and the particle sizes such that the hail embryos may grow for longer durations in a more favorable growth environment.

The probability that seeding may produce both positive and negative effects implies that: (i) the results achieved under one set of conditions or in one part of the world are not necessarily transferable to other conditions or meteorological regimes; (ii) without rational physical stratification of the data, a randomized statistical experiment may be statistically inconclusive because of the balancing positive and negative effects; (iii) without such stratification, we will probably be unable to improve our seeding methodology or "recipes" to optimize the beneficial effects and minimize or avoid the deleterious ones; and (iv) hail suppression programs may be jeopardized by legal injunctions against potentially hazardous activities or claims for damage unless some form of insurance can be provided or means are found to avoid the hazardous situations.

In order to enhance the chances of success of a statistical experiment, I propose a first approach to a scheme of stratification which should permit the physical discrimination between the conditions leading to increased or decreased hail. The strength of a statistical experiment would also be enhanced and its duration reduced by the use of a strong covariate; dynamic hail potential is one of the most likely candidates.

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42. The findings presented here have been strongly influenced by invaluable discussions with my colleague, Dr. K. A. Browning. The National Center for Atmospheric Research is sponsored by the National Science Foundation.

Analysis of Living Tissue by Phosphorus-31 Magnetic Resonance

Phosphorus nuclear magnetic resonance is a new method for observing the internal milieu of intact cells.

C. Tyler Burt, Thomas Glonek, Michael Bárány

Nuclear magnetic resonance (NMR) spectroscopy, originally a tool of the physicist and then, for more than a score of years, one of the most potent analytical methods of the chemist, has, within the last 10 years, found extensive application in the field of biochemistry (*1*). The growing use of the method has been principally stimulated by technological advances, which have steadily improved the spectroscopic sensitivity, permitting virtually every element of the periodic table to be experimentally accessible on a practical level.

It was inevitable that the method would eventually find application in the field of physiology, and this, in fact, is the principal new application of NMR technology in the 1970's. The use of NMR in the study of living tissues is at once simple and complicated—simple, in that the analysis of a piece of tissue is straightforward and requires little specialized equipment; complicated, in that the interpretation of the data poses a host of empirical and theoretical problems.

Our applications of NMR in basic biomedical research have involved detection of the phosphorus-31 nuclide, which, at 100 percent natural abundance, is the common isotope of elemental phosphorus. These studies (*2–21*) and those of others (*22–35*) conducted elsewhere have demonstrated that high-resolution ^{31}P NMR spectra of high information

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Table 1. Concentrations of phosphate metabolites determined in intact muscles by ^{31}P NMR. The average phosphate concentration over the 0- to 10-minute time span is given ± the standard error. Concentrations are expressed in terms of muscle water (see text).

Muscle	Concentration (m*M*)				*N*
	Sugar phosphates	P_i	Phospho-creatine	ATP	
Northern frog gastrocnemius	3.8 ± 0.6	2.1 ± 0.3	30.0 ± 2.0	3.0 ± 0.2	4
Normal chicken pectoralis	4.3 ± 2.3	7.9 ± 4.3	24.8 ± 2.3	6.2 ± 0.7	3
Dystrophic chicken pectoralis	10.5 ± 2.7	13.7 ± 1.6	14.9 ± 3.5	4.2 ± 0.3	3

content could be obtained not only from small molecules, such as inorganic phosphates (*3, 10, 11, 16, 23*), sugar phosphates (*16, 20, 31*), lipids (*2, 6*), and nucleotides (*9, 13, 15, 16, 22, 25*), and more complex organic systems, such as phosphorylated proteins (*24*), polyphosphates (*3, 4, 10*), phospholipid vesicles (*26*), polynucleotides (*17, 30*), cell walls (*5*), and circulating lipoproteins (*8, 29*), but even from a variety of intact tissues and cellular organelles, such as erythrocytes (*7, 18, 27*), reticulocytes (*18*), platelets (*18*), muscles (*12, 14, 19, 21, 28, 33*), yeasts (*32*), embryos (*34*), sperm (*36*), brain (*35*), malignant tumors (*35*), mitochondria (*36*), and nuclei (*36*). The spectra yield information about the phosphorus components and metabolic processes of such systems. In fact, the concentrations of "high-energy phosphates" such as adenosine triphosphate (ATP) and "low-energy phosphates" such as sugar phosphates, as measured by ^{31}P spectroscopy, have been used as criteria for the metabolic integrity of erythrocytes (*7*), platelets (*18*), and muscle tissue (*12, 14*).

Among the nuclides that can be utilized for biomedical studies, ^{31}P has several favorable characteristics, particularly for muscle. It is present in relatively few compounds whose cellular concentrations are in excess of 0.5 millimolar. These compounds, however, play important roles in the tissue's economy and can serve to differentiate a tissue's source and its metabolic state (*14, 19*). Furthermore, since all naturally occurring phosphorus exists as ^{31}P, spectra can be obtained relatively rapidly. This means that simple spectra can be obtained from biological material in 5 to 10 minutes. Moreover, since NMR is nondestructive, the intact tissue can be resampled, which enables determinations to be made of kinetic parameters in vivo.

The spectra may also uncover phosphates that previously had gone undetected by classical biochemical procedures. For example, in a variety of mammalian cellular types, a group of resonances have been detected at about 0.0 parts per million (ppm) (*37*) which do not correspond to any known common phosphate metabolites (*12, 14, 18–21, 28, 33*). There are several species associated with this family of resonances, one of which has been identified as arising from glycerol 3-phosphorylcholine; the sources of the other resonances (*20*) are unknown. In yeasts a number of signals arising from condensed phosphates (*32*) and phosphoramidates (*36*) have been detected. The precise molecular species associated with these resonances are also unidentified.

This article presents some of our recent applications of ^{31}P spectroscopy to the study of living tissues, in particular muscle, and demonstrates the advances that can be made by this technique in elucidating muscle function and disease.

Phosphate Spectra from Normal Intact Muscle

Intact muscles give rise to particularly detailed ^{31}P NMR spectra and are used here to illustrate the kind of spectroscopic data that can be obtained from intact living tissues. Figure 1 shows ^{31}P spectra from several intact muscles. The signals, proceeding from left to right, are assigned as follows: the external ^{31}P NMR reference, methylenediphosphonate, −16.3 ppm; sugar phosphates, the broad resonance at −3.7 ppm; inorganic orthophosphate (P*i*), the prominent resonance at −1.7 ppm; the group of newly discovered phosphate diesters which are particularly abundant in toad and frog and absent in abalone, 0.0 ppm; phosphocreatine (phosphoarginine in abalone), 3.2 ppm; and the γ, α, and β phosphate groups of ATP, 5.6, 10.7, and 19.1 ppm, respectively (*14*).

Beyond indicating the presence or absence of particular metabolites, the signals can be further characterized by their broadness, relaxation times, or other NMR parameters. Such an analysis has been applied to the orthophosphate signal from the human gastrocnemius muscle (Fig. 1). Instead of a single sharp resonance, as is usual for orthophosphate, this resonance from the human muscle is an overlapping doublet. The data have been interpreted (*33*) as indicating the presence of orthophosphate pools, most probably representing various hydrogen ion concentrations.

In all muscle spectra, the signals arising from the phosphate groups of ATP are displaced downfield from their resonance positions in simple aqueous solutions, the displacements being −2.1 ppm for the β, −0.8 ppm for the γ, and −0.4 ppm for the α group. For other phosphates, however, such as phosphocreatine or the sugar phosphates, similar relative shift changes between those in simple aqueous solutions and those in muscle are not observed. The shift changes for ATP have been interpreted (*12, 14*) as the result of complex formation between ATP and one equivalent of Mg^{2+}, and the spectrum of magnesium-ATP in water is in accord with that observed for ATP in the intact muscle. The binding of calcium to ATP does not produce the required spectrum. Moreover, other evidence (*38*) indicates that Ca^{2+} is entirely sequestered by the muscle's sarcoplasmic reticulum.

At physiological *p*H values, the chemical shifts of ATP, inorganic orthophosphate, and the sugar phosphates vary with the hydrogen ion concentration. By comparing the shift of a particular resonance in the tissue to that in a solution similar in composition to the myoplasm, a value may be assigned to the internal *p*H. Thus, we were able to ascribe an initial intracellular *p*H of 7.2 for frog muscle (*14*) and of 7.1 for human muscle.

Concentration of Phosphate Metabolites

With approriate calibration, the integral of the phosphate signals can be used to determine phosphate concentration in the intact muscle (*14*). Table 1 compares intact muscle ^{31}P phosphate profiles of three muscle types. Because the magnetic resonance technique only detects signals from compounds in solution, the concentrations are expressed in terms of the total free muscle water rather than the unit weight of the muscle. According to Dubuisson (*39*), various muscles contain 77 percent free water, and this value was used in calculating the molarities of the phosphates listed in Table 1.

The ATP, phosphocreatine, and P*i* contents of the frog muscle measured by ^{31}P spectroscopy agree well with data obtained by a variety of chemical methods (*40*). A higher concentration of ATP

is commonly encountered in animals in a warm environment (*41*), and Table 1 shows that the ATP contents of normal chicken muscles, which contract rapidly, are elevated and are in the range of values given by Farrell and Olson (*42*) and Malvey *et al.* (*43*). Moreover, significant decreases in the ATP and phosphocreatine contents of genetically dystrophic chicken muscle, as compared to the normal muscle, were reported by Farrell and Olson (*42*), and also appear in the data of Table 1. These studies validate ^{31}P spectroscopy as an analytical method for whole-tissue studies.

Isometric Caffeine Contracture

Because ^{31}P spectroscopy yields a variety of information about cellular phosphates in a single step and in a reasonably short period of time, the method has proved useful in the study of functionally different muscles. The experimental method thus far employed has involved comparison of the ^{31}P spectroscopic profiles of caffeine-treated and untreated intact frog muscle (*21*). Caffeine is a drug that causes muscle to develop tension and shorten. Considerable tension will be developed by the muscle if it is held at a fixed length. We have carried out isometric experiments by fixing the muscle between two hooks, one of which can vary the muscle's length. The stretching device can be spun in the NMR spectrometer to provide enhanced resolution of the ^{31}P signals.

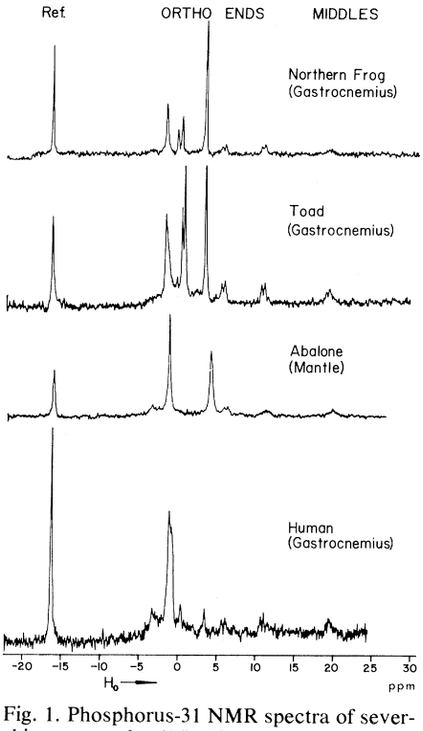

Fig. 1. Phosphorus-31 NMR spectra of several intact muscles (*14*). The spectra were accumulated over a period of 30 minutes and therefore show a time-averaged distribution of the phosphates. The reference compound, methylenediphosphonate, was contained in a sealed 1-mm capillary tube external to the sample. The chemical shift scale is relative to 85 percent inorganic orthophosphate (*44*); the temperature was 28°C. The magnetic field is denoted by H_0 and the arrow shows the direction of its increase. "Ends" and "middles" denote end and middle phosphate groups of ATP. For peak assignments, see text.

Figure 2 shows typical ^{31}P spectra obtained with a mounted muscle in an experiment designed to measure the effect of isometric caffeine contracture on muscle ^{31}P phosphate profiles. The pair of gastrocnemius muscles from a frog were used.

Figure 2A shows the ^{31}P profile of one of the muscles shortly after it was mounted in the NMR tube. The muscle is rich in phosphocreatine (the sharp resonance at 3.2 ppm), and the three resonance bands from ATP are visible (6, 11, and 19 ppm). The sugar phosphate (−3.7 ppm) and inorganic orthophosphate (−1.7 ppm) concentrations are low. After 45 minutes of caffeine-stimulated isometric contracture (Fig. 2B), the spectrum shows the complete exhaustion of phosphocreatine and barely detectable levels of ATP. On the other hand, the sugar phosphates and inorganic orthophosphate have increased markedly.

In the parallel experiment, carried out with the other gastrocnemius muscle (Fig. 2C), the ^{31}P phosphate profile shows high levels of phosphocreatine and ATP; the sugar phosphates and inorganic orthophosphate have increased compared to their concentrations at the beginning of the experiment; however, they are still much lower than in the caffeine-treated muscle. The striking changes involved in these experiments show clearly that we can actually observe the metabolism of chemical compounds as it occurs in intact tissue.

Comparison of Normal and Diseased Human Muscle

Phosphorus-31 NMR spectroscopy has also proved useful in the study of diseased muscle tissue. Once a human muscle biopsy has been taken, the endogenous metabolism continuously changes the phosphate profile. These changes reflect the various enzymic activities that take place in vivo. By measuring the ^{31}P NMR spectrum of intact muscles after excision as a function of time, the changes in the metabolic state can be observed (*14, 28*). It was expected that differences between diseased and normal muscle should manifest themselves, and this has, in general, been the case.

A typical plot of the time dependence of the phosphate metabolites of a normal human quadriceps muscle in the NMR tube is shown in Fig. 3A. The phosphocreatine concentration, which is initially about 10 m*M*, decreases linearly with time, reaching about 2.3 m*M* or 29 percent of its initial value in about 50 minutes, whereupon its rate of decrease diminishes. On the other hand, P_i shows a linear increase from its initial value of 10

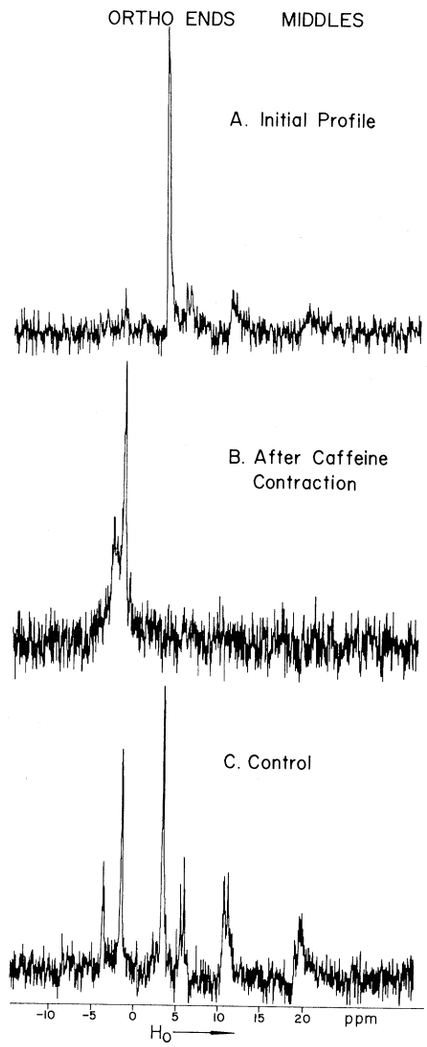

Fig. 2. Effect of isometric caffeine contracture on the ^{31}P phosphate profile of a frog gastrocnemius muscle at 31°C. (A) Spectrum from one of a pair of frog gastrocnemius muscles, obtained during the first 15 minutes after the animal was killed. After the spectrum was taken, 20 m*M* caffeine in a solution of 115 m*M* NaCl, 2.5 m*M* KCl, and 2.0 m*M* $CaCl_2$, *p*H 7.2, was added, and the muscle underwent isometric contracture for 45 minutes. After this time, the spectrum in (B) was obtained at the same instrumental settings and signal averaging time as the spectrum in (A). After this analysis, the ^{31}P phosphate profile from the other gastrocnemius muscle of the same frog (C) was determined, again with the same experimental conditions used for (A).

m*M* for about 50 minutes, after which its rate of increase diminishes. The ATP concentration, which initially is constant, begins to decline in a sigmoidal fashion at a time, T_m, the maintenance time, which is defined as the time corresponding to the intersection of the projected flat portion of the ATP curve with the projected slope of the rapidly decreasing portion. In this instance T_m occurs at 48 minutes. The ATP level is maintained so long as there is sufficient phosphocreatine to regenerate the ATP lost through metabolic processes. The concentration of sugar phosphates increases, rapidly at first and then more slowly.

A similar time course for the muscle of a patient with the signs and symptoms of a neuromuscular disease is shown in Fig. 3B. This time course exhibits the broad general features observed for all muscles—that is, decreases in the concentrations of phosphocreatine and ATP with progressive rigor, accompanied by increases in P_i and sugar phosphates. The total measurable phosphorus for this example, however, was 23 m*M*, which was about half the normal value, and the concentration of each phosphorus metabolite was lower than that in the normal muscle. By directly comparing the time course for the normal muscle in Fig. 3A with that for the diseased muscle in Fig. 3B, it can be seen that phosphocreatine is lower initially and decreases at a faster rate in the diseased organ than the normal one. Inorganic orthophosphate is lower initially but also increases at a higher rate than normal. In addition, ATP, which is initially lower, begins to decline earlier with a T_m of 24 minutes, about half the normal time. Furthermore, the phosphocreatine concentration at this point is 53 percent of the initial value, indicating that relatively higher levels of phosphocreatine are required to maintain ATP values in the diseased muscle.

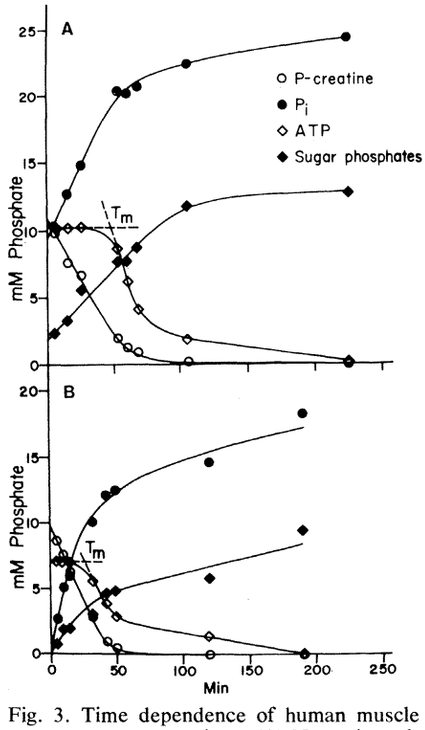

Fig. 3. Time dependence of human muscle phosphate concentrations. (A) Normal quadriceps. The maintenance time, T_m is the point at which ATP begins to decrease. (B) Quadriceps from nemaline rod myopathy.

Perchloric Acid Extracts of Diseased Human Muscle

In these comparative studies we have found the use of perchloric acid extracts to be particularly advantageous. The ^{31}P spectra of properly prepared extracts mirror the spectra obtained from the corresponding intact tissue; moreover, these extracts can be prepared near the location of the surgical biopsy, and it is not necessary to preserve the sample in the interim between biopsy and NMR analysis. The latter point is particularly significant; surgeons at a number of hospitals throughout the Chicago metropolitan area have participated in this aspect of the human muscle program, and the time required to transport some of the samples from the hospital where the biopsy was performed to the university for NMR analysis was prohibitively long for a fresh muscle analysis. Refrigerated neutralized perchloric acid extracts, however, are stable for several days.

Analysis of perchloric acid extracts has two other advantages over fresh muscle analysis for such types of comparative disease studies. Because the samples are chemically stable for long periods of time, smaller samples can be effectively analyzed. The advantage here is obvious when only 100 or so milligrams of tissue from a biopsy are available. In addition, ^{31}P spectra from perchloric acid extracts are usually much more highly resolved than those from the corresponding fresh muscle (*14*). This yields a greater information content for each spectrum, which frequently results in the detection of relevant minor differences that are obscured in the spectrum of the fresh muscle.

Figure 4 shows comparative spectra from perchloric acid muscle extracts; note the sharpness of the signals in Fig. 4B and the resulting additional detail observable in the sugar phosphate resonance band (−3.7 ppm). The progressed nature of the dystrophy is readily apparent in Fig. 4A. The resonances of ATP at 6, 10, and 21 ppm, are virtually unobservable, and the phosphocreatine signal cannot be detected. An interesting feature of this spectrum is the pronounced signal from nicotinamide-adenine dinucleotide (NAD^+) at 11 ppm, which is enhanced about fivefold with respect to that in the normal muscle. Insofar as we can determine, this observation has not been previously reported.

Detection of New Constituents

Normal human muscle shows a phosphodiester resonance (at 0.13 ppm for human gastrocnemius in Fig. 1) which appears to represent glycerol 3-phosphorylcholine; this compound has been isolated and characterized from a number of muscle sources (*20*). In various diseased muscles, however, the phosphodiester resonance is either missing or displaced about 0.5 ppm downfield, indicating that a different compound is present.

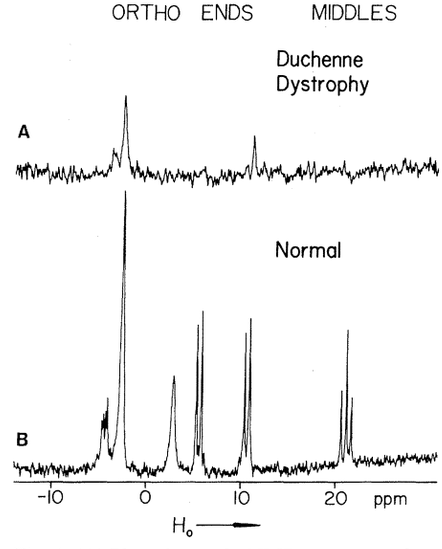

Fig. 4. (A) Phosphorus-31 NMR spectrum of a perchloric acid extract of a vastus lateralis muscle from a patient diagnosed as having Duchenne dystrophy. Before the spectrum was recorded the sample was passed through a Chelex-100 column to further sharpen the signals. Only three prominent resonances are noticeable. From left to right, these are due to sugar phosphates, −4.9 to −4.1 ppm; inorganic phosphates, −2.8 ppm; and NAD^+, 10.8 ppm. Two weak signals, which can be assigned to the β and α groups of ADP, can also be discerned after the spectrum signal areas have been calculated by a computerized integration process. (B) Phosphorus-31 NMR spectrum of a perchloric acid extract of a normal human gastrocnemius muscle. The resonances observable are due to sugar phosphates, −4.3 to −3.9 ppm; inorganic phosphate, −2.4 ppm; phosphocreatine, 3.0 ppm; and the γ, α, and β phosphates of ATP, 5.8, 10.9, and 21.9 ppm, respectively.

The pectoralis muscle of a chicken with hereditary muscular dystrophy is an extremely rich source of such unidentified phosphodiester compounds, which account for about 3 percent of the total phosphorus. These materials can be extracted with perchloric acid and isolated as a family by removing the common phosphate metabolites of the muscle with barium chloride precipitation. Glycerol 3-phosphorylcholine can be separated from the other phosphate diesters by ethanol fractionation. Column and paper chromatography are used to isolate a second phosphate diester. This compound contains serine as a subunit, but its precise chemical nature has not yet been established.

At least two other phosphate diester components are present in these extracts in greater than trace amounts. The detection of the four diester compounds by ^{31}P NMR in muscle opens the way for studying their possible physiological role and their connection with various disease states.

Spectra from Intact Cells Other than Muscle

Evidence that ^{31}P NMR spectra could be obtained from an intact biological sample was first obtained in research on doped rabbit erythrocytes by Moon and Richards (*27*). Further studies by Henderson *et al.* (*7*) showed that actively metabolizing human erythrocytes could be monitored as a function of time and their response to stimuli measured. Yeasts have also proved easy to analyze because of their sturdy nature (*32, 36*). Work with reticulocytes and erythrocytes of rabbit blood (*18*) and with sea urchin eggs (*36*) revealed a number of resonances that were similar to those seen in the spectra of intact muscles. For example, the sugar phosphate resonance band was found in each of the spectra, as well as the signal from inorganic orthophosphate. Adenosine triphosphate was readily observed in erythrocytes and reticulocytes, but it was absent in the sea urchin. The sea urchin spectrum showed the resonance from phosphocreatine, which was not present in the blood components. In erythrocytes and reticulocytes, 2,3-diphosphoglycerate was the major phosphate; however, additional resonances in the phosphodiester region allowed the further differentiation of these cells (*18*). These data indicate the scope of the analysis of intact tissues by NMR and suggest that this method will contribute significantly to furthering biomedical knowledge.

Summary

Nuclear magnetic resonance is a new method for assaying the content of phosphate metabolites in intact tissues. Its nondestructive nature allows simultaneous and repeated determinations of these compounds with a minimum perturbation of tissue. Changes in the concentrations of the phosphates as a function of time characterize the metabolic machinery of the tissue and reveal alterations in enzymic activity that result from drug treatment or disease.

The entire phosphate profile shows differences between normal and diseased muscle which should be of diagnostic value. Further, by examining phosphate profiles we detected a family of chemical compounds that were not previously known to exist as major constituents in muscle. Of these, two have been isolated and one has been identified as glycerol 3-phosphorylcholine.

Finally, shifts in the positions of resonances monitor the internal environment of the living system, its hydrogen ion concentration, the complexing of alkaline earth metals with ATP, and compartmentalization within the cell.

References and Notes

1. The NMR spectrophotometer is an electronic device in which a large magnetic field and radio-frequency energy of the type commonly employed in frequency-modulated radio communication interact with atomic nuclei of atoms within molecules to give signals which are then processed by special-purpose signal averagers and processors. These signals—that is, resonances which have parameters such as frequency, intensity, and characteristic decay times—monitor the local molecular condition and are interpreted to identify the structure and state of the chemical compound from which they arise.
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