NMR Research: Analysis of Living Cells and Organs

Although biochemists have successfully worked out many of the reactions by which cells obtain and use chemical energy, the investigations have frequently been hindered by the difficulty of making direct observations of biochemical events in living, intact tissues. The problems have been particularly acute, for example, in studies of the control of complex reaction pathways where simplified test-tube systems may not accurately represent the situation in the whole cell. Observation of the biochemical changes accompanying processes such as muscle contraction has also been difficult.

In order to study these changes researchers have had to flash-freeze muscles at various points in the contraction cycle and then extract and determine the concentrations of the chemicals of interest. This type of chemical analysis is not only tedious and time-consuming, but it is also subject to the uncertainty that the chemical composition of the tissue may be altered during the freezing process.

Now, however, researchers are taking advantage of improvements in nuclear magnetic resonance (NMR) technology to develop procedures for following biochemical reactions in whole cells and organs—in some cases even in organs in the living animal. With these methods they can observe increases and decreases in the concentrations of several compounds simultaneously and determine the kinetics of important reactions under normal physiological conditions. All in all, the techniques are proving useful for gathering biochemical information that would not otherwise be easily obtainable.

In addition, the research may eventually lead to the development of new noninvasive methods for medical diagnosis. The methods might be used, for example, to detect and measure brain or heart damage resulting from strokes or heart attacks, or to assess the condition of organs, such as kidneys, that are to be transplanted. Diagnosis by NMR may have the advantage of not being associated with the kind of radiation, such as x-radiation, that has harmful side effects.

In the past, NMR studies of living cells were difficult, if not impossible, because of the low sensitivity of the method. To obtain an NMR signal, a sample, which is under the influence of an applied magnetic field, is exposed to energy of the appropriate radio frequency. This energy perturbs the nuclei of the sample from their condition of equilibrium with regard to the magnetic field and the perturbation induces a very small electric voltage in detector coils placed around the sample. The signal from a given nucleus is influenced by its electronic environment, and thus nuclei of the same type that are located in different chemical environments may be distinguished from one another.

Protons give the biggest NMR signals, and although almost all biologically important molecules contain protons, their signals from living tissues would be obscured by those of the protons of water which constitutes some 90 percent of living cells.

The new techniques depend on the use of NMR to detect compounds containing phosphorus-31 or carbon-13. Because these nuclei give much weaker signals than do protons, advances in instrumentation were needed before their detection became possible. One advance was the development of more powerful, superconducting magnets. These magnets consist of a coil of wire cooled to very low temperatures at which the wire has no resistance to the passage of electric currents. Very high currents can thus be passed through the coil to generate intense magnetic fields that in turn generate larger NMR signals than less powerful conventional magnets.

Fourier-transform NMR is a second advance that has increased NMR sensitivity. To obtain NMR spectra in which the signals can be readily distinguished from the background noise, many scans must be made and the data averaged. (Because the noise obscuring the signals is random, it tends to cancel out in the averages while the nonrandom signals are enhanced.) With standard NMR instruments this is a time-consuming process because only a narrow region of the spectrum can be excited and recorded at any one time. But the Fourier-transform method uses a very intense radio-frequency pulse consisting of a spectrum of frequencies that excites the entire range of resonance frequencies in the sample simultaneously. A computer then sorts out the signals to obtain the spectrum. As a result, a given signal-to-noise ratio can be obtained in a small fraction of the time required by conventional NMR, and the sensitivity is thus increased by a corresponding factor. Decreasing the time required to obtain a spectrum is especially valuable in studies of isolated living tissue which deteriorates with time.

In an early experiment performed in 1974, David Hoult and his colleagues in George Radda's laboratory at the University of Oxford, England, took advantage of these technological improvements to show that important phosphate-containing compounds could be identified in whole muscles by NMR. The spectra they obtained contained peaks corresponding to inorganic phosphate, the sugar phosphates (key substances in the pathway for the breakdown of glucose), creatine phosphate, and adenosine triphosphate (ATP).

Moreover, these workers could observe changes in the concentrations of some of the phosphates as the muscle aged during the experiment. The concentration of creatine phosphate decreased until it eventually disappeared altogether. The concentration of ATP remained constant for a time and then it, too, decreased, while that of inorganic phosphate increased progressively.

These results were exactly as predicted by earlier biochemical experiments. In muscle, as in other tissues, ATP serves as a direct source of energy for cell activities, including muscle contraction. The energy for these activities is released by the splitting of ATP to yield inorganic phosphate plus adenosine diphosphate (ADP). As the ATP is split it is regenerated by the transfer of a phosphate to ADP from creatine phosphate, which serves as a source of stored energy until it is used up. Hoult says that the NMR techniques enabled them to acquire in a few hours data that would have taken weeks to accumulate by conventional biochemical analyses.

Several investigators* are now using NMR to follow the changes in phosphate compounds accompanying contraction of skeletal muscle and even of whole, isolated hearts. These studies are still in

*In addition to Radda and his colleagues these investigators include Michael Barany, C. Tyler Burt, and Thomas Glonek of the University of Illinois Medical Center; Britton Chance of the Johnson Research Foundation of the University of Pennsylvania School of Medicine; Raymond Damadian of the Downstate Medical Center of the State University of New York; Eric Fossel and Joanne Ingwall of Harvard Medical School; D. G. Gadian of the University of Oxford (England); Donald Hollis of the Johns Hopkins University Medical Center; and D. R. Wilkie of University College, London.

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a relatively early stage of development and many of the results confirm, rather than extend, what is known about muscle contraction. Nevertheless, unexpected findings are also turning up.

In one such case, C. Tyler Burt, Michael Barany, and Thomas Glonek of the University of Illinois Medical Center identified significant quantities of a phosphodiester called glycerol-3-phosphoryl choline in muscle. This compound was previously thought to be present in muscle in very low concentrations, if at all. The investigators found large amounts of another phosphodiester in muscle from chickens with hereditary muscular dystrophy (which is a model for the human form of the disease) although not in muscle from normal chickens. The functions of these diesters are unknown, but the differences in the composition of the normal and dystrophic muscles may provide some clue to the muscle defects characteristic of this disease.

NMR Studies of Whole Hearts

A major focus of the work on heart muscle is the identification of the chemical changes caused by oxygen deprivation—such as that resulting from a heart attack. The investigators are also using ^{31}P NMR studies of isolated hearts to assess the effectiveness of agents that may prevent the changes and possibly reduce the amount of tissue killed by lack of oxygen. A third goal is the development of a noninvasive method for measuring the dead cardiac tissue (infarct) in humans who have suffered a heart attack. Cardiologists who are attempting to devise therapies for limiting infarct size in heart attack patients are currently handicapped by the lack of an accurate noninvasive method for determining the efficacy of the experimental treatments.

The use of ^{31}P NMR to determine *p*H may provide a possible basis for such a method. Several investigators have noted that the position of the inorganic phosphate peak in NMR spectra depends on the *p*H of the tissue. Normally this is about 7.4, but the *p*H drops in tissue that has been deprived of oxygen. At *p*H's close to neutrality, small changes in hydrogen ion concentration greatly alter the relative proportions of the two phosphate species, HPO_4^{2-} and $H_2PO_4^-$, and thus also alter the position of the inorganic phosphate peak in NMR spectra.

According to Donald Hollis and his colleagues at the Johns Hopkins University School of Medicine, a shift in the phosphate peak position can be observed when the oxygen supply to isolated rat hearts is cut off. The peak moves back to the position corresponding to *p*H 7.4 when the oxygen supply is restored. If the oxygen supply to only a part of the heart is interrupted, then two phosphate peaks can be seen, one corresponding to the normal tissue at *p*H 7.4 and the other to the oxygen-deprived tissue with a *p*H around 6.4.

Britton Chance of the Johnson Research Foundation of the University of Pennsylvania School of Medicine, in collaboration with Radda and his colleagues, is currently trying to determine whether the size of infarcts in isolated hearts can be accurately measured from the relative sizes of the two phosphate peaks.

Of course, application of any such methods to human patients is still some time in the future. The researchers say the greatest limitation to the kind of experiments they can now do is the size of the magnets in their instruments. The maximum diameter of the sample tube that can be accommodated by commercially available instruments is only about 25 millimeters. The instrument manufacturers are starting to produce larger magnets, however.

In addition, other investigators are developing instruments for producing two- and three-dimensional NMR images of large objects—including the human body. Thus, the researchers working on ^{31}P NMR are confident that the techniques they are pioneering can be combined with the imaging techniques to produce new diagnostic instruments.

Potential applications, in addition to measuring heart infarct size, might include analogous determinations of brain damage resulting from strokes and assessment of the physiological conditions of organs destined for transplant. Damage to an organ is usually indicated by decreases in *p*H and in the concentrations of the energy-rich compounds ATP and creatine phosphate, all of which can be detected with ^{31}P NMR.

Chance and his colleagues have already demonstrated that ^{31}P NMR signals from brains are detectable through the skulls of mammals small enough to fit the sample tube. They obtained the signals from the brains of living mice, weighing 10 to 12 grams, that had been anesthetized and inserted into NMR sample tubes provided with air inlet and outlet tubes. Both creatine phosphate and ATP peaks can be distinguished in the resulting spectra, although the signal attributable to the sugar phosphate could not be distinguished from that of inorganic phosphate, which is normally present in very low concentrations in the brains of living animals. Chance says that the ratio of creatine phosphate to ATP is lower in spectra from the brains of living animals than in those from rapidly frozen brains. This may be because the animals must be confined in tight quarters which decrease oxygen transport to the brain. Nevertheless, the animals remain in good condition during the experiments. In fact, they sometimes scamper out of the sample tube if the anesthetic wears off.

A collaborative effort between the Chance and Radda groups is also under way to determine the feasibility of assessing kidney condition by ^{31}P NMR. Many transplanted kidneys fail not because they are rejected by the recipients' immune system but because the organs had already deteriorated before transplantation. The deterioration may be subtle and is currently hard to detect, but Radda says that ^{31}P NMR can be used to determine whether the organ responds to oxygen by making ATP and whether its *p*H is normal, two signs that the kidney is in good physiological condition. The ultimate goal would be to use NMR both before transplantation and then afterwards to monitor the function of the kidney in the recipient patient to make sure that the organ continues to work.

Pathways in Living Cells

Although the use of NMR to study biochemical pathways in isolated whole cells does not have the obvious potential medical applications of the research on whole organs, the cell work nevertheless has its enthusiastic proponents, notably Robert Shulman of Bell Laboratories (Murray Hill). His laboratory is actively pursuing the use of ^{31}P NMR to determine the distributions and concentrations of phosphorus compounds in several types of cells including liver cells, bacterial cells (*Escherichia coli*), yeast cells, and a number of different kinds of tumor cells, under varying conditions. The technique can even be used to determine the rates of reactions in living cells, a feat not possible by other means. Thus the control of complex biochemical reaction pathways can be studied under completely physiological conditions.

For example, Truman Brown and Kamil Ugurbil in Shulman's laboratory have used an NMR technique called saturation transfer to measure the rate of the breakdown of ATP to ADP and inorganic phosphate in *E. coli* cells. In saturation transfer the resonance signal of a reacting species—in this case that of the third phosphate of ATP—is destroyed with a pulse of electromagnetic radiation of the appropriate frequency. As the phosphate whose signal has been destroyed is split from ATP to produce in-

organic phosphate, the inorganic phosphate signal also decreases because the inorganic phosphate in the cell is diluted by the phosphate whose signal has been destroyed. This decrease can be measured and used to calculate the rate of the reaction. The rate of the reverse reaction can also be determined. Here the signal of inorganic phosphate is destroyed and the change in the signal from the third phosphate of ATP is monitored.

Another advantage of ^{31}P NMR is that it can be used to determine the pH's in two distinct cellular compartments. According to Sheila Cohen, Shulman, and their colleagues at Bell, the phosphate peak in spectra from isolated rat liver cells shows a shoulder on the side of the higher pH position. These spectra suggested to the investigators that they were seeing the results of a difference in pH between the mitochondria and the cell fluid (cytoplasm), with the main phosphate peak indicative of the cytoplasmic pH and the shoulder representing the mitochondrial value. This pH difference, incidentally, is predicted by the hypothesis put forward by Peter Mitchell of Glyn Research Laboratories in England to explain how mitochondria synthesize ATP. He proposed that the mitochondrial reactions result in the ejection of protons from the mitochondria, thus elevating the mitochondrial pH relative to that of the cytoplasm, and that this pH difference is the driving force for ATP production.

In any event, Cohen, Shulman, and their colleagues found that a compound known to enhance the pH gradient across the mitochondrial membrane causes the resolution of the peak shoulder into a separate phosphate peak, a result consistent with their suggestion that the shoulder is caused by the higher pH within the mitochondria. In contrast, treatment of the liver cells with a material that destroys the pH difference and also blocks ATP synthesis, produces a ^{31}P NMR spectrum with only a single phosphate peak. Thus, the Bell laboratory results provide direct support for the Mitchell hypothesis.

In addition to their studies of ^{31}P NMR, Shulman and his colleagues are also investigating the use of ^{13}C NMR to follow the passage of materials such as glucose through complex reaction pathways in living cells. Unlike ^{31}P, which is the naturally abundant isotope of phosphorus, ^{13}C constitutes only a small fraction of naturally occurring carbon atoms. Compounds labeled with additional ^{13}C atoms are readily, although somewhat expensively, available from commercial sources, however. The Bell workers have fed glucose labeled at carbon 1 with ^{13}C to *E. coli* and followed the results with NMR. Shulman says that the distribution of the ^{13}C among the various compounds formed from the labeled glucose can be readily followed and the relative rates of competing pathways evaluated. As he puts it, "the whole pathway unrolls before your eyes."

This article has described only a sampling of research in what is becoming a burgeoning new area of NMR investigation. Other work under way includes studies of liver biochemistry and the control of hemoglobin oxygenation in intact red blood cells. Moreover, the investigators doing the work are enthusiastic not just because of the biochemical information they are acquiring but also because they think their research, in combination with that on NMR imaging techniques, will lead to new noninvasive methods for medical diagnosis.

—JEAN L. MARX

The 1978 Nobel Prize in Physics

One-half of the 1978 Nobel Prize in Physics is to be awarded to Peter L. Kapitsa, director of the Institute for Physical Problems, U.S.S.R. Academy of Sciences, Moscow, for his basic inventions and discoveries in the area of low-temperature physics. A review of the record shows Kapitsa to be not only a very competent scientist, but also a talented engineer and a successful technical manager. As his career has, on occasion, become embroiled in Russian politics and the continuing struggle between the individual and the state in the Soviet Union, he enjoys considerable world renown beyond the narrow confines of the physics community. In the popular press, in fact, he has come to be somewhat lionized as a leading scientist who defied Stalin and yet survived to continue as an important scientific contributor. Kapitsa endured harrowing experiences in arriving at the condition of octogenarian in his Mother Russia, but he was also given many honors by successive regimes therein. With all these facts taken together, Kapitsa is a figure who looms large on the world stage, and it is presumably with this combination in mind that the Nobel Committee made its selection, 40 years after his most significant contributions to low-temperature physics.

P. L. Kapitsa, the son of a general of engineers, was born in Kronstadt in 1894, raised in Tsaritsyn, and educated in Petrograd. After graduating in 1918 from the Electro-Mechanical Faculty of the Polytechnic Institute in Petrograd he became a lecturer in the institute and carried out research under A. F. Ioffe. With the beginning of the Red Terror, Kapitsa fled to England and commenced a long association with the Cavendish Laboratory of Cambridge University. From 1921 to 1924 he worked under Sir Ernest Rutherford, in 1924 he was appointed assistant director for magnetic research, and from 1930 to 1934 he served as director of the Royal Society Mond Laboratory.

There is an aura of magic about Kapitsa's career, not least over his sojourn in Cambridge where, after 8 years and a fairly modest publication record, he not only was elected Fellow of the Royal Society but was the first foreigner to be accorded that honor in 200 years. Readers familiar with the British scene cannot fail to be impressed by the election of anyone under those career circumstances and doubly so by its uniqueness (*1*). Those not so familiar will perhaps take more notice of the fact that the Royal Society, in 1930, named him Messel research professor and then built the Mond Laboratory for him.

During these Cambridge years, Kapitsa traveled to Russia each summer to visit his mother. There government officials began urging him to return to Russia, offering working conditions similar to those he enjoyed in England. As these pressures built up, Kapitsa suspected that his freedom to go and come would not last forever, and he discussed these misgivings freely with his Cambridge colleagues. Nevertheless he was given guarantees of safe return (*2*), but when he attended a scientific conference in Moscow in 1934 he was detained on orders from Stalin. Although he had clearly foreseen this eventuality, Kapitsa, it is reported, refused to work for almost a year. He was then made director of the Institute for Physical Problems of the Academy of Sciences, a capacity in which he served until 1946. In addition, the Soviet government purchased all his equipment from Cambridge University

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