, hence, glycolysis does not proceed regularly. Consequently, ATP is maintained only by the phosphocreatine kinase reaction and, as Fig. 8 shows, this results in the net absorption of a proton.

**TABLE 1. CLASSIFICATION OF EXCHANGE PHENOMENA**

Case No.	Ratio of chemical exchange rate to peak separation	Nomenclature	Resulting spectra
1	High	Fast	One averaged peak
2	Approximately equal	Intermediate	Complicated
3	Low	Slow	Two separate peaks

$$S/N \propto C \cdot V \cdot (H_0, \gamma)^n \cdot \sqrt{N}$$

**FIG. 4.** Factors influencing signal-to-noise in NMR experiments. C is concentration of species of interest; V is volume being sampled and depends on surface-coil size; N is number of passes signal averaged; and  $(H_0, \gamma)^n$  is complex function that depends on nuclei chosen, since each will have its unique gyromagnetic ratio ( $\gamma$ ) and population distribution, which depends on main magnetic field strength ( $H_0$ ). Whole process is presented as function of arbitrary variable, n, because coil geometry will influence how these factors are expressed when they are measured.

By comparison, two patients with mitochondrial myopathy also show a rapid downfall of PCr (8). In this case, however, acidification was observed during the exercise, showing that glycolysis was proceeding. The subjects also showed much slower regeneration of PCr during recovery, which could be found by taking nine consecutive spectra over half an hour, capitalizing on the noninvasive nature of NMR. One could not imagine the alternative of demanding nine consecutive biopsies over a half hour.

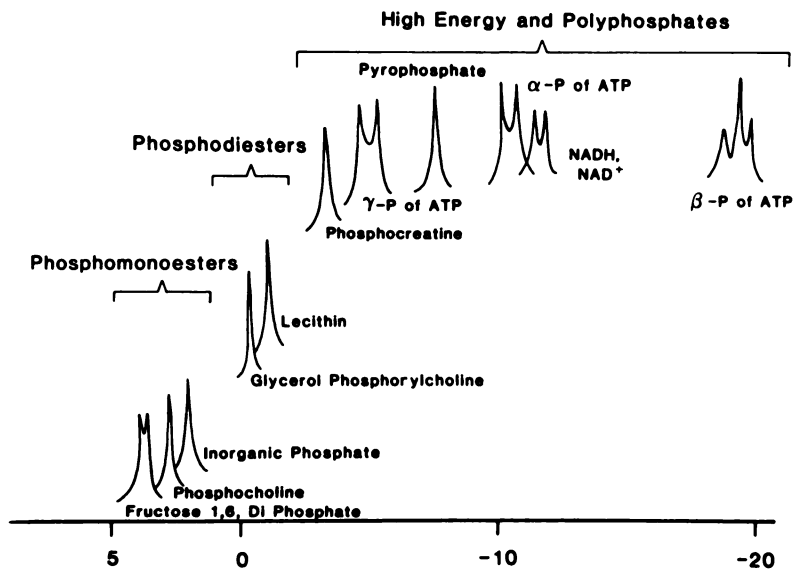
**Cardiac muscle.** No human studies have yet appeared, but a large number of bioenergetic studies have been performed on isolated perfused hearts exposed to various insults. Hearts of rats, rabbits, and guinea pigs (40-42,58) have all been used. In general, they show a lower ratio of PCr to ATP, compared with muscle (2-3:1 in heart against 4-6:1 for muscle). Two important areas of research (metabolite changes in working hearts and saturation transfer experiments) are discussed later. It is not clear how soon in vivo P-31 NMR analysis of human hearts will be possible. NMR has been used to grade the effectiveness of various cardioplegia solutions in maintaining high-energy phosphate reserves in isolated perfused hearts. Again, it is the noninvasive, repetitive-sampling feature of NMR that makes it so attractive.

**Kidneys.** Professor Radda's group has extensively studied the isolated perfused rat kidney (44). They are able to correlate high-energy phosphate level with the ion-pumping load imposed by perfusate. Of even more interest is the NMR examination of organs destined for transplantation. Using P-31 NMR, one can measure ATP levels and assess the extent of ischemia present before transplantation occurs (59). This has been done both in animal models and in human kidneys before use. With the growth of organ transplantation, NMR may provide a totally unexpected method of checking the status of a donor organ, complementing other tissue methods of characterization.

**Brain.** Brains have been investigated largely by surface coils (10,60,61). This is necessitated by the lack of a generally accepted isolated brain model. On animals the surface coils show interesting changes in the phosphate profile during development. This is discussed in the final section on marker compounds. Changes in ATP and PCr occur during various catastrophies such as stroke or epileptic seizure. Interestingly, the point initially raised about critical levels of ATP for cell function can be followed in the brain by comparing EEG recordings with ATP level. Pritchard et al. show that a flat-line EEG secondary to insulin-induced hypoglycemia corresponds to a reversible decrease in ATP and PCr (10).

**Tumors.** The study of energy metabolism in tumors has not been an extremely active area of research. NMR may change this because of the ease with which all the phosphorylated intermediates can be measured. Not enough tumor lines or different experimental conditions have been tried to warrant many general conclusions.

Of those that can be drawn, one is the effect discovered in the 1930s by Otto Warburg (62), that increased glucose levels can lead to increased tumor glycolysis, and the consequent decrease in pH is of value for P-31 NMR studies. This can be seen with P-31 NMR, even when



**FIG. 5.** Hypothetical spectrum, showing where various phosphorus-containing compounds resonate in P-31 NMR experiment (from Ref. 63).

Range Of Chemical Shifts For Some Classes Of <sup>13</sup>C Compounds

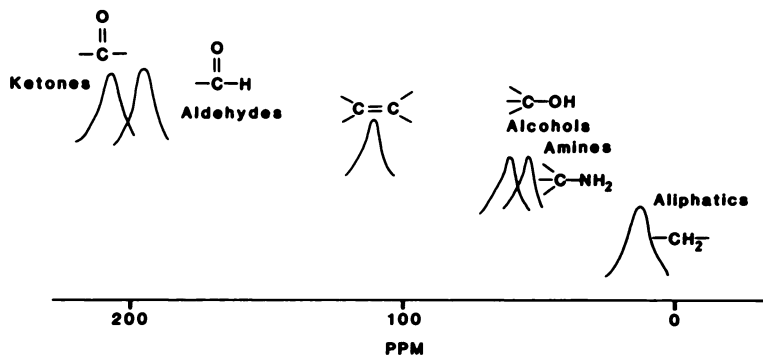


FIG. 6. Hypothetical spectrum showing where various carbon compounds resonate in C-13 NMR experiment. Note large range, in terms of parts per million, over which compounds are spread.

oxygen levels are high as in in vivo tumors (63). The diagnostic value of this is that most tissues do not show such an effect. An experimental tumor in a rat demonstrated a drop in pH when the rat was challenged with glucose, whereas pH in nearby muscle remained steady (64).

In addition, changes in the phosphodiester region (see Fig. 5) in P-31 NMR are seen in tumors challenged by hypoxia or hyperthermia (9,65,66). Since this region also contains the carboxyl phosphates like phosphoenolpyruvate, there may be energy pathways used by tumors that are not used by normal tissue. Such a circumstance is not unlikely, since tumors in vivo are often in an hypoxic environment where unusual glycolytic pathways may be necessary to ensure cell survival. This kind of speculation shows how NMR's ability to detect phosphorus compounds may eventually point toward new pathways in the design of oncolytic drugs.

QUANTIFICATION AND SPIN-LATTICE RELAXATION TIMES

Typically the area under a peak is taken as a measure of the amount of a compound present. However, as was mentioned in our first paper, a delay of about 5 spin-lattice relaxation times (T<sub>1</sub>) between pulses is required

to ensure that the free induction decay from each pulse is the same as the preceding (i.e., that the system returns to equilibrium between pulses). If one pulses at a more rapid rate and doesn't change the pulse angle (i.e., the amount of energy absorbed by the sample), one will eventually measure a steady-state magnetization that is less than equilibrium, thus being led to believe that there is less compound present than is actually there.

One refers to this situation as one in which the signal is partially saturated. If one pulsed very rapidly or exposed the sample to a continuous-wave signal at the resonance frequency of the peak of interest, one could completely eliminate a signal, i.e., completely saturate it. After achieving this situation, one has effectively equalized the population of the two spin states.

Depending on the T<sub>1</sub> of the sample and the pulse repetition rate, under certain conditions one can improve S/N faster by repeating the pulse too rapidly for equilibrium to occur. The decrease in signal due to the magnetization not returning to equilibrium is offset by increased signal because of the increased number of signal averages. Such experiments are called partially relaxed FT NMR or PRFT. The texts on NMR in the first article offer a fuller description of this phenomenon (67).

The most common way to avoid this difficulty is to

Range Of Chemical Shifts For Some Classes Of <sup>1</sup>H Compounds

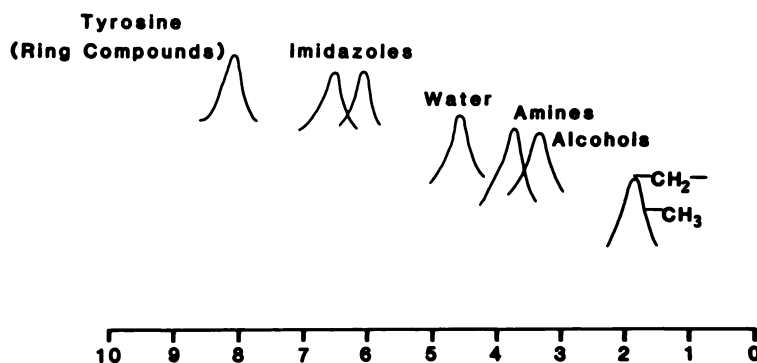
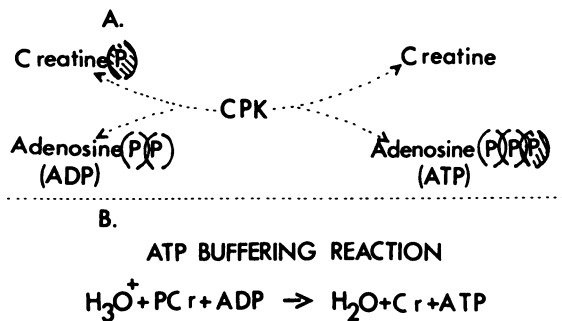


FIG. 7. Hypothetical spectrum, showing where various proton-containing compounds resonate in <sup>1</sup>H experiment.



**FIG. 8.** (A) Schematic of phosphocreatine kinase reaction showing its reversible nature. It represents catalysis of transfer of phosphate on phosphocreatine (creatine-P) to ADP by enzyme creatine phosphokinase (CPK) to form creatine and ATP. Double arrows show that reaction can be run in either direction. Shaded phosphate is one transferred. One can compare Figs. 5 and 3 to appreciate relative difference between those phosphate resonance positions. (B) Chemical equation showing proton absorption during ATP-buffering nature of phosphocreatine kinase. Reaction pictured is for Mg-chelated ATP, where net proton is absorbed by reaction.

decrease the pulse angle (i.e., expose the sample to less energy), so that it takes less time for the magnetization to return to its equilibrium value. Even with this adjustment,  $T_1$  may be long enough to cause saturation. It is therefore necessary to have some measure of  $T_1$  in tissue to be able to quantify metabolite concentrations. This can be done on live tissue with surface coils (68,69), or in cell suspensions similar to the tissue being measured. One other possibility is to construct a model solution of chemical composition similar to the tissue's. Solution  $T_1$ 's are equal to or greater than in vivo ones. From the  $T_1$  values of the above, one constructs calibration tables that adjust the area of the peak under consideration to what it would be if complete relaxation had been permitted. Only then can true metabolite quantification be ensured. Even when the peak area ratios are used, differences in relaxation times between various chemical species can influence the results if only PRFT spectroscopy is used.

The above concerns are given added impetus since relaxation times of ATP are reported to change between stimulated and resting samples of isolated frog muscle (70). Additionally, in a work that has yet to be duplicated, Fossel et al. report that ATP, PCr, and inorganic phosphate ( $P_i$ ) vary during the cardiac cycle in an isolated working heart (71). If these results carry over to surface-coil studies in heart, they will significantly influence the interpretation of ATP and PCr levels found during bouts of ischemia in heart. We mention this since relaxation times must again be considered in the interpretation of any data where quantification is necessary.

**Relaxation times—effect of shortened  $T_2$ .** An additional vexing problem with NMR measurements is that compounds bound in a matrix or to a large macromolecule often show a shortened  $T_2$  (48). Thus protons in the

form of water bound to protein have a very short  $T_2$ . In such a situation the signal peak from the bound species broadens until it is no longer observable, and merges with the baseline. This is why the phospholipids in the membrane are generally not visualized by P-31 NMR. They are bound very tightly in the membrane bilayer and have a very low  $T_2$ . Paramagnetic ions also can lower the  $T_2$  of peaks to the point where they are broadened and cannot be observed.

#### PROBLEM SPECTRA—H-1

The high concentration of  $\text{H}_2\text{O}$  protons in biological entities ( $\approx 80 \text{ M}$ ) is a major advantage for NMR imaging. Unfortunately, it complicates high-resolution NMR spectroscopy by virtue of its overwhelming concentration, which results in a very strong peak intensity. The concentration of other chemical species present—such as the metabolites of the Krebs cycle or the glycolytic pathway, which are approximately  $10 \text{ mM}$ —are almost insignificant when compared to  $\text{H}_2\text{O}$ . Stated in another way, a factor of almost 10,000 exists between the signal of water and that of most of the other metabolites. This is generally referred to as a dynamic range problem in that the water signal is not a single sharp peak (see the discussion on  $T_2$ ) but rather has a broadened line shape that has “wings” extending several parts per million on each side of the main resonance. For a resonance the size of a water peak in tissue, the wings tend to obscure all resonances within their range.

Practical observation of a nearby peak requires some method of suppression of the water signal. Several methods of doing this exist. One would be to irradiate selectively at the resonance frequency of water. As discussed previously, this would saturate the resonance and allow other smaller peaks to be seen. The technique has been used, for instance, in the examination of isolated frog muscle to monitor metabolites at the millimolar level (72).

Such a technique has also been used in a surface-coil study of rat brain to portray millimolar metabolites in the brain (73). The Yale group was even able to see concentrations of neurotransmitters such as gamma aminobutyric acid (GABA). There exist other special techniques to suppress the water signal, involving multiple pulse sequences. One potential problem with such saturation or multipulse techniques for water elimination, however, is that they all require transmission of microwave power into the sample.

#### DETERMINATION AND THE CONCEPT OF EXCHANGE

Nuclei and atoms are constantly in motion. There is Brownian motion and there is directed motion due to ionic or steric interactions. This motion involves actual physical displacement of the atoms through space. Thus, the atoms or nuclei in one area will change places or

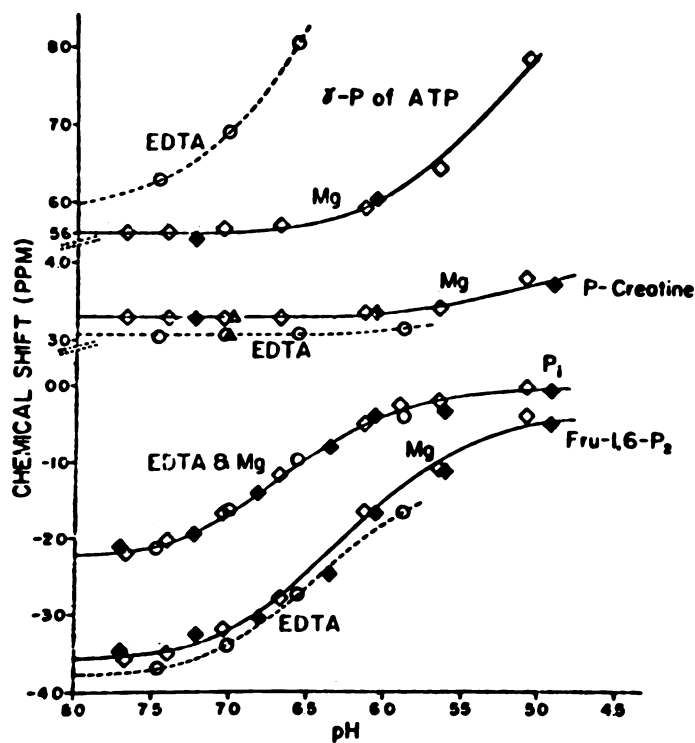


FIG. 9. Example of how chemical shifts can vary as function of pH and metal-ion concentration. Solution was made to model intracellular milieu of muscle. In particular: Mixture A ( $\diamond$ — $\diamond$ ) was made to approximate concentration of metabolites in frog muscle; 3 mM ATP, 20 mM PCr, 2 mM  $P_i$ , 2 mM 1,6-fructosediphosphate, 0.1 M KCl, and 12 mM  $Mg^{2+}$ . Mixture B ( $\blacklozenge$ — $\blacklozenge$ ) was mixture A with omission of KCl. Mixture C ( $\circ$  . . .  $\circ$ ) was mixture A with added 1 mM EDTA and omitted 12 mM  $Mg^{2+}$ . Mixture D ( $\Delta$ ) contained 30 mM PCr and 12 mM  $Mg^{2+}$ . Mixture E ( $\blacktriangle$ ) contained only 30 mM PCr. One notes that chemical shift of ATP is drastically affected by presence of Mg, due to change in effective pK for terminal phosphate. Note also that there are small effects due to ionic strength. Of all resonances, however, that of inorganic phosphate seems least affected. Its chemical-shift position varies significantly with pH through critical 6.5–7.5 region; hence its chemical shift is most often used as intracellular pH monitor (from Ref. 36).

exchange with others. Hence, the origin of exchange phenomena.

Critical in the NMR aspects of these exchanges is the time constant of exchange compared with the peak separation for the nuclei in each situation.

Several cases suggest themselves, and are highlighted in Table 1. Measurements of pH generally fall into Case 1. Here the proton that determines the pH shuttles back and forth between a protonated and a deprotonated form of the acceptor. This occurs so rapidly that the observed chemical shift is an average of the protonated and deprotonated chemical shift weighted by the fraction of the species in each form. This situation has been exploited to determine the pH in a sample because the average pH follows a Henderson-Hasselbalch type of equation (74), and one can write:

$$pH = pK - \log \frac{\delta_{obs} - \delta_B}{\delta_A - \delta_{obs}} \quad (2)$$

where  $\delta_{obs}$  is the observed chemical shift and  $\delta_B$  and  $\delta_A$  are the extreme values of the chemical shift.

This situation allows calibration curves to be constructed for pH compared with chemical shift (36). As Eq. 2 shows, the determination of pH demands not only the proper limits of exchange rate relative to peak separation, but that the pK being studied must lie in the region of intracellular pH. Figure 9 highlights such a calibration procedure and shows how some of the factors such as the presence of metal ions and the pK value can affect such curves (75–77).

Intracellular salt concentrations are relatively constant, and concentrations of divalent metals (Ca, Mg)

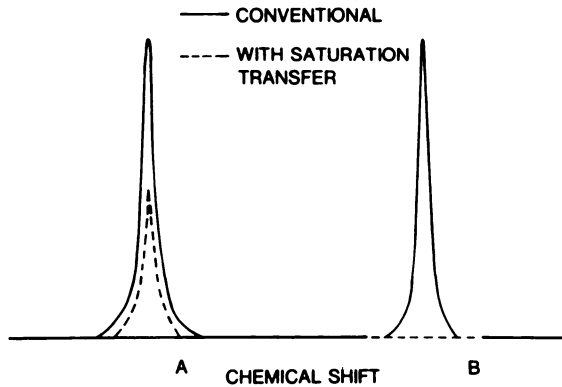
are low. However, there are tissues—like the adrenal gland or synaptic vesicles—where some compounds are in such high concentration that they can influence the chemical shift of phosphorus compounds present (78). In general, as with the calculation of  $T_1$ , a calibration solution as close to that observed intracellularly must be used to obtain a correct calibration chart.

Two general classes of naturally occurring biologic compounds have values of pK that lie in the physiologic range: the phosphates and the histidine types of residue. Of these, the phosphates have been used most extensively. Several articles exist on how they are used to measure pH movements during normal developmental changes and during pathological ones such as ischemia (79–81). No surprising results have been found, and in general, pH changes are just as would be predicted based on classical studies.

One interesting sidelight is the use of deoxyglucose to provide an additional measure of pH. Cells will take up this compound and phosphorylate it. Once phosphorylated, it is only slowly degraded and, hence, builds up in tissue. Curiously, the pH reported by the phosphorylated deoxyglucose is often more acid than that of the phosphate system. The reason for this is unclear (65).

The other naturally occurring compound that has a pK (6.5–7.5) in a region relevant to intracellular pH is the imidazole group of natural peptides, such as carnosine (present in muscle). The signals from the protons on the imidazole ring have been used effectively as a pH measure in the same way as inorganic phosphate, although only a few studies on this have been published (72). The imidazole resonance can be seen in some





**FIG. 10.** Example of how saturation transfer experiment would work. Two resonances would be that of PCr and  $\gamma$  peak of ATP. Since they exchange (Fig. 8), saturation of  $\gamma$  peak (dotted line) leads to decrease of intensity of phosphocreatine peak (dotted line of resonance on left). One should be aware that many variants of this experiment exist (from Ref. 93).

proton studies on cells, so the technique may have broader applications.

One must bear in mind that the concept of fast and slow exchange also comes into play with chelators such as ATP. The chemical shifts of the phosphates of ATP vary dramatically (at near-neutral pH) with the addition of magnesium. This has led some to estimate free magnesium levels by the chemical shifts of ATP in intact tissue (82). Since ATP binds to so many other cellular constituents that might effect its chemical shift, this method requires particularly rigorous models of cytosolic solutions, perhaps even including relevant enzymes. Once achieved, however, free magnesium levels remain a possible parameter that could be measured.

**Relaxation times and chemical exchange—saturation transfer studies.** One class of experiments of particular interest is that of the saturation transfer type (83). They have been done only on excised tissue and are complicated experiments for which one can refer to the reference above for further details. The pulse sequences essentially involve the generation of transient NMR species by radiation that saturates one peak or puts it at some specific flip angle.

If such a species is in chemical exchange between two different chemical sites—such as the phosphate group on the terminal end of ATP and that on phosphocreatine—then one measures chemical transfer in the non-saturated peak by a change in its signal intensity. The situation is illustrated in Fig. 10. The equations are developed in the previous reference, but the significant point is that one-way rate constants are available for appropriate chemical reaction.

These experiments have been used to try to understand the bioenergetics at the molecular level in isolated hearts and in brains (84,85). The inclusion of these results shows how much information NMR can extract from a piece of tissue, beyond simple concentration studies. It also pinpoints the theoretical complexity involved in

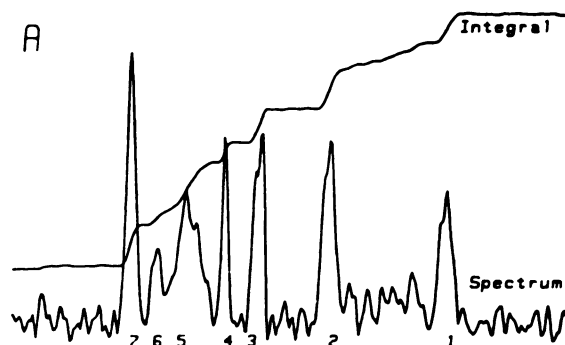
intricate NMR experiments, and how far the technique still has to go in evaluating cellular function and pathology.

**Specific species—phosphorus.** Beyond bioenergetics and the determination of pH, there is the classification of the specific profile of a tissue as either normal or pathological. This has been done in intact tissues (6,9,28,30,37,61,72,86), and is now under investigation with surface coils. This may eventually lead to the development of a catalog of characteristic compounds in tissue as a function of normal or pathological development.

The mere appearance of such a compound in a surface-coil study may be exciting. One such example is the peak in the phosphomonoester region of neonatal brain, which decreases with development (see Fig. 11) (60). Such a developmental tag would be of interest in following normal and abnormal growth, but the compound must be identified to provide a full explanation of its importance. Cady et al. (61) have tentatively identified the peak as ribose-5-phosphate, based on extract studies in guinea-pig brain by Glonek et al. (87).

The identification of a particular peak in a spectrum must be approached rigorously. This is done using tissue extracts obtained from the tissue in question, which can be studied by NMR. By adding various compounds to the extract under different conditions (pH, ionic strength, different metal ions, etc.) one tries to find the chemical species that behave identically to the unknown peak. The various peaks can often be identified in this manner.

The above discussion is highlighted by the fact that regions such as the phosphomonoester region can be especially rich in signals, and the identification of peaks such as ribose-5-phosphate (rib-5-P) must be done with care. For example, one can note from earlier extract work that, over the pH range 7 to 10, phosphorylethanolamine has chemical shifts (30) similar to those in the peak identified as rib-5-P. It is noted in the *Biology Data Handbook* that phosphorylethanolamine does vary in



**FIG. 11.** Surface-coil study of neonate brain. Most of peaks seen in Fig. 3 are seen in this spectrum as well. Large peak farthest left (Peak 7) represents potential marker of development (from Ref. 61).

the millimolar range during development (88). Only careful analysis of extracts over a large pH range can differentiate the two.

Another class of marker compounds contains the phosphodiesteres such as glycerolphosphorylcholine (GPC) and glycerolphosphorylethanolamine (GPE). Their presence seems to mark certain tumorous states and also to mark development in man and other species in such diverse circumstances as red-white muscle differentiation and sperm maturation (89,90).

Part of the power of surface-coil studies lies in following changes in these marker compounds as a function of experimental stress or treatment. The safety of the method implies that follow-up studies can be repeated at whatever intervals are required. A demonstration of this can be seen in the sequential studies of Griffiths et al. of a tumor on the arm (91).

Specific knowledge of biochemical changes may be sufficient to offset poor spatial localization. One can confidently expect that biochemical information will be of such value as to prompt better methods of spatial localization leading to better biochemical diagnoses. Some methods by which better localization might be achieved will be described in our next article.

**Specific species—carbon.** Although carbon is present in substantial concentration in proteins and other biological structures, its natural abundance spectra (i.e., C-13 NMR, since C-12 does not have a magnetic moment) are hampered by the fact that only 1% of carbon is the isotope C-13. This means that only very concentrated species can be seen in a natural abundance mode, mainly  $-\text{CH}_2-$  in fat and protein, and  $\text{C}-\text{OH}$  in glycogen granules (92). Similar results are noted in surface-coil results on human arms (6). The use of isotopically enriched C-13 metabolites holds promise for the future; this will be discussed in our last chapter.

#### SUMMARY

This section has introduced several concepts—such as identification of metabolites through NMR, saturation, saturation transfer, chemical exchange, and surface-coil studies—that are peculiar to high-resolution NMR studies of chemical shift. They generate biochemical or physiologic information. This chapter should acquaint the reader with the range of techniques available to NMR spectroscopists for studying tissues (both in vivo and in vitro) and generating biochemical and physiologic data. It should be apparent that the results presented thus far are far more of a scientific than clinical nature, but it should also be apparent that the signposts pointing to clinical applications are visible.

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