Analysis of Phosphate Metabolites, the Intracellular pH, and the State of Adenosine Triphosphate in Intact Muscle by Phosphorus Nuclear Magnetic Resonance*

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31P nuclear magnetic resonance spectra recorded from intact muscles showed resonances for ATP, phosphocreatine, inorganic orthophosphate, and the sugar phosphates. Quantitation of these metabolites by 31P nuclear magnetic resonance was in good agreement with values obtained by chemical analyses.

The spectra obtained from various muscles showed considerable variation in their phosphorus profile. Thus, differences could be detected between (a) normal and diseased muscle; (b) vertebrates and invertebrates; (c) different species of the same animal.

The time course of change in phosphate metabolites in frog muscle showed that the ATP level remains unchanged until phosphocreatine is nearly depleted. Comparative studies revealed that under anaerobic conditions the Northern frog maintains its ATP content for 7 hours, while other types of amphibian, bird, and mammalian muscles begin to show an appreciable decay in ATP after 2 hours.

Several lines of evidence indicated that ATP forms a complex with magnesium in the muscle water: (a) the phosphate resonances of ATP in the muscle were shifted downfield as compared to those in the alkaline earth metal-free perchloric acid extract of the muscle; (b) the coupling constants of ATP measured in various live muscles closely corresponded to those for MgATP in a solution resembling the composition of the muscle water; (c) in the muscle the γ-phosphate group of ATP exhibited no shift change over a period of 10 hours under conditions where resonances of other phosphate compounds could be titrated. This behavior is similar to that of MgATP in model solutions in the physiological pH range, and it is different from that of CaATP.

The chemical shifts of the phosphate metabolites were determined in several relevant solutions as a function of pH. Under all conditions only inorganic orthophosphate showed an invariant titration curve. From the chemical shift of inorganic phosphate observed during aging of intact muscle the intracellular pH of frog muscle was estimated to be 7.2.

31P nuclear magnetic resonance has been applied in these laboratories to various complex biological systems (1-3) including intact red blood cells (4). It seemed, therefore, that the use of this technique could be of value to the study of muscle as well, since this tissue is extremely rich in phosphate compounds, and phosphate compounds play dominant roles in the function and metabolism of muscle. The recent article of Hoult et al. (5), as well as our reports (6, 7), are preliminary steps in this direction. In this communication we wish to describe the use of 31P NMR as an analytical tool for the characterization of muscles from a broad range of animals, for the identification of ATP as its magnesium complex in muscle, and for the determination of pH in the muscle cell.

METHODS AND MATERIALS

31P Nuclear Magnetic Resonance—The spectroscopic analyses were performed in a manner analogous to that which has been developed in these laboratories for the study of macromolecular complexes (1) and other systems of intact cells (2). The NMR spectrometer employed was a Bruker HFX-5 with "D stabilization operating at 36.43 MHz for 31P (21 kG magnetic field, 1H frequency 90.00 MHz), and incorporating facilities for all modes of Fourier transform signal-averaging and broad band and continuous wave heteronuclear 1H decoupling (1, 8, 9). Large bore spinning sample tubes (10 to 13 mm) were used. These permitted useable spectra to be obtained from intact muscles in as little time as 1 min although for the greater bulk of the 31P data gathered, spectrum accumulation times of 5 to 30 min were employed. 31P analyses of the perchloric acid extracts were performed by routine methods (10); the spectrometer probe temperature was 25°.

Chemical shift data are reported relative to the usual standard of 85% inorganic orthophosphate (11, 12); however, the primary standard
was a capillary (1 mm diameter) containing 1.0 M methylenediphosphonic acid, (HO)₂OPCH₂PO(OH)₂, in D₂O (pD = 9.5, Na⁺ counter cation) co-axially mounted in the sample tube. Stabilization of the magnetic field was achieved through the homogeneity signal from the capillary; the area of the phosphonic acid signal (−16.3 ppm) served as the reference for relative area measurements. Once the capillary had been standardized, the concentration of the various known compounds could be determined by comparing the integral of their signals to that of the capillary. The capillary was calibrated against a 5-ml volume of 0.02 M Na₂HPO₄. This sample volume was used in the determination of the muscle phosphate levels and ensures that all portions of the sample sensed by the receiver coil of the spectrometer probe contained the material to be analyzed. When only relative phosphorus concentrations were required, volumes as small as 0.3 ml could be profitably analyzed by positioning the sample in the center of the receiver coil through the use of Teflon plugs. It was found that the capillary gave a constant chemical shift value regardless of the nature of the sample. P-Creatine which might serve as an internal standard, however, showed some dependence on ionic conditions (see Fig. 6 and Ref. 13) and hence was not used as the primary reference.

The water abrasion conditions were used in this study; however, those described below which were used in the analysis of dystrophic chicken muscle, are typical: sweepwidth, 2500 Hz (200 μs/data point, 4096 data points), cycling time, 832 ms, filter bandwidth, 2560 Hz, pulse width, 4.5 μs; number of spectra accumulated per signal, 50; Bruker 80 Hertz. In addition, it was found that the generated filter time constants introducing 1.2 Hz broadening was applied. Peak areas (analog as well as digital integration), intensities, and chemical shifts were also calculated using the spectrometer’s computer. The coupling constants (J values) for the α-/β- and β-γ-phosphate groups of ATP are given in Hertz units.

**Preparation of Samples**—Muscles from frogs were dissected at 2° from animals previously chilled to this same temperature, with the exception of toads which were killed at room temperature. Muscles from warmblooded animals were dissected after previous intravenous injection of Nembutal (Abbott) and were chilled to 2°. Chilling allowed for the mounting of the muscles in the sample tubes without pronounced degradation of the tissue. After the sample tube was prepared (about 3 min) it was placed in the spectrometer probe. Warming to the probe temperature of 28°C required about 6 min, and during this time a spectrum could be obtained of the cold muscle; the data of Table I were obtained from such spectra. However, the spectra of live samples, shown on the figures, were taken for time periods ranging from 20 to 45 min.

Excised muscles were inserted directly into cold NMR tubes which contained an open 1-mm capillary. The muscles were worked to the bottom of the tube with a large glass plunger, the entrapped air escaped capillary. After the capillary was withdrawn, the remaining phosphorus resonances were complete and sharp. The capillary was then replaced and positioned through the capillary, and the NMR reference capillary inserted through this plug and muscle to the bottom of the tube. The tube was then capped and placed in the spectrometer probe for analysis; anaerobic conditions prevailed. The best spectra were obtained from mountings where the region of the sample tube sensed by the spectrometer’s receiver coil (about 1 cc volume) was filled with a single muscle. Nearly all the muscle samples contained an unknown resonance, the data of Table I were obtained from such spectra. However, the spectra of live samples, shown on the figures, were taken for time periods ranging from 20 to 45 min.

The spectra obtained from mountings where the region of the sample tube sensed by the spectrometer’s receiver coil contained a single muscle were taken for time periods ranging from 20 to 45 min. The spectra obtained from mountings where the region of the sample tube sensed by the spectrometer’s receiver coil contained a single muscle were taken for time periods ranging from 20 to 45 min. The spectra obtained from mountings where the region of the sample tube sensed by the spectrometer’s receiver coil contained a single muscle were taken for time periods ranging from 20 to 45 min.

The spectra obtained from such extracts, for the most part, showed all the peaks. The only exception, however, was the peak corresponding to the end group phosphate and the middle phosphate end group phosphate, one esterified end group phosphate, and middle group phosphate (ionized), these interacting with each other to produce the characteristic doublet, doublet, triplet pattern of ATP. Further evidence contributing to the assignment of peaks is the characteristic shift behavior of each resonance as the muscle pH lowers with aging. Thus, monosterified phosphates possess a more or less weakly dissociable proton which will exhibit characteristic upfield changes with increasing acidity. Orthophosphate diester, monosterified phosphate end groups, and middle phosphates are strong acids and show little chemical shift pH dependence in the physiological pH range. Similar behavior is also exhibited by compounds like P-Creatine which are unusually strong phosphoric acids (pK = 4.6). Peak assignments can be complicated by the presence of pollutants, high ion concentrations, or acids in the medium containing the phosphate. We have not, however, found such effects to interfere to a degree which renders peak assignments uncertain. Instead, chemical shift data from other factors, such as pH, ionic strength, or both, and by changing the counter cation (2°, 14) in solution. Through such procedures it was possible to identify all the resonances found in the perchloric acid extracts for which the appropriate purified compounds were available.

Phosphates can be identified in perchloric acid extracts of muscle by simply adding a known quantity of a pure compound to the sample and observing the position of resonance. Coincidence of the resonance of the added material with the signal of the sample is usually sufficient to identify an unknown resonance. However, in regions where a number of closely spaced resonances are formed, e.g. the hexose phosphate and the orthophosphate region, the assignment can be reinforced by selectively shifting the resonance of interest with respect to those from other compounds in the sample by judicious adjustments of pH, or ionic strength, or both, and by changing the counter cation (2°, 14) in solution. Through such procedures it was possible to identify all the resonances found in the perchloric acid extracts for which the appropriate purified compounds were available.

The spectra thus far presented are all 31P broad-band-decoupled so that the multiplicity resulting from proton-phosphorus coupling is eliminated and the only observable couplings are P-O-P. These spectra can also be obtained without proton decoupling so that the fine structure of each resonance resulting from 31P-31P coupling is retained. However, in this case the phosphorus multiplets overlap to a considerable degree and where many of the regions will be reduced to a single broad resonance band. Nevertheless, the proton-coupled spectra may aid in the identification of a compound in intact muscle if that compound is present in large amount. Thus, proton-coupled spectra assisted in the identification of inosine in between phosphocreatine, P in all muscle, and the phosphate diesters in amphibian muscle.
Frogs—For most of the experiments the Northern frog (Rana pipiens) was used. A large batch of frogs was purchased in the fall and were kept in the cold room without any feeding. In some experiments the Southern K. pipiens was used. These frogs were kept at room temperature and supplied with mealworms. The toads (Bufo bufo) were also kept at room temperature.

Chickens—The normal New Hampshire chicken, Line 200, and the genetically dystrophic New Hampshire chicken, Line 304, were obtained from the Department of Avian Sciences, University of California, Davis. They were 10 weeks old at the time of killing; and dystrophic birds were not able to arise when placed on their backs.

Abalone—A Pacific coast abalone (Haliotis cracherorrr) was kindly supplied by the Shedd Aquarium in Chicago. It was separated from its shell, and a plug of muscle which just fit into the NMR tube was excised from the mantel muscle by a piece of steel tubing.

RESULTS

Comparison of Frog Muscle with Its Extract—A typical $^3$P NMR spectrum for freshly dissected Southern frog muscle is shown in the upper part of Fig. 1. The major resonances, from left to right, are the external reference, $P_n$, P-creatine, and the $\gamma$, $\alpha$, and $\beta$ groups of ATP, with parts per million values as listed in the legend to the figure. The sugar phosphates, which in the intact muscle represent the overlapping peak of hexose and triose phosphates, appear as small signals around -3.7 ppm. The lower part of Fig. 1 illustrates the spectrum of the perchloric acid extract of the frog muscle. With the removal of alkaline earth metals by Chelex resin, the resolution of the resonances is improved and the characteristic multiple patterns of ATP and the signals of the sugar phosphates are clearly seen. The relative area under the $P_n$ peak is larger in the extract than in the intact muscle, due to the breakdown of P-creatine. Furthermore, in the end phosphate group region of the perchloric acid extract a minor resonance is seen on the high field side of the $\gamma$ group doublet of ATP and on the low field side of the corresponding $\alpha$ group doublet. These small signals arise from ADP; they can be observed in the perchloric acid extract but not in intact fresh muscle. No signals were detected for AMP with certainty in any case. Addition of AMP to perchloric acid extract localized its resonance between those of the sugar phosphates and $P_n$.

The resonance positions of the phosphate groups of ATP in the spectra of intact muscle relative to those in the perchloric acid extract are displaced downfield. The chemical shift displacements are: -2.1 ppm for the $\beta$, -0.8 ppm for the $\gamma$, and -0.4 ppm for the $\alpha$ group. No appreciable shift change is observed for P-creatine, $P_n$, or sugar phosphates. It is most probable that the changes observed for the phosphates of ATP are caused by complex formation between ATP and Mg$^{2+}$ in the muscle, since according to current views, Ca$^{2+}$, the other alkaline earth metal ion, is entirely sequestered by the sarcoplasmic reticulum (15, 16).

Change of Phosphate Metabolites during Aging—Fig. 2 depicts the changes in the relative concentrations of various phosphate compounds of frog muscle during aging at 28°C. At the earliest measurement (10 min after placing the muscle into the NMR tube) P-creatine comprises as much as 68% of the total phosphate recorded (this represents the average P-creatine content at 5 min). Quantitation of this P-creatine peak leads to a concentration of about 30 mM P-creatine in the muscle water. In contrast, the $P_n$ concentration in the fresh muscle is extremely low; 4% of the total phosphate, or 2.1 mM. One notes a rapid decrease in the P-creatine level as a function of time. Parallel with this decrease is the increase in the $P_n$ content. In addition, one finds an increase in the amount of sugar phosphate, from 3 to 15% for the time period from 10 to 400 min. It takes over 10 hours to deplete P-creatine in frog muscle, whereas the curve for $P_n$ rises throughout the experiment, reaching 72% value at the end of the experiment, 15 hours. The elevated sugar phosphate content of the muscle remains.

Fig. 2 shows that the ATP level remains constant until only a trace amount of P-creatine is present, about 500 min. ADP can be detected in the spectrum with certainty only after 750 min (not shown). It is noted that in Fig. 2 ATP is plotted according to its phosphate content, and thus its actual percentage concentration is only one-third that of P-creatine. The ATP content (6.3% of the total phosphate) corresponds to 2.8 mM ATP in the muscle water of the frog used.

In control experiments we measured the concentrations of ATP, P-creatine, and $P_n$ by conventional methods (6, 17-19). No change was found in the ATP content of muscles left in the NMR tube for several hours as compared to that of contralateral muscles which were analyzed immediately after killing the frog. On the other hand, the breakdown of P-creatine could be
demonstrated already after 20 min in which time a decrease of about 15% was noted. A closely equivalent increase in Pi was observed for the same interval. These data of chemical analyses show the same pattern as Fig. 2.

Not shown in Fig. 2 is the NAD content, which remained constant at the level of 1% throughout the 15 hours of incubation of dissected frog muscle. In addition, there were other phosphate fractions omitted from Fig. 2. In the course of these studies, we observed two resonances (-0.4 ppm and +0.2 ppm) between the signals of Pi, and P-creatine, particularly in the winter frogs (see Fig. 3). These resonances have not yet been matched positively with any common organic phosphorus compound. Nevertheless, we could measure their amount, which varied between 6 to 10% of the total phosphorus. Furthermore, it could be established that there was no change in the content of these unknown compounds under the conditions of Fig. 2.

The pattern of Fig. 2 for intact frog muscle resembles that of Fig. 4 of Hoult et al. (5) for the rat leg muscle. However, there is one significant difference: the lifetime of ATP and P-creatine in the frog at 28° is 5 to 6 times longer than in the rat at 20°. This difference may be related to the poikilothermic nature of the frog.

Distribution of Phosphate Metabolites in Various Muscles—Fig. 3 compares the spectra of various intact muscles. The gastrocnemius muscle of the North American winter frog shows the two unknown peaks described previously, which lie in a region normally assigned to phosphate diesters. Similar peaks are also found in the toad gastrocnemius, apparently in a rather large amount. The mantle muscle of abalone shows no outstanding difference as compared to the frog leg muscle (see Fig. 1). A closer examination of the peaks in abalone, however, reveals an upfield shift in the peak which would be normally assumed to be P-creatine. This will be discussed subsequently in connection with Fig. 4. Finally, Fig. 3 shows a human gastrocnemius muscle from a gangrenous leg. In this case, all the characteristic peaks of the phosphate compounds are visible, although their absolute amount is significantly reduced. The peak for P, is not only broadened but it also is split.

Further investigation on the composition of the abalone muscle was carried out on its Chelex-treated perchloric acid extract (Fig. 4). In the upper part of the figure from left to right, two small peaks (-4.8 ppm, and -4.1 ppm) for sugar phosphates, the large peak (-2.9 ppm) for Pi, and a small unknown peak (-1.6 ppm) are seen. The novel peak in this spectrum is the very pronounced signal at 3.2 ppm, which is followed by the multiple signals derived from the phosphate groups of ATP. If abalone and frog muscle extracts are mixed (lower part of Fig. 4), P-creatine is the only new peak which appears. Its position, 3.0 ppm in the perchloric acid extracts, clearly differentiates it from the novel peak in the abalone extract. This peak may be assumed to be arginine phosphate, since this is the only high energy storage phosphate known in molluscs (20).

An interesting difference between normal and dystrophic chicken muscle was also detected by 31P NMR. A typical experiment is shown in Fig. 5. In the upper part of the figure the phosphorus resonances from the normal chicken muscles are seen: a small amount of sugar phosphates and Pi, and a relatively high concentration of P-creatine and ATP. The lower part of the figure for the dystrophic muscle shows approximately the same concentrations for these compounds. However, a significant new signal (see arrow), between 0.4 to 0.5 ppm, appears in the dystrophic muscle. This result was reproduced in all the three experiments involving dystrophic and normal muscles.

Concentration of Phosphate Metabolites in Various Muscles 31P NMR offers a new method for simultaneous determination of the concentration of the most common phosphate compounds in muscle. Table I shows the results of a series of determinations averaged from 0 to 10 min after killing the animal. Since the NMR readily detects only signals from
The values were in the same range as given in Table I.

Our value for the ATP and P-creatine content of toad gastrocnemius (Table I) is close to that reported for toad sartorius (29). A higher level of ATP is commonly accounted in animals in a warm environment (23) and in Table I it is found that the ATP content of fast chicken muscles are in the same range as those given by Farrell and Olsen (24) and Malvey et al. (25). Furthermore, a statistically significant decrease in the ATP and P-creatine content of genetically dystrophic chicken muscle, as compared to the normal muscle, was reported by Farrell and Olson (24), which also appears in the data of Table I.

Our values for the sugar phosphates in Table I include hexose monophosphate, fructose 1,6-bisphosphate, glycerol 3-phosphate, 3-phosphoglyceric acid, and other triose phosphates. All of these compounds have resonances so close to one another that in intact muscle they must be determined as a single group, as it may be seen most clearly in Fig. 5. Nevertheless, our data are in the same range as those reported for some individual sugar phosphates in various muscles: 2 mM hexose monophosphate in the frog (21); 4.5 mM hexose monophosphate in ox (26); 5 to 9 mM glucose 6-phosphate, 0.8 to 0.9 mM fructose 6-phosphate, 3 to 4.5 mM fructose 1,6-bisphosphate, 2 to 2.5 mM glycerol 3-phosphate, and 0.3 to 0.4 mM triose phosphates in pig (27). Furthermore, it appears from Table I that sugar phosphates are elevated in the dystrophic chicken muscle.

A statistical analysis of the data, making up Table I, shows that 31P NMR is a satisfactory technique for determination of ATP and P-creatine in muscle. The standard error values compare favorably with those seen in classical methods of analyses. However, the error for sugar phosphates is rather large; this is caused by the broadness of the overlapping peaks.

Not listed in Table I are the following observations: Southern frogs contained more ATP than Northern ones, and they hydrolyzed their P-creatine at a more rapid rate. More ATP was found in rabbit fast muscle (extensor digitorum longus) than in slow muscle (soleus) or in the heart. The breakdown of P-creatine in the extensor muscle was rather rapid. More ATP was found in the dystrophic muscle, as compared to that in free solution. In the course of this study, we have followed this phenomenon systematically. Table II lists the constants for various muscles and animals. Comparable values for coupling constants of ATP in solution, corrected for some individual sugar phosphates in various muscles: 2 mM hexose monophosphate in the frog (21); 4.5 mM hexose monophosphate in ox (26); 5 to 9 mM glucose 6-phosphate, 0.8 to 0.9 mM fructose 6-phosphate, 3 to 4.5 mM fructose 1,6-bisphosphate, 2 to 2.5 mM glycerol 3-phosphate, and 0.3 to 0.4 mM triose phosphates in pig (27). Furthermore, it appears from Table I that sugar phosphates are elevated in the dystrophic chicken muscle.

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**Coupling Constants of ATP in Muscle**—In our previous work (6) we noted a difference in the coupling constants for both the α-β- and the β-γ-phosphate groups of ATP in the muscle as compared to that in free solution. In the course of this study, we have followed this phenomenon systematically. Table II lists the constants for various muscles and animals. Comparable values for coupling constants of ATP in solution, corrected for some individual sugar phosphates in various muscles: 2 mM hexose monophosphate in the frog (21); 4.5 mM hexose monophosphate in ox (26); 5 to 9 mM glucose 6-phosphate, 0.8 to 0.9 mM fructose 6-phosphate, 3 to 4.5 mM fructose 1,6-bisphosphate, 2 to 2.5 mM glycerol 3-phosphate, and 0.3 to 0.4 mM triose phosphates in pig (27). Furthermore, it appears from Table I that sugar phosphates are elevated in the dystrophic chicken muscle.

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**TABLE III**

*Coupling constants for phosphate groups of ATP in solution*

| Additions                           | pH | \(\delta_{P_i} \) | \(\delta_{P_j} \) |
|-------------------------------------|----|-------------------|-------------------|
| 1 mM EDTA and 0.1 mM K\(^+\)        | 7.5| 19.5              | 19.5              |
| 7.0                                 | 19.5| 19.5              | 19.5              |
| 6.5                                 | 19.5| 19.5              | 19.5              |
| 12 mM Mg\(^{2+}\)                  | 7.2| 16.0              | 15.0              |
| 6.8                                 | 16.0| 17.0              |
| 6.3                                 | 14.0| 15.0              |
| 12 mM Mg\(^{2+}\) and 0.1 mM K\(+\)| 7.2| 16.0              | 16.0              |
| 6.6                                 | 16.0| 14.0              |

Corresponding to the artificial milieu of muscle, are shown in Table III. These latter coupling constants were determined at various pH values in the physiological range. No systematic difference was found as a function of pH, although the values in the presence of EDTA are significantly higher than those with Mg\(^{2+}\). By comparing Table II with Table III, it is apparent that the coupling constants for ATP in the intact muscle are in the same range as those of the MgATP solutions. Even the highest value (17.0 in case of abalone) is significantly lower than that of ATP in the presence of EDTA which is 19.5.

Intracellular pH of Frog Muscle—Currently direct and indirect methods are used for measuring the value of pH in the muscle cell: insertion of microelectrodes may give the pH inside the muscle directly. Among the indirect methods, the distribution of DMO between the total water of the muscle and its extracellular space is the most popular, but the distribution of carbonic acid (CO\(_2\)) is also frequently applied for the estimation of intracellular pH (28). Direct intracellular pH measurement in rat and crab muscle gave conflicting results. For the rat a value as low as pH 6 was found by Carter et al. (29), as opposed to the pH of 6.7 of Paillard (30) although the discrepancy for the crab was much less, pH 7.2 (31) versus pH 7.0 (30). The question of identity of the pH values obtained by the microelectrodes in the deep and superficial layers of muscle cells was first raised by Paillard (30) and recently by Wiggins (32). Calculations of intracellular pH of skeletal muscles from cat, rat, dogfish, and human, and of heart muscles from dog and turtle by measuring the distribution of CO\(_2\) and DMO in muscle gave values which ranged from 6.6 to 7.6 (28, 33). In contrast to the detailed etude on mammalian muscles, determinations of frog muscle are rather scarce. With intracellular electrodes, Kostyuk and Sorokina (34) measured a pH of 7.12 in frog sartorius, whereas with the DMO technique Izutsu (35) has found 7.16 in bullfrog toe muscle.

We have used \(^{31}P\) NMR for the determination of intracellular pH in frog muscle. Moon and Richards (36) were the first to show how the titration of model compounds can lead to an estimate of intracellular pH in erythrocytes. In Fig. 6 we show an artificial mixture (A) of the relevant organic phosphates and inorganic ions present in the plasma of frog muscle, in which we measure the chemical shifts of the various phosphates as a function of pH. It should be pointed out that at each pH there is a separate chemical shift for each phosphate metabolite; this is the reason for the several curves in Fig. 6. In the physiological pH range there is no shift change in the resonances of either the \(\gamma\)-phosphate of ATP or P-creatine in the presence of magnesium. Upfield shifts become apparent for ATP with magnesium only below pH 6.0 and for P-creatine below 5.5. On the other hand, \(P_i\) and Fru-1,6-P\(_2\) exhibit upfield shifts from pH 7.5 to 5.0. This titration behavior of \(P_i\) and Fru-1,6-P\(_2\) makes them suitable for use in determining the intracellular pH.

In order to study the various parameters which may affect the chemical shift we have modified the artificial mixture as described in the legend of Fig. 6. Omission of KCl (Mixture B) has no effect on the shape and position of the curves. Omission of Mg\(^{2+}\) by the addition of EDTA (Mixture C) leads to profound differences in the position and shape of the titration curve for ATP and smaller changes in P-creatine and Fru-1,6-P\(_2\), whereas \(P_i\) remains insensitive. Because P-creatine comprises the main part of the phosphates in muscle, a separate experiment was carried out to examine the effect of Mg\(^{2+}\) on P-creatine alone at neutral pH (Mixtures D and E in Fig. 6). The points from this experiment lie on the respective titration curves (12 mM Mg\(^{2+}\) or EDTA) mentioned above. From this finding it can be concluded that P-creatine does not contribute significantly to the chemical shifts in the total artificial mixture of muscle. The main conclusion which can be drawn from the data of Fig. 6 is that, of all the metabolites studied \(P_i\) should be the most reliable indicator of intracellular pH.

Not shown in Fig. 6 are our results of the effect of Ca\(^{2+}\) on the model compounds studied. In these experiments the titration mixture contained 3 mM ATP, 20 mM P-creatine, 2 mM Pi, 2 mM Fru-1,6-P\(_2\), and 0.1 mM EDTA, and 12 mM Mg\(^{2+}\), the same as the basic mixture used in Fig. 6. The Ca\(^{2+}\) concentration was 2.5 mM which is the upper limit found in frog and other muscles (15, 37). The pH...
titration was carried out between pH 6.5 and 5.5, under conditions where Ca\(^{2+}\) does not form a precipitate with phosphates. The change in chemical shift for CaATP was appreciable already at 6.5 and became more pronounced with decreasing pH. In this range MgATP showed much less change (Fig. 6). This difference in the magnetic resonance pH profile between MgATP and CaATP is in agreement with the higher affinity constant of MgATP as compared to CaATP (38). Cohn and Hughes (39) have also observed a difference in the chemical shifts of MgATP versus CaATP as a function of pH. Concerning other phosphate metabolites, the curve for P1 was not affected by Ca\(^{2+}\) (in accord with that for Mg\(^{2+}\)). Fru-1,6-P2 showed the same titration curves as Mixtures A and B in Fig. 6, whereas P-creatine possessed values closer to that of Mixture C.

Fig. 7 shows that in the intact muscle the chemical shifts of the \(\gamma\)-phosphate of ATP, P-creatine, P\(_3\), and Fru-1,6-P\(_2\) correspond to those shown in Fig. 6 (with the exception that the sugar phosphates in the muscle represent the superposition of several peaks). The shifts were analyzed for four Northern and two Southern frogs. The initial chemical shift of the \(\gamma\)-phosphate group of ATP lies in the region of 5.6 to 5.8 ppm which places ATP in the muscle into the MgATP class of the model system (see Fig. 6). Furthermore, the lack of any appreciable change in the shift over a period of 600 min, which undoubtedly leads to rigor and thus acidification under anaerobic conditions, also corresponds to the insensitivity of the chemical shift of MgATP in the model system between pH 7.5 and 6.0. P-creatine shows essentially the same value in the intact muscle as it does in the model titrations. For the first 3 hours the shifts of sugar phosphates are constant after which there is a continuous upward migration.

The chemical shift in case of P\(_1\) shows a steeper slope for the Northern frogs than for the Southern ones. The data for the two Southern frogs fall on the same straight line, whereas for the Northern frogs one line can be drawn for three frogs and another for the fourth.\(^1\) In our detailed analysis, actually, a line was drawn for each animal; by extrapolating these lines back to zero time the chemical shifts for five frogs laid between -2.0 and -1.8 ppm while one was -1.6 ppm. From the shifts for P\(_1\) in Fig. 6 the pH values corresponding to these chemical shifts in the intact muscle were estimated. For the six frogs examined these values were 6.95, 7.18, 7.18, 7.25, 7.30, and 7.40, averaging to a pH value of 7.2 in the fresh intact frog muscle. Since chemical shifts for P\(_1\) were measured on sharp peaks there can be no systematic errors in this pH value.

**DISCUSSION**

\(^{31}\)P nuclear magnetic resonance spectra recorded from intact muscles showed resonances for ATP, P-creatine, P\(_3\), and the sugar phosphates. These metabolites can be quantitated in good agreement with the chemical analyses. Moreover, \(^{31}\)P NMR enables the simultaneous determination of the phosphate compounds on a single intact muscle without the extensive precautions which are necessary to avoid breakdown of phosphocreatine in such destructive methods as freeze-clamping. The simplicity, accuracy, and comprehensiveness of this method is very attractive.

Most of our \(^{31}\)P NMR measurements were carried out on frog muscles for relatively short experimental time, so that the

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\(^1\) One line of the Northern frogs delineates after 600 min of aging.
of identification were also found. In free solution, there is a definite change in the coupling constants of ATP in the presence of Mg$^{2+}$ (Table III). The coupling constants of ATP measured in various live muscles (Table II), closely correspond to those for the MgATP in solution (Table III). The γ-phosphate group of free ATP undergoes a chemical shift over 3 ppm in the pH range 7.5 to 6.0, while the same group in MgATP shows little change (Fig. 6). This is because free ATP has a pK of 6.95 and hence undergoes a characteristic sigmoidal shift in the region of pH 7 of the titration curve (Fig. 6) while MgATP has a pK of 5.18 and will not show appreciable changes in shift until below pH 6 (41). The pH-induced chemical shift can be used to distinguish MgATP from CaATP since the latter enters its transition region at a higher pH than does the magnesium complex. In the muscle, the γ-phosphate group of ATP exhibits no shift change over a period of 10 hours (Fig. 7) when the muscle reached the state of rigor and the consequent lowering of pH to the neighborhood of 6. This provides additional proof for MgATP in the muscle. The idea that ATP is magnesium-bound in the muscle has persisted for a long time. From the frequencies of the ATP peaks in the muscle, Hoult et al. (5) have also suggested that ATP in the muscle forms a complex with magnesium ions.

We have shown that $^3$P NMR is a reliable method to determine the pH in intact muscles. This is based on the correlation between the chemical shift of all significant phosphate compounds and the pH of the medium in several relevant solutions. Under all conditions only inorganic phosphate gave an invariable titration curve (Fig. 6). From the extrapolated value of the chemical shift for P$_i$ at zero time, the pH within the frog muscle was estimated to be 7.2. This value agrees with that reported by two other methods (34, 35).

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