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STRUCTURAL CHANGES IN MYOSIN DURING CONTRACTION AND THE STATE OF ATP IN THE INTACT FROG MUSCLE

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The reactivity of myosin to [^{14}C]-labeled N-ethylmaleimide ([^{14}C] NEM) or to tritium was determined in functionally different frog muscles. The incorporation of [^{14}C] NEM into myosin decreased during isotonic or isometric contractions, as compared to resting muscle. The cysteine residues which were protected during contraction were not involved in the ATPase activity or the actin-binding ability of myosin. Peptide mapping revealed that several residues were protected simultaneously. The incorporation of tritium into the peptide N-H groups of myosin was also decreased during muscle activity. These data support the idea that activation and subsequent contraction of muscle are correlated with structural changes in the myosin molecule.

The reactivity of myosin to [^{14}C] NEM was increased when the muscle was stretched to 140% rest length and treated with iodoacetate to deplete ATP. Based on in vitro experiments and on literature data, it is suggested that in the resting muscle myosin contains bound MgATP which decreases the rate of incorporation of [^{14}C] NEM into myosin and that upon the irreversible loss of ATP the rate increases.

^{31}P nuclear magnetic resonance signals from a number of phosphates were detected in the intact frog muscle. The data indicated that the minimum concentration of ATP in the muscle is 3 mM, a value which agrees with that of chemical determination. The characteristic chemical shifts, coupling constants, and line widths of ATP in the muscle were considerably altered from that of either free ATP in aqueous solutions or ATP in perchloric acid extracts of muscle.

INTRODUCTION

A natural goal of muscle biochemistry is the investigation of the molecular mechanism of contraction at the level of intact muscle. Experiments on this line became feasible with the extensive knowledge which has been accumulated during the past two decades about the structure and activity of contractile and regulatory proteins in vitro. The properties of myosin are so unique that they can be used to study changes in the structure of this protein during muscle activity.

At physiological ionic strength and pH, the reactivity of myosin to [^{14}C]NEM * is markedly influenced by ATP (Fig. 1A). The rate of incorporation of [^{14}C]NEM into myosin in the presence of MgATP is considerably reduced as compared to that of "None" addition. In contrast, when the bivalent cations are complexed with EDTA, ATP enhances the rate about two fold. These effects require the native structure of myosin, since the dual response to ATP is abolished after a mild denaturation of myosin with KI (Fig. 1B).

Both the protecting effect of MgATP and the activating effect of free ATP on the [^{14}C]NEM incorporation are localized in the globular head of the myosin molecule (Fig. 2). This finding fits well to a substrate-induced conformational change, since the head section of myosin carries the ATPase site.

Another indication of the specificity is that incorporation of [^{14}C]NEM into several ATP-utilizing enzymes, other than myosin, is not modified by ATP.

[^{14}C]NEM is a small, neutral molecule which penetrates into the muscle water. When used at 0.1 mM concentration, it has only a negligible effect on the contractility of frog muscle at 2°C for about 1 hr (1–3). Therefore [^{14}C]NEM can be used as a probe for studying changes in the reactivity of myosin during muscle contraction.

Recent studies in these laboratories involving a number of biological systems (human circulating lipoproteins (4), lipopolysaccharides, high molecular weight bacterial condensed phosphate (5), phosphohexosans, human erythrocytes (6), human sperm, yeast, rat brain) have shown that ^{31}P nuclear magnetic resonance spectra of high information content could be obtained from large macromolecular structures and intact cells or tissues (7). In these spectra it was possible to identify ^{31}P resonances arising from discrete molecular species and to observe changes in these signals which correlated with the metabolic state of the sample. Information relating to the environment of these molecules in the intact, undisturbed systems could also be obtained from the spectra. These observations suggested that similar studies involving intact muscle would also yield useful information, and in this paper our initial findings regarding the application of this method are reported.

METHODS

^{31}P Nuclear Magnetic Resonance (7, 8)

The spectrometer employed was a Bruker HFX-5 operating at 36.43 MHz for ^{31}P and 90 MHz for ^{1}H and containing modules for gathering Fourier transform (4) ^{31}P NMR spectra and broad-band decoupling of protons. Ten-millimeter spinning sample tubes were used containing 2.5–3.0 ml samples, and from 10 min to 2 hr of signal-averaging time were required for each spectrum. The probe temperature was 28°C.

Chemical shift data are reported relative to the usual standard of 85% inorganic orthophosphate (8); however, the primary standard was a capillary (1 mm diameter) containing 1.0 M methylenediphosphonic acid, $(HO)_2OPCH_2PO(OH)_2$, in D_2O (pD = 9.5, Na^+ counter cation) coaxially mounted in the sample tube. Stablization of the magnetic field was achieved through the deuterium signal from the capillary; the phosphonic acid

*Abbreviations: [^{14}C]NEM, N-ethyl-[1-^{14}C] maleimide; PCA, perchloric acid; THO, tritiated water.

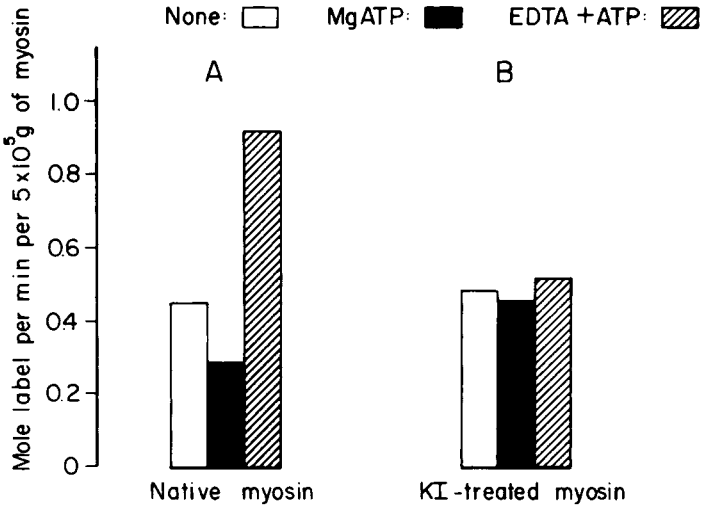

Fig. 1. Rate of incorporation of [^{14}C] NEM into rabbit skeletal myosin. Myosin, 1.0 mg per ml, suspended in 50 mM KCl and 25 mM potassium phosphate buffer, pH 7.0, was treated with 10 moles of [^{14}C] NEM per 5 × 10^5 g of myosin in the presence of the following additions: "None", 50 mM KCl; MgATP, 10 mM $MgCl_2$ and 5 mM ATP; EDTA + ATP, 1 mM EDTA and 5 mM ATP. After stirring at 25°C for 1 min, the reaction was stopped by the addition of 400-fold molar excess of cysteine over [^{14}C] NEM and the myosin was isolated after six reprecipitations from 0.6 M KCl. The determination of incorporation was described (1). "KI-treated myosin" was prepared by incubation in 1.0 M KI at 25°C for 1 hr. The myosin was freed from KI by repeated reprecipitations and then treated with [^{14}C] NEM as described before.

signal (−16.55 ppm) served as the reference for the relative area measurements. The capillary was calibrated against a 3 ml vol of 0.02 M solution of Na_2HPO_4. This volume was used in the determination of the muscle phosphate levels and insured 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. The usual (7, 9, 10) ^{31}P NMR procedures were used for the analysis of the PCA extracts (adjusted to pH 7 with NaOH) and the EDTA-treated (7) PCA extracts.

Other methods are described in figure and table legends.

RESULTS AND DISCUSSION

Change in the Incorporation of [^{14}C] NEM into Myosin during Muscle Contraction

Figure 3 shows the set-up for the muscle experiments. The paired semitendinosus muscles of the frog, Rana pipiens, are incubated under oxygen in Ringer's solution containing [^{14}C] NEM, at 2°C, for 30 min. During the [^{14}C] NEM treatment one muscle is stimulated 25 times per min with a duration time of 1 msec at 25 V (Model 104-A stimulator of the American Electronic Laboratories, Inc.) under a load of 1.5 g, while

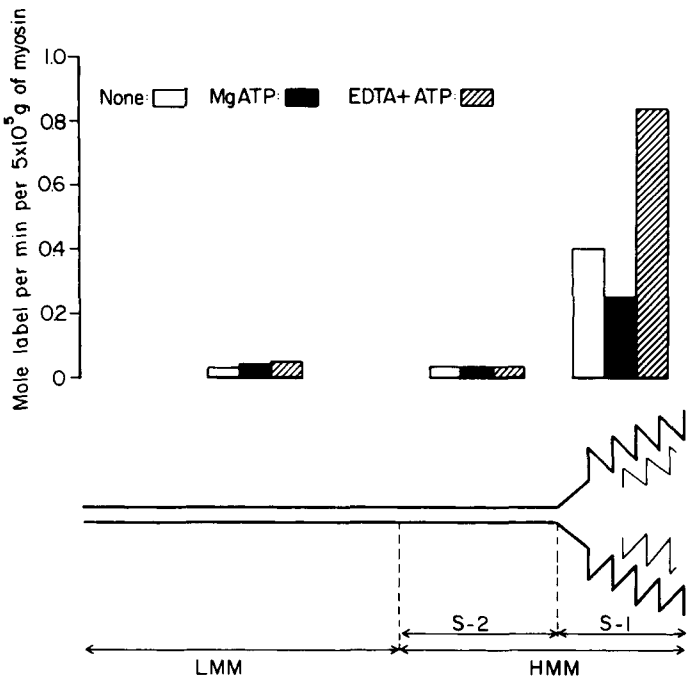

Fig. 2. Distribution of label in the substructure of rabbit skeletal myosin after treatment with [^{14}C]NEM in the presence of various additions. The "native myosin" treated with [^{14}C]NEM, as described in the legend to Fig. 1, was used for the preparation of the proteolytic subunits of myosin (1).

the other muscle is resting under the same load. At the end of the experiment unreacted [^{14}C]NEM is removed from the muscle by repeated washings with normal Ringer's solution. Finally, myosin is isolated from each muscle and its radioactivity determined. In these experiments less label was found in myosin of contracting muscle, as compared to the myosin of resting muscle (1–3). This indicates that the reactivity of myosin to [^{14}C]NEM decreases during muscle contraction.

In order to determine the distribution of label in the substructure of myosin, we pooled the myosin from several [^{14}C]NEM-treated resting or contracting muscles, prepared the proteolytic fragments from both types of myosin, and determined their specific radioactivity. Figure 4 shows that most of the label is in the head of the myosin molecule and only a little label is in its tail. Furthermore, the decreased incorporation during contraction is localized entirely in the head. This 25% decrease correlates with the 21% of the total time which is spent by the stimulated muscle in the shortening phase of the contraction cycle. Since in the structure of muscle the head corresponds to the crossbridges and the tail to the backbone of the thick filaments, these results indicate that during muscle contraction the reactivity of crossbridges is changing, whereas the reactivity in the backbone of the myosin filaments remains the same.

The decreased incorporation of [^{14}C]NEM into the myosin-head of contracting

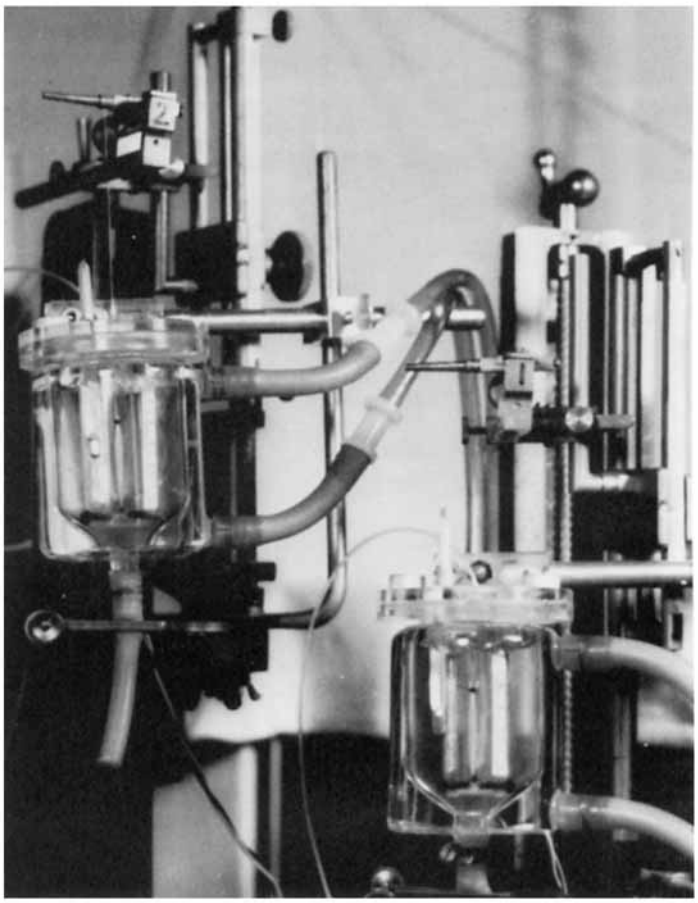

Fig. 3. The set-up for muscle experiments. For details see text and reference (2).

muscle (Fig. 4) resembles that caused by MgATP in pure myosin (Fig. 2). However, in the working muscle actin also interacts with the crossbridges in addition to MgATP. We were looking for evidence to demonstrate that MgATP, or actin, or both are protecting the "active sites" of the crossbridges from reaction with [^{14}C]NEM during contraction. In this case one would expect a higher ATPase activity, actin-binding ability, or both in myosin of contracting muscle compared to the resting one. No difference in any of these properties between myosins of contracting and resting muscle was found (1). Thus, it appears that the sulfhydryl groups which are protected during contraction are not in the active site area of the myosin-head.

For further clarification of the reason for the decreased reactivity, we compared the radioactive peptides from myosins of the two different muscles (Fig. 5). Four to five peptides from contracting muscle show less label than the corresponding peptides from resting muscle, indicating that the decreased reactivity is spread to four to five cysteine

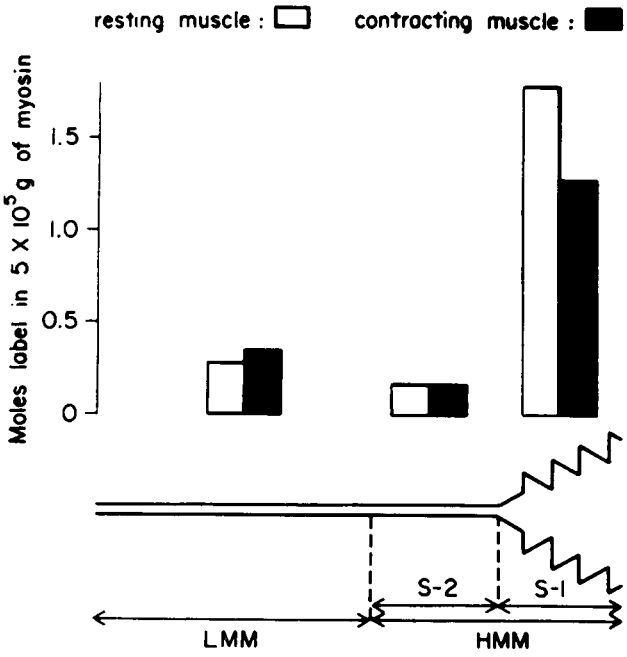

Fig. 4. Distribution of label in the substructure of myosin of resting and contracting frog muscle. For details see Bárány and Bárány (3).

residues. In total, 11–14 sulfhydryl groups are labeled per mole of myosin in the muscle; this large number shows clearly that not a specific but rather an overall reaction takes place. Apparently in the resting muscle many cysteine residues, on the surface of the myosin-head, are available for reaction with [^{14}C]NEM; during contraction some of these groups move from the surface to the interior of the protein thereby making them inaccessible to [^{14}C]NEM.

The Reactivity of Myosin to [^{14}C] NEM in Functionally Different Muscles

The reactivity of myosin to [^{14}C]NEM is dependent on the functional state of the muscle (Fig. 6). The reactivity, compared to the resting state, is generally decreased but an increase is also found. Maximal decrease occurs during isotonic contraction, followed by isometric contraction, and rigor. An increased reactivity is found when the muscle is stretched to 140% rest length and treated with iodoacetate to deplete ATP in the muscle. However, no change in the reactivity was detected when the muscle was stretched to 140% length and stimulated, that is, under conditions when the actin filaments could not interact with the myosin filaments (2, 3).

From these data it appears that the simultaneous interaction of actin, ATP, and the myosin-head, which occurs in muscles contracting isotonically or isometrically, is

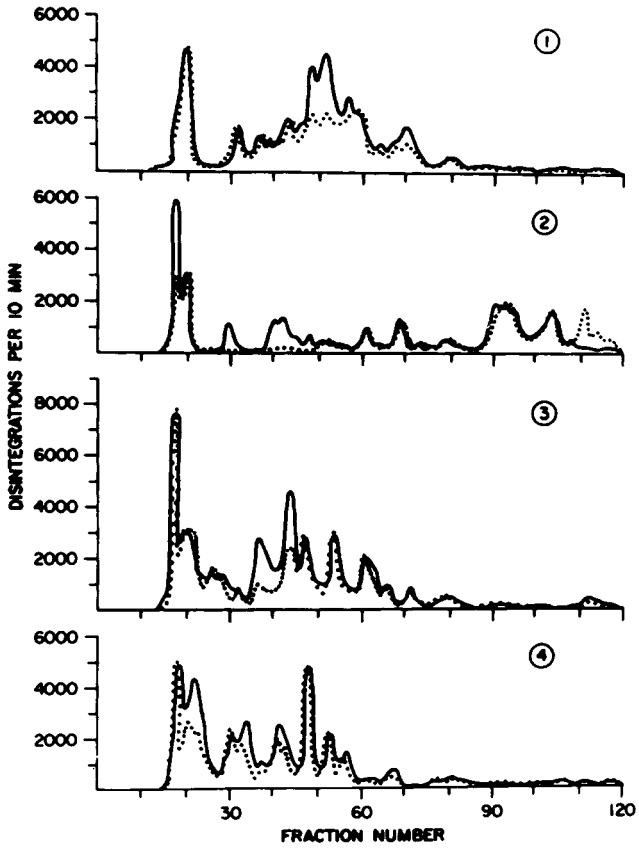

Fig. 5. Dowex-50 chromatography of myosin of resting (——) and contracting (- - - -) gastrocnemius muscle, digested with the following proteolytic enzymes: 1, trypsin; 2, pepsin; 3, pepsin-trypsin-chymotrypsin; 4, subtilisin. For details see Bárány et al. (1).

necessary to produce a full decrease in the reactivity of myosin with [^{14}C]NEM. The increased incorporation in stretched and iodoacetate-treated muscle reveals that in the resting muscle the reactivity of myosin to [^{14}C]NEM is not maximal. This will be further discussed in the section, "Interaction of Myosin with ATP in the Intact Muscle."

The Reactivity of Myosin to Tritium in Functionally Different Muscles

[^{14}C]NEM is a specific –SH group reagent and has the limitation that changes only in the environment of cysteine side chains can be detected. However, the exchange of hydrogen atoms in proteins with the tritium in the medium may be used to follow possible functional changes in the entire protein molecule.

Tritiated water is essentially an ideal reagent for studying the mechanism of contraction, since 80% of the muscle cell is water. With the high sensitivity of tritium determination, it is enough to label only 1 of 10^8 hydrogen atoms in the Ringer's solution

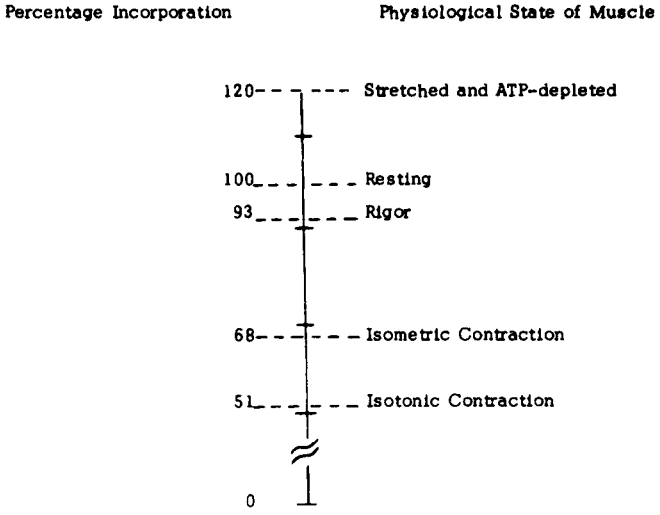

Fig. 6. Levels of incorporation of [^{14}C]NEM into myosin of functionally different muscles. For details see Bárány and Bárány (3).

bathing the muscle, thus avoiding any side effect of the isotope on the contractile properties of the muscle.

There are a few problems which have to be solved for quantitation of the rate of tritium incorporation into proteins of intact muscle. The first is the rapid stopping of the hydrogen-tritium exchange. This was achieved by dropping the muscle into a large volume of dry acetone (11), a procedure which immediately removes the intracellular water containing free tritium but leaves the protein-bound tritium in the dehydrated muscle. The second problem is the separation of the tritium incorporated into the protein side chains from the tritium which has exchanged with the protein peptide N-H groups. The former class of tritium is bound in large quantities and nonspecifically, whereas the incorporation of the latter class of tritium depends on the functional state of the muscle (11). In our method, advantage was taken of the great differences in the rates of exchange of the two classes of tritium with the hydrogen in the medium (11). Thus, the acetone-dried muscle was homogenized in a dilute salt solution, then centrifuged, leaving in the supernatant all of the rapidly exchangeable tritium (derived from protein side chains), while the residue contained the slowly exchangeable tritium (derived from peptide N-H groups). This procedure is outlined in Fig. 7. It may be seen that the sarcoplasmic proteins of the tritiated muscle also remain in the supernatant, so that, ultimately, one isolates the myofibrillar proteins labeled with tritium in their peptide hydrogen.

Table I shows the percentage rate of tritium incorporation into the peptide N-H of myofibrils of functionally different muscles. The rate decreases as a result of muscle stimulation. The most pronounced difference is found during isotonic contraction. A significant difference also occurs during isometric contraction, and a decrease is detectable in stretched and stimulated muscle.

The question may now be raised as to which component of the fibrils is responsible

TABLE I. Incorporation of Tritium into the Peptide N-H of Myofibrillar Proteins of Functionally Different Frog Semitendinosus Muscles as Compared with Resting Muscles

Functional state of muscle	Pairs of muscle	Percentage tritium incorporation
Isotonic contraction	15	82.7 ± 2.9 ($p < 0.001$)
Isometric contraction	25	86.9 ± 2.7 ($p < 0.001$)
Stretched to 140% rest length and stimulated	30	92.7 ± 2.1 ($p < 0.005$)
Resting (control)	23	97.7 ± 2.7 (no significant difference)

Muscles were incubated in Ringer's solution containing 15 μCi of THO per ml, at 2°C, for 30 min. During the incubation period one of the paired muscles was stimulated 30 times per min, while the other muscle was resting. The 100% tritium content varied between 424 and 496 moles of tritium per 5×10^5 g of myofibrillar proteins.

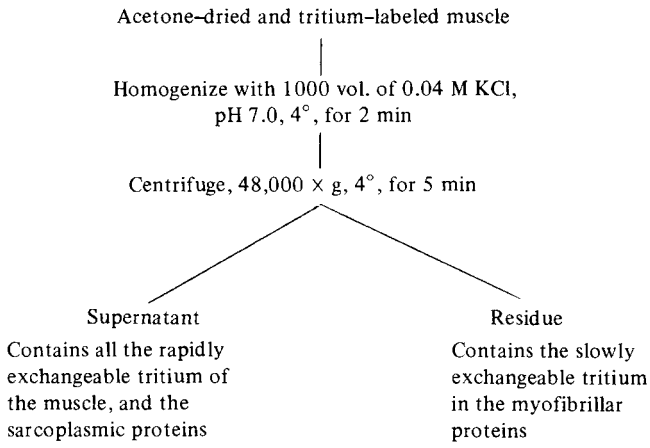

Fig. 7. A scheme for the separation of rapidly and slowly exchangeable tritium of dehydrated muscle.

for this decrease? In order to obtain an answer, however, we must solve the "third problem," that is, the fractionation of myofibrillar proteins in anhydrous media. The following organic solvents were found to be suitable (11): dimethylsulfoxide for actin; a mixture of acetone and formic acid, 5 : 1 v/v, for the combined regulatory proteins tropomyosin-troponin; and propionic acid for the myosin light chains. From the tritium content of these proteins and from that of the original myofibrils one can also calculate the incorporation of tritium into myosin. Hence, one can measure the tritium incorporation into the individual proteins of myofibrils.

Figure 8 shows the specific changes in the rate of tritium incorporation, moles tritium per 30 min per 10^5 g of protein, into each of the peptide N-H groups of myosin, actin, and tropomyosin-troponin of stimulated muscles as compared to that of the same entities from resting muscles. (For myosin light chains no change in incorporation was found.) During isotonic and isometric contractions the rate of incorporation decreases for both myosin and actin, whereas for tropomyosin-troponin it increases. In stretched and stimulated muscle the change is restricted to one protein, myosin, which exhibits a decreased incorporation. By comparing the extent of changes in the various proteins, the greatest differences are found in myosin. The following numbers illustrate the changes, in terms of moles of hydrogen atoms per 30 min per mole of myosin: 30–35 for stretched and stimulated muscles; 55–60 for isometric contraction; and 72–85 for isotonic contraction.

From the data of Fig. 8 the following conclusion may be drawn. Upon stimulation of the muscle, a rearrangement in the backbone of the myosin molecule takes place, and consequently the peptide N-H becomes sterically shielded from the solvent. Thus, the ease with which peptide hydrogens may hydrogen bond with THO in the medium will be reduced proportionally. These effects are amplified in muscles which are stimulated and are allowed to develop tension or to shorten. The realignment of so many hydrogens in the primary structure of myosin, as a result of muscle activity, supports the idea that conformational changes occur in myosin during muscle contraction (1–3). Localization of the altered tritium incorporation in the subunits of myosin would be of great interest; this would require proteolytic digestion of myosin in the absence of water.

Figure 8 also demonstrates the dynamic changes which occur in the structure of several myofibrillar proteins in activated muscle. The merit of the tritium exchange technique is that it is capable of measuring the extent of this submolecular motion.

The Interaction of Myosin with ATP in the Intact Muscle

Unexpectedly, the reactivity of myosin to [^{14}C]NEM was increased in stretched and ATP-depleted muscle, as compared to stretched and ATP-containing muscle (Fig. 6). Following this finding, we investigated the effect of stretch on [^{14}C]NEM incorporation into myosin of normal fresh muscle; no dependency on muscle length was found. Hence the conclusion was affirmed that in resting muscle, ATP per se decreases the rate of covalent binding of [^{14}C]NEM to myosin, and upon an irreversible loss of ATP, as in the stretched and iodoacetate-treated muscle, the rate increases.

In vitro experiments with isolated frog myosin, at physiological ionic strength and pH, demonstrated that the decreased incorporation of [^{14}C]NEM is absolutely specific for the presence of MgATP (2). Therefore, it was suggested that in the resting muscle myosin contains bound MgATP. The alternative hypothesis for a myosin-ADP complex in muscle (12) was not borne out by our analytical determinations, which showed that all the ADP found in fresh muscle can be accounted for exclusively as bound to actin (2, 3).

In Table II we compare our data for the ATP and ADP content of frog semitendinosus with those published in the literature for frog and other muscles. Good agreement may be seen. Both the low ADP contents and the high ATP/ADP ratios support our previous suggestion that ATP is the nucleotide which is bound to myosin in the resting muscle (2, 3).

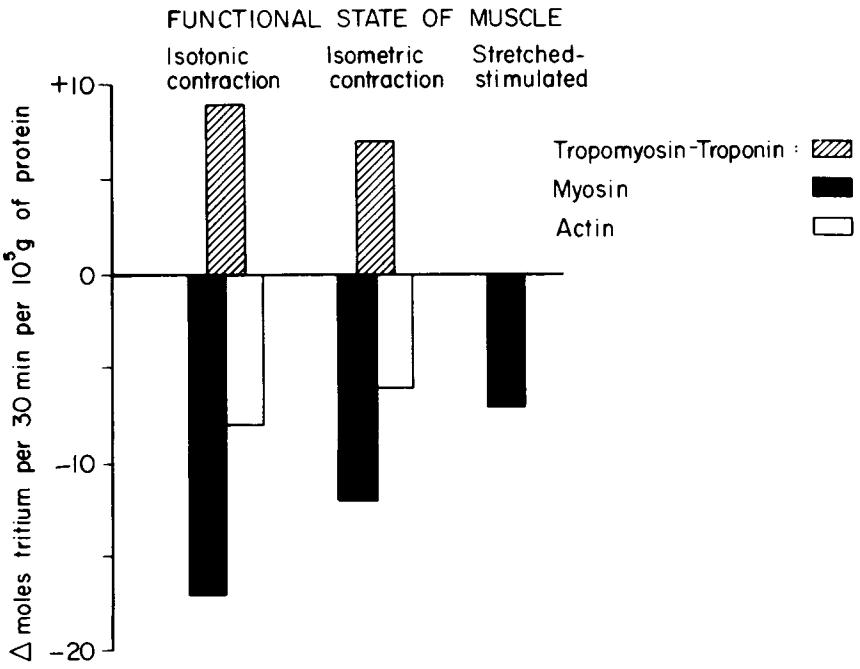

Fig. 8. Changes in the rate of tritium incorporation into the peptide hydrogen groups of various myofibrillar proteins of functionally different frog semitendinosus muscles as compared with resting muscles. The incubation of the muscles in Ringer's solution containing THO and their stimulation are described in the legend to Table I. Determination of tritium incorporation into the proteins is described in the text and in reference (11). The average data from about 15 experiments are shown for each protein.

ATP interaction and ATP content of intact frog muscle, as studied by ^{31}P NMR, will be described in the next section.

^{31}P NMR Spectra of Intact Muscle and PCA Extracts

Figure 9 shows proton broad-band decoupled spectra obtained from intact frog gastrocnemius muscle, a PCA extract of another fresh sample of this muscle, and the PCA extract after the addition of EDTA (7). The signals, from left to right in the muscle spectrum, have been assigned as follows: the external methylenediphosphonate reference compound, −16.55 ppm; the resonance band of the sugar phosphates (orthophosphate monoesters) of which the predominant signal is attributed to fructose-1, 6-diphosphate, −3.75 to −2.64 ppm; inorganic orthophosphate, −1.73 ppm; two signals in the orthophosphate diester region which have not been positively identified but which show proton coupling in proton-coupled spectra, which do not shift with changing pH within the physiological pH range, and which do not appear to coordinate with polyvalent cations (7) (see the PCA spectra); −0.48 and 0.10 ppm; creatine phosphate, 3.28 ppm; the γ, 5.57 ppm; α, 10.72 ppm, and β, 19.05 ppm phosphate groups of ATP. In addition, a small shoulder is often observed on the high-field side of the α group of ATP which has several spectral features of the signal from NAD (P, P′ diesterified pyrophosphates, see PCA extract with EDTA).

Except for the absence of the reference signal these same resonance bands are observed in both of the PCA extract spectra. Because treatment with EDTA improves resolution (7), it is possible in this case to detect additional minor resonances which are observable, but difficult to discern in the muscle spectrum and are obliterated in the PCA extract spectrum. For example, the characteristic ab multiplet from ADP is resolved and appears as the minor resonances overlapping the γ group multiplet of ATP on the high-field side and the α group multiplet of ATP on the low-field side. In the subsequent discussion, only the resonances of the condensed phosphates, ATP and ADP, will be considered. A detailed analysis of the entire phosphorus profile from muscle, including time-course data, effects of various treatments, and comparisons of several muscle types will be reported in a forthcoming publication.

The ATP spectrum of the EDTA-treated extract is characteristic (8, 13) of decoupled ATP in neutral aqueous solution, and alterations in this multiplet pattern can be interpreted in terms of interactions between ATP and other factors in the medium (14–16).

In comparing ATP spectra from the three types of samples, several differences were observed. It must be recognized, however, that in considering such changes in ^{31}P spectra of phosphates, several factors may affect any given spectral parameter. Three parameters which have been shown (10) to be important for condensed phosphates are pH, the nature of the counter cation, and the ionic strength of the medium. Each of these may result in chemical shift changes as large as 5–7 ppm, line broadening to the extent of obliviating the signal, and orders of magnitude changes in T_1 relaxation times. They have even resulted in coupling constant changes approaching 50%. Interaction with proteins may also result in large spectral changes (6, 16).

In the muscle and the neutral PCA extract, the chemical shifts of all three phosphate signals are displaced downfield relative to those of the EDTA-treated PCA extract. In the muscle the displacements are as follows: γ, -0.41 ppm; α, -0.16 ppm; β, -1.78 ppm. In the untreated PCA extract these displacements are: γ, -0.47 ppm; α, -0.25 ppm; β, -1.95 ppm.

A pH vs. shift study in the muscle, which will be reported elsewhere, indicated that these downfield shift changes do not result from pH effects. A more likely possibility is that the downfield shifts result from interactions between ATP and alkaline earth cations such as Mg^{++} or Ca^{++}. Such spectral changes have, in fact, been observed for the interaction of ATP with these ions. At this stage in the investigation, it is not possible to distinguish between these two cations. The PCA extract contains both ions, and the shift effects observed reflect the equilibrium between these ions and ATP. Since differences are observed in the muscle as compared with the untreated PCA extract, at a minimum this equilibrium condition has been modified. Interactions with protein and ionic strength effects must be contributing to the total shift change, but the magnitude of each contribution is unknown at this time.

The most striking feature of these spectra, other than the fact that signals from discrete molecules can be obtained from an intact organ, is the marked differences from one spectrum to another in the line widths of the individual signals (width at half-height, $\nu_{1/2}$). Consistent with previously reported results on condensed phosphates (10), phosphate spectra obtained from samples with excess EDTA show sharp, well-defined signals as seen in the EDTA-treated PCA extract. The interactions with divalent ions broaden these

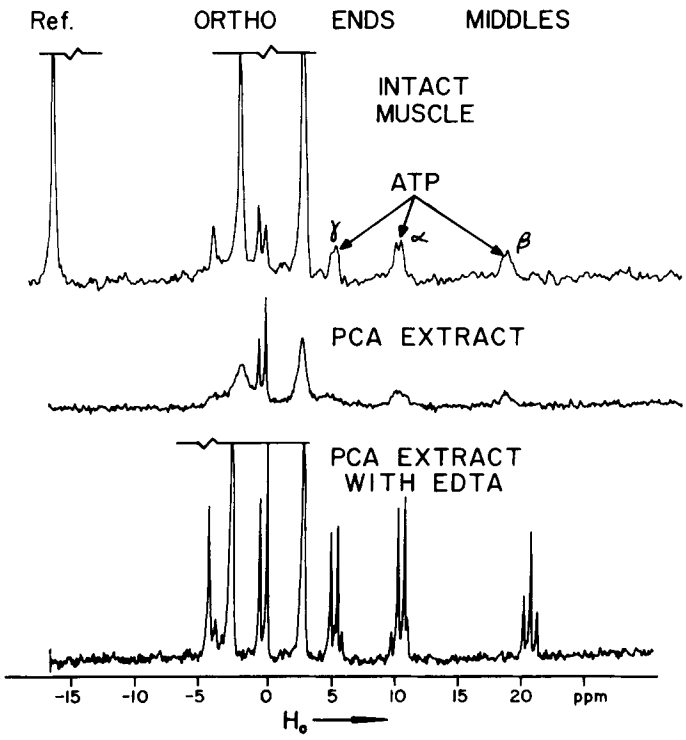

Fig. 9. ^{31}P NMR spectra from fresh, intact frog gastrocnemius muscle, a perchloric acid extract of fresh frog muscle mince adjusted to pH 7 for ^{31}P analysis (Na^+ counter cation), and the above perchloric acid extract to which sodium EDTA had been added (0.2 M, pH = 7). The gastrocnemius muscle of the frog, Rana pipiens, was dissected at 2°C and placed in the NMR tube and shaken to the bottom to remove entrapped air. The sample was covered with a Teflon plug and the reference capillary inserted through this plug into the sample. Gastrocnemius muscles from other frogs were minced at 2°C, and treated immediately with 0.1 vol of 60% perchloric acid. After a thorough mixing in ice, the extract was separated from the residue by high speed centrifugation at 0°C, and neutralized with KOH. After removal of the perchlorate precipitate, the extract was evaporated to near-dryness, dissolved in a small volume of water, filtered, and adjusted to pH 7 with NaOH. The EDTA-treated extract was prepared by adding solid trisodium EDTA (Sigma Chemical Co.) to the sample so that the final concentration was 0.2 M, and the pH was similarly adjusted to 7. The spectra were gathered through the use of Fourier transform technology (4) – sweep width, 2,500 Hz; cycling time, 832 msec; 4,096 data points per free induction decay; temperature, 28°C – and the characteristic regions of the phosphate ^{31}P spectrum are indicated in the figure. Ref. is the position of resonance of the intensity reference, methylenediphosphonate, in the phosphonate region of the ^{31}P spectrum. Signal-averaging processes were used, and this muscle spectrum was gathered over a period of 90 min. Also, a filter time constant introducing 4.66 Hz line broadening was used. Much less time was required to obtain the other two spectra, and little or no filter time constant required. In the figure, positive chemical shifts are associated with increasing magnetic fields, as is customary in ^{31}P NMR, with 0 ppm being the resonance position of 85% inorganic orthophosphoric acid; the prominent resonances have been truncated for the purpose of the illustration.

signals extensively, as is observed with the untreated PCA extract. The $\nu_{1/2}$ values of the signals obtained from muscle appear to be approximately midway between those of the extensively coordinated (untreated PCA extract) and totally uncoordinated phosphates (EDTA-treated extract). The α and β groups appear to be affected to approximately the same degree while the γ group appears to show relatively less broadening.

The decreased $\nu_{1/2}$ values in muscle relative to those from the untreated PCA extract probably do not result from altered hydrogen ion concentration or ionic strength. Two likely explanations are either that the metal ion broadening has been modified through the interaction of other cellular constituents, such as protein, or that the local concentration of the interacting ion is lower in the muscle due to physical compartmentalization.

Spectra from the intact muscle almost always are sufficiently resolved so that the coupling constants, J values, of the α-β and the β-γ phosphate couplings can be determined. J values for the α-β and the β-γ couplings observed from freshly prepared muscle are the same and have the value of 14.8 Hz. This may be compared to the couplings in aqueous solutions where $J_{\alpha\text{-}\beta} = J_{\beta\text{-}\gamma} = 19.5$ Hz (pH = 7, Na^+ counter cation). The J values in muscle have been observed to change with the lowering of the pH upon aging so that a sample aged for 10 hr at 28°C exhibits $J_{\alpha\text{-}\beta} = 22.8$ Hz and $J_{\beta\text{-}\gamma} = 14.8$ Hz. To our knowledge, this latter divergence in the values of the α-β and β-γ coupling constants has not been observed with ATP in aqueous media. However, a similar change has been observed from tetra-n-butylammonium ATP in anhydrous N, N, N′, N′-tetramethylurea at proton concentrations equivalent to pH 5 or lower (17). For example, in this anhydrous medium at proton concentrations of 10^{-4} M, $J_{\alpha\text{-}\beta} = 27.5$ Hz and $J_{\beta\text{-}\gamma} = 17.0$ Hz.

We have no firm interpretations to account for the 4.7 Hz lowering of the J values of ATP in the fresh muscle or divergence in the values of the coupling constants in aged muscle. We would like to suggest, however, that these unusual spectroscopic observations may arise from the fact that the ATP molecules reside in a structured aqueous and/or a hydrophobic medium. Changes in J values are best interpreted in terms of distortion of the geometry of the linkage between the interacting groups (18), in this case the POP bonds.

Because the magnitude of NMR signals are proportional to the amount of atoms giving rise to the resonance, the molarity of the ATP in the muscle could be determined through appropriate calibration procedures. The concentration of ATP in the fresh muscle was found to be 3.0 mM. This compares well with the 2.6–2.8 mM values determined through chemical analyses (Table II). This amount is essentially sustained as the muscle ages at 28°C for 400–600 min, at which time the rate of disappearance accelerates.

Relative differences in the areas of the β and γ resonance bands can be used to evaluate the relative amount of ADP present in the sample when the individual resonances cannot be resolved. The β group of ADP and the γ group of ATP overlap (see the EDTA-treated PCA extract where the ADP resonances are resolved), so that increasing the ADP concentration will result in increasing the area of this resonance band. In the intact muscle no signals have been observed overlapping the β group resonance of ATP, so that its intensity accurately reflects the quantity of ATP present.

In comparing the relative areas of the β and γ resonance bands of ATP, it can be

TABLE II. ATP and ADP Contents of Various Muscles

Muscle	μmole/g fresh muscle ATP	ADP	ATP/ADP	Authors
Frog semi-tendinosus	2.61	0.47	5.5	Bárány and Bárány (2)
Frog sartorius	2.72	0.60	4.5	Canfield and Marechal (19)
Frog sartorius	2.55	0.42	6.1	Mommaerts and Wallner (20)
Frog rectus	2.75	0.47	5.9	Cain, Kushmerick, and Davies (21)
Hamster hind leg	5.97	0.35	17.0	Dhalla, Fedelesova, and Toffler (22)
Ox sterno-mandi-bularis	2.6	0.4	6.7	Newbold and Scopes (23)
Rabbit psoas	–	–	6.7[1]	Mommaerts and Rupp (24)
Rat rectus	6.09	0.74	8.2	Hohorst, Reim, and Bartels (25)
Pigeon pectoralis major	7.35	0.8	9.2	Arese, Kirsten, and Kirsten (26)
Rabbit heart	2.78[2]	0.51[2]	5.4	Liu and Feinberg (27)

[1] Authors list only the distribution of ATP and ADP as a percentage of the total nucleotide which was the basis for the calculation of ATP to ADP ratio.
[2] These values were calculated from the data of Liu and Feinberg, expressed as μmole/g dry heart weight, assuming 16.67% dry weight for the heart.

stated that in the fresh muscle the concentration of ADP observed is less than 2% of that of ATP, i.e., the areas of the β and γ groups were always found to be the same within experimental error. The presence of ADP was observed after muscle had been aged at 28°C from 750 to 1,100 min. In this case it was frequently possible to detect the separate ADP resonances.

In contrast to the situation which exists in fresh muscle, the PCA extract does contain ADP. The quantity of ADP, however, cannot be obtained from the untreated PCA extract but it is readily obtained from the PCA extract after the addition of EDTA. The amount detected corresponds to 0.43 μmole per gm muscle which is in the same range as that determined chemically in frog PCA extracts (Table II). This ADP corresponds to that bound to F-actin in the muscle. It should be mentioned in this connection that highly purified F-actin containing bound ADP does not give rise to high resolution ^{31}P NMR resonances.

After this paper was submitted for publication we learned of the work of Hoult et al. (Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E., and Seeley, P. J., Nature 252:285, 1974) who used ^{31}P nuclear magnetic resonance for the determination of metabolite levels in intact rat muscle. Their findings are in general agreement with ours.

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