A Procedure for the Quantitative Determination of Protein of the Rat Heart

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A procedure has been developed for the quantitative determination of the extractable proteins of the rat heart. Utilizing extraction with a sucrose-versene solution and employing differential centrifugation, followed by extraction with a phosphate-pyrophosphate buffer, low-ionic-strength extractives, a mitochondrial fraction, and high-ionic-strength extractives have been obtained. Electrophoretic analysis has shown five subfractions to be present in the low-ionic-strength extractives and three sub-fractions in the high-ionic-strength extractives.

There has been considerable interest in the fractionation of the proteins of muscle. However, such fractionation has been mainly concerned with skeletal muscle. Low-ionic-strength extractives, which contain much of the glycolytic system, have been studied electrophoretically and have been shown to contain several characteristic protein fractions. High-ionic-strength extracts include three electrophoretically demonstrable components of the contractile system which have been labeled α-myosin, β-myosin and contractin.1

Less attention has been directed to the proteins of the heart.2, 3, 4 Not until recently has there been an attempt to determine quantitatively the protein present in extracts of this tissue.3 Benson and co-workers4 have determined protein fractions of heart muscle in dogs with experimental valvular lesions and chronic heart failure as compared with those of normal control dogs.

This communication reports the results of experiments directed to the development of a reproducible procedure for determining quantitatively the protein present in the low-ionic-strength extracts, high-ionic-strength extracts and the mitochondria of a single rat heart.

The rat is ideally suited for such a study. It can be subjected to a variety of experimentally induced cardiac myopathies and hypertrophies. Furthermore, because of its size and low fat content, the heart of this species offers certain advantages in the manipulations involved in the protein extractions.

Experimental Procedure

Rats used in these studies were females of the Addis-Slonaker strain weighing between 167 and 192 Gm. and maintained on a stock diet.4

Four of the extraction procedures investigated are described below. The solutions employed are as follows:

1. 0.25M Sucrose; 0.01M Versene; pH 5.0;
2. 0.25M Sucrose; 0.01M Versene; 0.02M NaOH; pH 7.4;
3. 0.8M Sucrose; pH 7.4;
4. 0.60M KCl; 0.00573M KH2PO4; 0.00427M K2HPO4; 1 g. per cent Na3P04.10 H2O; 0.01M Versene; pH 6.5;
5. 0.60M KCl; 0.00573M KH2PO4; 0.00427M K2HPO4; 1 g. per cent Na3P04.10 H2O; 0.003M Cysteine; pH 8.3;
6. 0.049M Na Acetate; 0.029M Sodium Diethylbarbiturate; 0.003N HCl; 1 Gm. per cent Na3P04.10 H2O; pH 9.1.

Fraction I obtained by the following procedures is defined as that protein extracted by low-ionic-strength solutions and soluble in a 0.03M KCl solution in the pH range 6.5–7.5. Fraction II is defined as that protein extracted by high-ionic-strength solutions and precipitated when the ionic strength is adjusted to 0.03 (KCl) in the pH range 6.5 to 7.5. The mitochondria are contained in fraction III.

The rat was anesthetized with ether and the heart immediately removed. While still beating, the heart was trimmed of non-cardiac tissue and dipped repeatedly into ice water to wash it as free from blood as possible. The tissue was then blotted, weighed and stored in the deepfreeze until it was analyzed.

Procedure A. The heart was ground with four times its weight of purified sand in a mortar im-
homogeneous paste was obtained. The fractionation was continued according to the following steps:

1. The homogenate was transferred quantitatively from the mortar to a lustron tube with approximately 4 cc. of ice-cold sucrose-versene solution of pH 5.0. The mixture was spun at 19,000 g. for four minutes. To the residue remaining in the centrifuge tube were added 1.5 cc. of sucrose-versene solution, pH 5.0. Extraction was continued by placing the tube on a refrigerated rotor for one hour. (The rotor used for extraction consisted of a drum 8 cm. in diameter revolving around a horizontal axis at the rate of 68 revolutions per minute. The tube was attached to the drum at a 45 degree angle permitting a rolling contact between the extracting solution and the material being extracted.) Two additional rotor extractions were made, one for 20 minutes and one for 20 minutes. The residue was then washed. After each extraction and washing, the mixture was spun at 19,000 g. All supernatants from the centrifugations in (1) were combined to form Fraction I. (2) All supernatants from the centrifugations in (1) were combined to form Fraction I. (3) After the removal of Fraction I, 3 cc. of a sucrose-versene solution of pH 7.4 were added to the residue. Extraction was carried out on a refrigerated rotor for 20 minutes. The residue was again rotor-extracted for 30 minutes and washed at least two times. After each washing and extraction the mixture was spun at 650 g.; the supernatant was removed to a lustron centrifuge tube. (4) The extract obtained under (3) contained Fractions IA and III. To separate these fractions, the lustron tube containing the extract was centrifuged at 19,000 g. The supernatant contained Fraction IA; the precipitate, Fraction III. (5) Three cc. of a 0.6M KCl-phosphate-pyrophosphate-versene buffer of pH 6.5 were added to the residue remaining after removal of Fractions I, IA and III. Overnight extraction was made on the refrigerated rotor. The following morning the residue was again rotor-extracted for one hour, then washed with 0.6M KCl-phosphate-pyrophosphate-versene buffer. The residue was again rotor-extracted with 3 cc. of a 0.6M KCl-phosphate-pyrophosphate-versene-cysteine buffer. All extracts were centrifuged at 19,000 g. The supernatants were combined and contained Fraction II. (6) After removal of Fractions I, IA, III and II, the protein remaining in the residue was designated Fraction IV.

**Procedure B.** Procedure B was similar to procedure A with the exception that the primary extractant was 0.85M sucrose. An aliquot of the extract was dialyzed against 0.03M KCl. A precipitate formed. The precipitated protein is designated as Fraction IB. After removal of Fractions IB plus IB, the residue was suspended in 0.88M sucrose and centrifuged at 650 g. Fraction III (mitochondria) was thereby obtained. Further protein extraction was continued as outlined in steps (5) and (6) of procedure A.

**Procedure C.** Procedure C was similar to procedure A with the exception that the primary extractant was 0.25M sucrose and 0.01M versene of pH 6.5. After removal of Fraction I, Fraction III was obtained by differential centrifugation at 650 g. with sucrose-versene solution of pH 6.5 as the suspending solution. Further protein extraction was continued as outlined in steps (5) and (6) of procedure A.

**Procedure D.** (1) After grinding the heart with sand and obtaining a homogeneous pink paste, the ground heart was transferred quantitatively from the mortar to a lustron centrifuge tube with approximately 4 cc. of ice-cold 0.6M KCl-versene-pyrophosphate buffer of pH 6.5. Extraction was continued by rotor for one hour. Two additional rotor extractions were made, one for 50 minutes and one for 20 minutes. The residue was then washed with 0.6M KCl-versene-pyrophosphate buffer (pH 6.85). After each extraction and washing, the mixture was spun at 19,000 g. for four minutes. To the residue remaining in the centrifuge tube were added 2 cc. of 0.6M KCl-versene-pyrophosphate buffer (pH 6.85). Extraction was continued by rotor for one hour. Two additional rotor extractions were made, one for 50 minutes and one for 20 minutes. The residue was then washed with 0.6M KCl-versene-pyrophosphate buffer (pH 6.85). This extract contained Fractions I and II. (3) To separate Fraction II from Fraction I, 5.00 cc. of the extract obtained under (2) were allowed to drain slowly into 110 cc. of ice-cold glass-distilled water to which had been added 0.10 cc. of 1N HCl. The final pH of the solution was 6.65. The final ionic strength was 0.03. To allow for complete precipitation of Fraction II, the mixture was put in the refrigerator and swirled every half-hour for several hours. The gel-like precipitate (Fraction II) was packed by spinning at high speed. The precipitate was washed with a total of 1 to 2 cc. of 0.03M KCl. (4) Five cc. of the extract obtained under (2) containing Fractions I and II were dialyzed against 0.03M KCl overnight. The following day the contents of the dialyzing bag were centrifuged at high speed. The supernatant contained Fraction I. (5) After Fractions I and II had been removed, 3 cc. of sucrose-versene solution, pH 7.4, were added to the residue. Rotor extraction was made for 60 minutes. The residue was washed two times. After each extraction and washing, the mixture was centrifuged at 650 g. All extracts were combined and designated Fraction III. (6) After removal of Fractions I, II and III, the protein remaining in the residue was designated Fraction IV.

Protein content of all Fractions obtained by procedures A, B, C and D was determined. Protein contained in aliquots of each fraction was precipi-
tated by the addition of trichloroacetic acid solution to give a final concentration of 10 per cent. Nitrogen determinations were made using a micro-Kjeldahl procedure involving aeration and titration. A factor of 6.25 was used for conversion to protein.

Aliquots of Fractions I, IA and II were concentrated and dialyzed against Dubuisson's phosphate buffer, pH 7.6 (Fraction I) and a veronal-phosphate buffer of pH 9.1 (Fraction II). Electrophoretic analyses were made with a Kern micro-electrophoresis apparatus (type LK 30). Each interference band was assumed to represent a concentration gradient of 0.031 Gm. per cent.

Cytochrome-c-oxidase activity of the mitochondria (Fraction III) was determined by the Nadi reaction. Mitochondrial morphology was determined by staining with Janus Green B.

RESULTS

Quantitative analysis of protein fractions obtained by the procedures described are tabulated in table 1.

When the initial extraction was made with 0.28M sucrose and 0.01M versene, pH 5.0 (Procedure A), Fraction I was present in a crystal-clear amber solution. Fraction IA obtained by increasing the pH to 7.4 had a slightly turbid appearance. Fraction I obtained by Procedure C was slightly turbid. When the initial extraction was made with 0.88M sucrose, the extracted protein solution was extremely turbid. In Procedure D, Fraction I obtained by dialyzing the high-ionic-strength extract against 0.03M KCl was slightly turbid.

When dialyzed against a 0.03M KCl solution, no precipitate was observed in Fractions I obtained by Procedures A and C. Upon dialyzing the fraction obtained by Procedure B, however, 22.0 per cent of the protein extracted by 0.88M sucrose precipitated. According to the definitions set forth in the second paragraph of the experimental procedure for Fractions I and II, this precipitate cannot be precisely designated as Fraction II since the protein was extractable by 0.88M sucrose. Nor can it be designated precisely as fraction I since it is precipitated by 0.03M dialysis. Since it is uncertain at this time that contractile protein is partially extractable by 0.88M sucrose the precipitated protein is designated Fraction IB. The protein remaining in solution quantitatively corresponded to the protein extracted by Procedure C.

Fraction II was quantitatively greatest when obtained by procedure D. Fraction III, the mitochondria, was obtained in greatest yield by Procedure A. When stained with Janus Green B, the mitochondria obtained by procedures A and C were spherical. Those obtained by procedure B were rod-shaped. Cytochrome-c-oxidase activity was determined by the Nadi reaction. Fraction III contained cytochrome-c-oxidase activity. Fractions I and II showed no cytochrome-c-oxidase activity.

The proteins of Fractions I, IA and II were subjected to electrophoretic analysis. The results of this analysis are tabulated in table 2.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No. of Rats</th>
<th>Fraction I (% Tot. Prot.)</th>
<th>Sub-Fraction as % of Fraction I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>8</td>
<td>24.7 ± 1.8</td>
<td>14.1 ± 2.7</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>32.3 ± 2.2</td>
<td>8.3 ± 0.9</td>
</tr>
</tbody>
</table>

* Standard deviation
Table 3.—Quantitative Analysis of Electrophoretic Partition of Fraction II Obtained by Procedures A and D.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No of Rats</th>
<th>Fraction II (% Total)</th>
<th>Sub-Fraction as % of Fraction II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>29.1 ± 1.4</td>
<td>20.6 ± 4.1</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>46.9 ± 3.7</td>
<td>22.3 ± 3.0</td>
</tr>
</tbody>
</table>

and 3. For dialysis and electrophoresis of Fraction II, pyrophosphate was found to be a necessary constituent of the buffer. If pyrophosphate was omitted, a portion of Fraction II precipitated. The precipitate could again be solubilized by adding pyrophosphate.

Fraction I was resolvable into five protein sub-fractions (table 2). These components have been arbitrarily designated 1, 2, 3, 4, and 5. Component 1 corresponds to that sub-fraction migrating toward the anode with the greatest mobility at pH 7.6. At this pH components 2, 3, and 4 migrated toward the anode, component 5 migrated toward the cathode.

Fraction II was resolvable into three protein sub-fractions (table 3). These components have been designated α, β, and γ. Although Fraction II obtained by procedure D contained a greater proportion of the total cardiac protein than the fraction obtained by Procedure A, the relative proportions of components α, β, and γ were nearly alike.

Fraction IA obtained by procedure A was sub-fractionated by electrophoresis into three components. These components contained 6, 55, and 39 per cent of this fraction.

Discussion

The purpose of this investigation was to develop a fractionation procedure that would be suitable for the quantitative determination of the protein fractions contained in low-ionic-strength extracts and high-ionic-strength extracts. The former are known to contain most of the enzymes involved in the glycolytic system. The high-ionic-strength extracts are known to contain most of the proteins involved in the contractile system. It was also required to obtain the mitochondria in quantitative yield. The mitochondria are known to house most, if not all, of the enzymes involved in the aerobic oxidative system.

Relatively, Procedure D yielded the largest Fraction II. Fraction III, however, was not obtained in satisfactory yield (table 1). Using Procedure A the maximum Fraction III was obtained. The use of a sucrose-versene solution for obtaining intact mitochondria is based on the studies of Cleland and Slater.8

The incorporation of pyrophosphate was necessary for the most quantitative extraction of Fraction II and in order to maintain its components in solution. In the absence of pyrophosphate, the γ-component is relatively larger and the α-component smaller.

Fraction IV presumably contains nuclear protein, insoluble connective tissue protein, and protein insoluble in or denatured by the extracting solutions used.

No attempts have been made to accurately correlate the electrophoretic fractions obtained in this investigation with fractions obtained by other investigators since the extraction procedures are radically different. However, components 1, 2, 3, 4, and 5 of Fraction I seem to correspond to fractions h, j-k, k, l, and m of Dubuisson and components α, β, and γ of Fraction II seem to correspond to Dubuisson's α-myosin, β-myosin, and contractin.1

Attempts were made to obtain actin from the rat heart by the method of Straub.9 After extraction with high-ionic-strength buffer, the residue was suspended in acetone several times as prescribed. After removal of all traces of acetone from the residue, a distilled water extract was made. No protein was extracted. Several attempts were made to obtain actin but in every case negative results were achieved.

Summary

Several procedures are described for the fractionation of the proteins of a single rat heart. One of these procedures employed primary extraction with 0.28M sucrose and 0.01M versene at pH 5.0, then at pH 7.4. By differential centrifugation a mitochondrial fraction was obtained. The residue was treated with 0.60M KCl containing a phosphate-pyrophosphate-versene buffer system. This enabled the
extraction of proteins of the contractile system. The extracts were subjected to electrophoretic analysis which demonstrated the presence of five proteins in the low-ionic-strength extractives and three proteins in the high-ionic-strength extractives. This procedure gave quantitatively reproducible results.

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