Molecular Characteristics of Canine Cardiac Myosin

By Robert J. Luchi, M.D., Eve Marie Kritcher, A.B., and Hadley L. Conn, Jr., M.D.

Contraction of the heart is dependent upon the proteins myosin and actin. Studies of these contractile proteins have therefore been of interest in the understanding of the normal contractile process, and attempts have been made to relate the diminished cardiac contraction observed in congestive heart failure to alterations in the myosin molecule.1 Cardiac myosin has been prepared generally by modification of the basic Szent-Györgyi technique used for isolation of skeletal muscle myosin. It is now clear from chemical and immunological evidence that solutions of skeletal muscle myosin prepared by such techniques are not homogenous.2-6 Recently, Marshall (personal communication) has shown that conventionally prepared solutions of chicken skeletal “myosin” can be separated into three components. He presents cogent evidence for the belief that the two minor components are impurities and that the major component represents myosin freed from such impurities.

If skeletal muscle myosin prepared by modification of the Szent-Györgyi technique is inhomogeneous, cardiac myosin prepared by related techniques is also suspect, as are comparisons between cardiac myosin in normal and diseased states, and comparison of cardiac myosin with skeletal myosin.

Using a modification of the Szent-Györgyi technique, we and others4 have been unable consistently to prepare cardiac myosin that meets a minimum criterion for homogeneity, i.e., a single, sharp sedimentation peak in the ultracentrifuge. Adapting the technique Marshall applied to skeletal myosin preparation, we were able to separate into three components solutions of cardiac myosin prepared by faithful adherence to published techniques.7-8 The major component, herein termed myosin, had a distinctly higher adenosine triphosphatase (ATPase) activity than previously reported for cardiac myosin. Additional differences include a greater reproducibility of other molecular characteristics and absence of detectable aggregation (as determined by ultracentrifugation and stability of ATPase activity) during storage for five to eight days. We believe that this new method of preparation allows a narrower biochemical definition of cardiac myosin, and permits a more accurate description of its molecular characteristics as well as a more valid comparison with other myosins.

Methods

All solutions were prepared with reagent grade chemicals. Those lots containing the smallest amounts of phosphate, nitrogen, and heavy metals were selected. Both the lithium chloride and ammonium sulfate were recrystallized in the presence of disodium ethylenediaminetetraacetate and adjusted to a final pH of 7.0. Deionized water, with a conductivity of $1.20 \times 10^{-6}$ mhos/cm², was used in the preparation of solutions and in the rinsing of glassware and dialysis tubing. The procedure to be described for the extraction and fractionation of myosin was done at 0 to 4°C.

A. PREPARATION AND FRACTIONATION OF MYOSIN

Healthy mongrel dogs were lightly anesthetized by an intravenous injection of sodium pentobarbital (20 mg/kg). An endotracheal tube was
inserted through a tracheostomy and positive pressure respiration was begun when the chest was opened. The beating heart was rapidly excised and chilled in iced saline. The procedure for extracting myosin outlined by Davis et al. or Ellenbogen et al. was followed initially. In later experiments the following procedure was used. After chilling the heart for 45 minutes at 0°C, the ventricles were cut into small chunks, weighed, and minced for 10 seconds in a Waring blender with a salt solution (300 ml/100 g) of the following composition: 0.3 M KC1 and 0.15 M potassium phosphate buffer, the final pH adjusted to 6.8 with KOH. One mg of ATP was added per ml of extracting solution prior to blending. Extraction was allowed to proceed for 20 minutes with slow magnetic stirring. The muscle extract was then centrifuged in a Servall RC-2 refrigerated centrifuge for 20 minutes. To precipitate the myosin, the ionic strength of the supernatant fluid was reduced to 0.05 by adding deionized water. The precipitate was collected by centrifugation and dissolved in a salt solution containing 0.4 M KC1 and 5.0 x 10^-4 M potassium borate adjusted to a pH of 8.8. The protein solutions were prepared for fractionation (and subsequent analysis) by dialysis against repeated changes of this solvent.

By means of an immersed buret an equal volume of 4.0 M lithium chloride was added to a 1 to 2% protein solution which was stirred continuously. Enough saturated ammonium sulfate was then added to reach 38% saturation. The ammonium sulfate was added slowly during continuous stirring of the protein solution to prevent gelation near the immersed buret tip. The slightly turbid solution was stirred slowly for one hour and then centrifuged. The precipitate was recovered as the first or "38%" fraction. Enough saturated ammonium sulfate was added to the supernatant fluid to reach 45% saturation. During 60 minutes of slow stirring, a dense white precipitate appeared which was collected by centrifugation and dissolved in the KCl-borate solution. Solid ammonium sulfate (25 g/100 ml) was added to the supernatant fluid of the 45% fraction to reach 90% saturation. After standing for 24 hours at 0°C, a precipitate formed. This was collected as the third or "90%" fraction.

Separation of the other two components from the 45% fraction was completed by a second and third cycle. During the third cycle, enough ammonium sulfate was added to reach 39% saturation. If no turbidity was noted, ammonium sulfate was again added to 40% saturation. The solution was stirred for one hour, centrifuged, and the supernatant fluid was taken to 45% saturation. The precipitate formed was collected, dissolved in KCl-borate solution, and dialyzed against repeated changes of this solvent. The 45% fraction was then centrifuged at 40,000 rpm for three hours in a Spinco model L preparative centrifuge.

B. ANALYTICAL PROCEDURES

Protein concentration was determined by the micro-Kjeldahl method. A nitrogen content of 16% was assumed for myosin.

1. Adenosine Triphosphatase Activity

The enzymatic activity of the protein fractions was determined by measuring the amount of inorganic phosphate liberated over a five-minute period. The reaction rate was essentially linear over this interval. The reaction mixture contained 0.16 M tris-maleate buffer (pH 6.5), 1.0 x 10^-2 M CaCl2, 1.6 x 10^-3 M ATP and 200 to 900 μg of myosin in a total volume of 6.1 ml. The ATP was added last. The reaction was allowed to proceed for five minutes at 30°C with constant agitation in a Dubnoff shaker. The reaction was stopped by the addition of 2 ml of cold 15% trichloroacetic acid. The amount of phosphate liberated was determined spectrophotometrically by the method of Martin and Doty, and the ATPase activity expressed as μmoles phosphorus liberated/mg of protein/5 minutes. Approximately 8% of the ATP was hydrolyzed during the five-minute incubation. With each group of determinations a standard solution of phosphate and a dialysate blank were analyzed. All determinations were done in duplicate.

2. Viscosity

Viscosity determinations were made at 15.00 ± 0.01°C in a capillary viscometer. The charging volume was 2 ml, the shear gradient was 395 sec^-1, and the outflow time for 0.4 M KCl-borate was 274.5 sec. Results were similar using a viscometer with a shear gradient of 284 sec^-1. All protein dilutions were equilibrated at 15°C for 15 minutes prior to pipetting into the viscometer to minimize bubble formation. It was determined in separate experiments that the viscosity of myosin solutions was stable for up to one hour at this temperature.

3. Spectrophotometry

Ultraviolet absorption spectra were determined in a Zeiss spectrophotometer PMQ II, using matched 1-cm cells and a slit width of 0.06 mm. An 0.1% solution of protein in 0.1 N NaOH (resulting mixture pH 13) was centrifuged at 12,000 rpm for 20 minutes at room temperature. The optical densities at 2800, 2944, 3400, and 3600 Å were measured. Corrections for irrelevant absorption were calculated by linear extrapolation from optical densities at 3400 and 3600 Å. Tyrosine and tryptophan contents were calculated...
according to the method of Goodwin and Morton.10

4. Sedimentation Velocity and Molecular Weight

Sedimentation velocity studies were done in a Spinco model E analytical ultracentrifuge at a speed of 50,740 rpm and a temperature of 14.0° C. A standard 12 mm cell was used for concentrations of protein above 1 mg/ml, with a Kel-F centerpiece to prevent protein denaturation. A synthetic boundary cell, capillary type, was employed for protein concentrations below 1 mg/ml. Schlieren patterns were photographed at eight-minute intervals for one to two hours, and measurements were made in a Bausch and Lomb optical comparator. Corrections for the density and viscosity of water at 20°C were made by the standard procedure. A partial specific volume of 0.73 was assumed.

Molecular weight determinations were done in a Spinco model E ultracentrifuge using the Archibald approach to equilibrium method as described by Schachman.11 In the approach to equilibrium runs, rotor speeds of 4609, 5784, 6995, 7447, and 8225 rpm were used. Cw values were determined from separate runs made in a capillary-type synthetic boundary cell. Temperature was 14.0°C for runs of two hours or less; temperature of 1°C was used when runs of longer than two hours were made. Initial protein concentration varied from 6 mg/ml to 1.5 mg/ml. Most molecular weight determinations were made from calculations at the meniscus. Calculation of molecular weight from the bottom of the cell gave values similar to those made from meniscus calculations. When the bottom of the cell was used for calculations, 0.1 ml of a heavy silicone oil was added to the cell before introduction of the protein solution. This resulted in a sharper boundary at the bottom of the cell and facilitated calculations.

Results

EFFECT OF LITHIUM CHLORIDE AND AMMONIUM SULFATE ON CONVENTIONALLY PREPARED MYOSIN SOLUTIONS

Ammonium sulfate alone did not separate conventionally prepared myosin solutions (i.e., three times precipitated and subjected to preparative ultracentrifugation by the methods of Ellenbogen or Davis) into components. Separation occurred only when the addition of ammonium sulfate followed that of lithium chloride. Only a limited number of lithium chloride concentrations were investigated. Lithium chloride in 1.5 M concentration did not produce as complete a separation of the components, as judged by absorbancy studies, as did 2.0 M lithium chloride. ATPase activity was inconstantly reduced by 2.5 M lithium chloride; 4.0 M and 5.5 M lithium chloride resulted in complete loss of ATPase activity.

As 38% ammonium sulfate saturation was reached, warming the solution to 10°C caused a dense flocculent precipitate to appear which almost completely redissolved on cooling to 0°C. In common with Marshall's observation, this increase in precipitation with warming served as an indication that the first point of separation was reached. Warming to 10°C with subsequent recooling was not done routinely. The 38% fraction was always collected at 0°C. There was no overlap in the zones of precipitation of these fractions. The 38% fraction precipitated over the range of 30 to 38%, but sometimes at lower percentages of ammonium sulfate in crude myosin extracts. The 45% fraction precipitated over the range of 42 to 45%, and the 90% fraction covered the widest range with precipitation beginning at 55 or 60% ammonium sulfate saturation.

In all studies the protein precipitating with 45% ammonium sulfate saturation was by far the largest component. In earlier studies the total nitrogen in the 38% and 90% fractions

![Figure 1](image-url) Calcium activation of cardiac myosin adenosine triphosphatase activity expressed as micromoles of phosphate/mg protein/5 min. Two hundred micrograms of protein were present in the reaction mixture.
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Figure 2 shows the stability of ATPase activity with time. ATPase activity did not diminish significantly up to six days following death of the dog. After this period ATPase activity decreased slightly, the viscosity increased slightly, and a leading edge or small second peak appeared during ultracentrifugation. Storage for one or two days longer resulted in a great decrease of ATPase activity, a sharp increase of viscosity, and the appearance of a large, rapidly sedimenting component during ultracentrifugation.

Lithium chloride in $10^{-12}$ to $10^{-4}$ molar concentrations failed to increase the ATPase activity of conventionally prepared myosin solutions; nor did dialysis decrease enzyme activity, making it unlikely that the high ATPase activity noted can be attributed to the presence of lithium ions.

**Viscosity**

The graph of reduced viscosity as a function of protein concentration is shown in figure 3. The intrinsic viscosity by extrapolation is 2.08 deciliters/g. Most of the determinations were done with protein concentrations below 1 mg/ml. There is no upward or downward deflection of the line at protein concentrations below 0.5 mg/ml as noted by others.

**Spectrophotometry**

The absorbance index (i.e., $A_{\text{abs}}$, the optical
Spectrophotometric Studies, pH 13

<table>
<thead>
<tr>
<th>A. 2800</th>
<th>A. 2944</th>
<th>Tyrosine</th>
<th>Tryptophan</th>
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<td>4.45</td>
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<td>4.56</td>
<td>5.11</td>
<td>1.80</td>
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</tr>
<tr>
<td>4.63 ± 0.02 SE</td>
<td>5.06 ± 0.02 SE</td>
<td>1.78 ± 0.01 SE</td>
<td>3.48 ± 0.03 SE</td>
</tr>
</tbody>
</table>

*Corrected for background scatter by the method of linear extrapolation from 3400 Å and 3600 Å.

Absorbance Indices of Native and Denatured Myosin, and of 38% and 90% Fractions, pH 13

<table>
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<th>Native myosin</th>
<th>Denatured myosin</th>
<th>38%</th>
<th>90%</th>
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</thead>
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<tr>
<td>2800 Å</td>
<td>4.83</td>
<td>6.00</td>
<td>4.08</td>
</tr>
<tr>
<td>2944 Å</td>
<td>5.06</td>
<td>6.01</td>
<td>3.79</td>
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</tbody>
</table>

The absorbance indices proved to be a simple and reliable guide to the separation of the 38% and 90% fractions from the 45% fraction. These indices decreased progressively after the first and second cycle but remained constant after a third and fourth cycle. As an example, for dog 51 the prefractionation absorbances at 2800 and 2944 Å were 7.94 and 7.92 respectively; after the first cycle, the values were 4.85 and 5.32, after the second cycle, 4.64 and 5.12, after the third cycle, 4.59 and 5.01, and after the fourth cycle, 4.60 and 5.01.

Absorbance indices for the 38% and 90% fractions are shown in table 2. These values are distinctly different from absorbance indices on myosin denatured either by heat or by 4.0 M lithium chloride.

Sedimentation Velocity Studies

Figure 4 shows the pattern of the sedimenting myosin solution as seen on the ultracentrifuge. The run was continued for two hours and a single sharp peak was noted throughout. Figure 5 shows corrected sedimentation coefficients plotted as a function of protein concentration. The intrinsic sedimentation coefficient by extrapolation is 6.0.

Molecular weight determinations by the Archibald method are plotted in figure 6 as the reciprocal of apparent molecular weight versus protein concentration. The molecular weight was not dependent on either the initial protein concentration or the protein concentration calculated to be present at the meniscus. The molecular weight obtained from the intercept of the upper plot is 500,000. The
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FIGURE 4

Sedimentation patterns of cardiac myosin. Protein concentration is 1.95 mg/ml in 0.4 M KCl; pH 6.5; temperature 14°C; rotor speed 80,740 rpm; bar angle 75°. Pictures taken at 16-minute intervals.

FIGURE 5

Sedimentation coefficients of cardiac myosin.

intercept of the lower plot gives a molecular weight of 493,000, a value not significantly different from 500,000.

Discussion

A. FRACTIONATION OF CONVENTIONALLY PREPARED SOLUTIONS OF CARDIAC MYOSIN

Fractionation of myosin solutions by ammonium sulfate alone or in combination with other agents is not new. Tsao and Tenow, as well as Marshall, have shown that two or more fractions can be obtained from solutions of skeletal myosin which are supposedly pure. The per cent saturation of ammonium sulfate required to precipitate myosin was identical to, or not very different from that reported in this communication. In our experience only one fraction resulted when ammonium sulfate alone was added to cardiac myosin prepared by three precipitations and preparative ultracentrifugation. In contrast, three fractions were identified when lithium chloride was added before ammonium sulfate fractionation. The role of lithium chloride is not clear. Edsall and Mehl observed the effects of 1.0 M lithium chloride on solutions termed “myosin” but which almost certainly were solutions of actomyosin. They observed a decrease of viscosity, loss of flow birefringence and no change in detectible SH groups. They concluded that the effect
of lithium chloride was one of "denaturation," but these results could also be interpreted as representing dissociation either of actomyosin aggregates or dissociation of myosin from contaminating impurities, including actin. Shulman et al.14 noted that 2.0 M lithium chloride reversed the polymerization of fibrin, yielding apparently undenatured activated fibrinogen. They attributed the effect of lithium chloride to disruption of electrostatic bonds. By analogy, we suggest that lithium chloride separates tightly adherent, contaminating molecules from myosin, thus permitting their separate precipitation by ammonium sulfate.

Before one accepts this tentative conclusion, consideration should be given to the nature of the three fractions isolated. Specifically, one may ask whether the addition of lithium chloride and ammonium sulfate partially denatures myosin, thus accounting for the three components; or alternatively, whether the addition of one or both of these compounds results in a disruption of the molecule yielding three components.

There seems little reason to doubt that the largest or 45% fraction represents myosin. Although this protein differs in some respects from those of conventionally prepared myosin, the differences are not so great as to obscure the relationship. That the two remaining fractions represent denatured myosin is unlikely in view of the absorbance studies. Myosin, denatured by standing at room temperature or by exposure to high concentrations of lithium chloride, showed the same absorbances at pH 13 as native myosin. The absorbances of the 38% and 90% fractions, on the other hand, are clearly different from the 45% fraction.

The 38% and 90% fractions may represent subunits of myosin but the following evidence suggests they do not. As noted, evidence from several sources indicates that conventionally prepared myosin solutions contain one or more impurities, and there is some evidence to suggest that one of the components in the 38% fraction is actin. Furthermore, the molecular weight, intrinsic viscosity, and $S_{20,w}$ of the 45% fraction were similar to those described for cardiac myosin by Davis et al.7 and hence it would be unlikely that large subunits were separated from the parent myosin molecule. As judged by their appearance in the ultracentrifuge, both fractions were grossly inhomogeneous, unlike the result of trypsin digestion of myosin which yields two reasonably homogeneous components, light and heavy meromyosin. Moreover, if one assumes that the action of lithium chloride and ammonium sulfate was to remove an enzymatically inactive subunit of myosin amounting to, on the average, 15% of the total nitrogen content, an increase of 15% in ATPase activity would be anticipated. The ATPase activity of the myosin solution prior to fractionation was 1.5 µmole P/5 min/mg protein. The ATPase activity of the 45% fraction is more than twice this value. This suggests that the material separated in the 38% and 90% fractions is tenaciously held foreign material which may either: (1) promote rapid aggregation of the protein at the temperature used during the measurement of ATPase activity, or (2) in some way interfere with substrate-enzyme interaction.

B. RELATIONSHIP OF THE 45% FRACTION TO OTHER PREPARATIONS OF CARDIAC MYOSIN

The relationship of the 45% fraction to other preparations of cardiac myosin has already been touched upon. In addition to a higher ATPase activity than previously reported, reproducibility of ATPase activity and viscosity results were better, and the protein showed much less tendency to aggregate with time. Whereas Davis et al.7 and Ellenbogen et al.8 noted wide fluctuation in viscosity determinations at protein concentrations below 0.5 mg/ml, we found no tendency for the curve to deviate either upwards or downwards at these low concentrations. Molecular weight determinations by the Archibald technique gave results consistent with that reported by Davis but higher than originally reported by Ellenbogen et al. However, a recent report from their laboratory indicates that they now accept a molecular weight of 500,000 for myosin.15 Our findings for cardiac myosin are
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TABLE 3
Cardiac Myosin Adenosine Triphosphatase Activity Adapted from Various Authors

<table>
<thead>
<tr>
<th>Authors</th>
<th>Buffer</th>
<th>pH</th>
<th>Temp.</th>
<th>Ca conc.</th>
<th>ATPase activity</th>
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<tbody>
<tr>
<td>Luchi et al.</td>
<td>Tris-maleate</td>
<td>6.5</td>
<td>30°C</td>
<td>1 x 10^-2 M</td>
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<tr>
<td>Brahms and Kay</td>
<td>Tris</td>
<td>8.0</td>
<td>25°C</td>
<td>4 x 10^-8 M</td>
<td>0.84</td>
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<tr>
<td>Ellenbogen et al.</td>
<td>Veronal</td>
<td>8.6</td>
<td>25°C</td>
<td>1 x 10^-2 M</td>
<td>1.30</td>
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TABLE 4
Comparison of Canine Cardiac and Chicken Skeletal Muscle Myosin

<table>
<thead>
<tr>
<th></th>
<th>Canine cardiac myosin</th>
<th>Chicken skeletal myosin</th>
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</thead>
<tbody>
<tr>
<td>Absorbance index</td>
<td>2800 A</td>
<td>2944 A</td>
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<td></td>
<td>4.63</td>
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<td>S20, w</td>
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<td>6.25</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
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<td>2.08</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>500,000</td>
<td>460,000</td>
</tr>
<tr>
<td>ATPase activity,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μmoles P/mg prot./5 min</td>
<td>3.9</td>
<td>5.8</td>
</tr>
</tbody>
</table>


similar to those of Brahms and Kay except for a significantly higher ATPase activity and a lower molecular weight. Their result for cardiac myosin ATPase activity was 0.84 μmole P/mg protein/5 min and a molecular weight by the Archibald technique of 758,000.

Table 3, taken from the papers of various authors, lists cardiac myosin ATPase activity expressed as μmole P/mg protein/5 min. A strict comparison is not possible because of variations in technique of determining ATPase activity, particularly hydrogen ion concentration. The high value for ATPase activity at pH 6.5 reported in this paper takes on added significance since myosin ATPase activity tends to be higher at pH 8 or 9.

The relative stability of this myosin preparation obviates the need for haste in preparing and analyzing the protein suggested by Brahms and Kay. Indeed by this technique, myosin with the characteristics herein described could be recovered from conventionally prepared myosin solutions stored at -20°C in 50% glycerol for as long as three months, as originally demonstrated by Marshall for skeletal muscle myosin.

C. RELATIONSHIP OF CARDIAC MYOSIN TO SKELETAL MUSCLE MYOSIN

Analyses of simultaneously prepared canine and skeletal muscle myosin in our laboratory did not reveal differences in any of the molecular characteristics measured except ATPase activity. By the same experimental procedure used to measure cardiac myosin ATPase activity, canine skeletal myosin ATPase activity was 5.6 μmole P/5 min/mg protein. Therefore skeletal muscle myosin had an ATPase activity approximately 30% higher than that found for cardiac myosin. Comparison between canine cardiac and chicken skeletal muscle myosin (as prepared by Marshall) is shown in table 4. Cardiac myosin ATPase activity is approximately 65% of skeletal myosin ATPase activity. Furthermore, there appears to be a difference in the effect of ionic milieu on ATPase activity in that cardiac myosin ATPase activity is inhibited by 0.15 M potassium whereas skeletal myosin ATPase activity is not. The observation that on a weight basis much more skeletal myosin (four or five times the amount of cardiac myosin) is extracted with the KCl-potassium phosphate buffer solution may also be the result of differences between these two molecules, although it could result from differences in the milieu of cardiac and skeletal myosins.

The major advantage of this method of
preparing myosin is its ability to separate substances that are held tenaciously to the myosin molecule and that resist removal by repeated precipitation and preparative centrifugations. These impurities in cardiac myosin solutions apparently have been responsible for considerable variability in certain physicochemical properties, in particular enzyme activity, molecular weight, and viscosity determinations. We believe that this method of preparing myosin will aid future studies of the relationships between myosins obtained from normal animals and from animals with disease.

Summary

Cardiac myosin was obtained from the hearts of normal dogs by conventional extraction and precipitation techniques, followed by ammonium sulfate fractionation in the presence of 2.0 M lithium chloride. The molecular characteristics of this protein preparation are presented. Cardiac myosin prepared by this technique shows less tendency than conventionally prepared solutions to denature when stored at 0 to 4°C, and has a significantly higher ATPase activity. Comparison of canine cardiac myosin and skeletal muscle myosin, obtained under identical conditions, revealed no differences between these two proteins in their intrinsic viscosities, S20,w, molecular weight, and UV absorbances, although ATPase activity was lower for cardiac myosin.

References

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