Purification and Some Properties of Rabbit Skeletal Muscle Phosphofructokinase*

KUO-HUANG LING,† FRANK MARCUS,‡ AND HENRY A. LARDY

From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706

(Received for publication, December 10, 1964)

In spite of an abundant literature (1-15) indicating that phosphofructokinase plays a key role in the regulation of carbohydrate metabolism, few attempts have been made to purify the enzyme (4, 17, 18) since the subject of P-fructokinase was last reviewed (19). The apparent instability of the enzyme makes purification difficult, and a number of suggestions have been offered to explain this property (20-23). Experiments directed to the problem of stabilization of the P-fructokinase activity led us to restudy the conditions for the extraction of the enzyme from rabbit skeletal muscle tissue, and to develop an improved procedure for its purification. This new and simplified procedure yields a preparation somewhat more active than that consistently obtained by the method reported earlier from this laboratory, but the maximum activity obtained then (specific activity = 130) (24) is comparable to that reported here. In addition, data are presented to explain the apparent heterogeneity of the purified enzyme.

EXPERIMENTAL PROCEDURE

Materials—AMP and the sodium salts of ADP and ATP were obtained from Pabst Laboratories and prepared solutions were adjusted to pH 8 with KOH. The sodium salt of DPNH was from Boehringer und Soehne, and solutions were made in 0.01 M Tris-HCl, pH 8. Tricyclohexylammonium salts of fructose 1,6-diphosphate and 2-phosphoenolpyruvic acid, and the barium salt of fructose 6-phosphate were also from Boehringer und Soehne. The latter was converted to the potassium salt by passage through a column of Dowex 50, K+ form. The fructose 6-phosphate concentration was estimated in the presence of excess P-fructokinase by the method described later for the estimation of P-fructokinase activity.

All these solutions were prepared in small amounts, kept at 4°, and usually used within a 10-day period.

Disodium ethylenediaminetetraacetate was obtained from Fisher Scientific Company; solutions were adjusted to pH 8 with NaOH and are referred to in the text as EDTA. Tris(hydroxymethyl)aminomethane was purchased from Sigma Chemical Company, and Tris-phosphate buffers were prepared by adjusting the pH of Tris base solutions with H3PO4. Ammonium sulfate, Special Enzyme Grade (Mann Research Laboratories), and isopropyl alcohol (Fisher Scientific Company) were used without further purification.

Aldolase, pyruvate kinase, lactate dehydrogenase, and a mixture of triose phosphate isomerase and α-glycerophosphate dehydrogenase were crystalline suspensions in ammonium sulfate from Boehringer und Soehne.

DEAE-cellulose (Cellex-D) from Bio-Rad Laboratories with an exchange capacity of 0.97 meq per g was washed as described by Peterson and Sober (25). Sephadex G-100 and G-200 were from Pharmacia, Uppsala. Agar gels were prepared according to Andrews (26).

All other reagents were of analytical grade. Twice distilled and deionized water was used for the preparation of all reagents.

Methods—P-Fructokinase activity was measured by coupling the reaction

\[ \text{Fructose 6-phosphate} + \text{ATP} \rightarrow \text{fructose 1,6-diphosphate} + \text{ADP} \]

with aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase, and recording DPNH oxidation by dihydroxyacetone phosphate.

Assay mixtures (3 ml, final volume) were prepared at room temperature in absorption cells with a 1-cm light path and contained 33 mM Tris-HCl, pH 8; 2 mM fructose 6-phosphate; 2 mM ATP; 2 mM MgSO4; 6.6 mM mercaptoethanol; 0.16 mM DPNH; 0.20 ml of auxiliary enzymes (see below); and 0.10 ml of a solution containing the P-fructokinase. The reaction was initiated by the addition of P-fructokinase, and the optical density at 340 mμ was continuously recorded, against a water blank, with a Cary model 11 spectrophotometer equipped with a thermostated cell compartment maintained at 28°. Under these conditions the change of absorbance at 340 mμ was linear with time and was directly proportional to P-fructokinase concentration.

Dilutions of P-fructokinase solutions were made in cold 0.1 M potassium phosphate, pH 8, containing 10-3 M EDTA, so that when added to the reaction system a change of absorbance at 340 mμ of about 0.1 per minute was obtained (for stability of diluted P-fructokinase see "Results").

Auxiliary enzyme solutions were prepared by diluting 0.25 ml of crystalline aldolase (10 mg per ml) and 0.05 ml of a mixture of crystalline triose phosphate isomerase and α-glycerophosphate dehydrogenase (10 mg per ml) to 5 ml with 0.01 M Tris-HCl, pH 8, containing 2 mg per ml of crystalline bovine serum albumin. This solution was stable for at least 1 week at 4°.

Pyruvate kinase was assayed as described by Bücher and Pfleiderer (27).
were centrifuged for 30 minutes at 10,000 X g at 0°, and the clear supernatant solutions were assayed immediately for P-fructokinase activity as described under "Methods." The protective effect of fluoride was also shown by adding 10⁻³ M KF to a freshly prepared phosphate extract, in which case the high initial level of P-fructokinase activity again remained unchanged for at least 1 week.

**Results**

**Study of P-Fructokinase Extraction from Rabbit Skeletal Muscle**

In order to obtain a stable and highly active extract, a restudy of the extraction of rabbit skeletal muscle was undertaken. It was observed that the activity of many of the extracts tested varied depending on the interval between preparation and assay. Assays conducted immediately on 0.03 M K₃HPO₄ extracts disclosed much higher activities than were found in extracts prepared with 0.03 M NaOH or KOH as reported by Taylor (23) and also in the procedures of Ling, Byrne, and Lardy (24), and Gatt and Racker (30). Phosphate buffers have also been used for extracting P-fructokinase by Guerritore, Pette, and Biccher (17), but no time effect was mentioned in their report.

In view of these differences, methods of stabilizing the P-fructokinase activity were sought. The addition of diisopropyl fluorophosphate, a useful agent for blocking proteolysis during enzyme purification (31), was found to prevent P-fructokinase destruction, but rather high concentrations were required. In testing whether liberated fluoride ion might be responsible for the effectiveness of diisopropyl fluorophosphate, it was found that extracting muscle with 0.03 M KF yielded far greater amounts of P-fructokinase activity than had been found previously. Table I summarizes the results obtained with three different extraction procedures. Extraction with KF yielded not only the greatest activity, but also the most stable. No loss of activity was observed during storage of the KF extract for at least a week.

**TABLE I**

**Extraction of rabbit skeletal muscle phosphofructokinase**

A rabbit was decapitated after deep anesthesia with Nembutal and the muscles from the hind legs and back rapidly removed and chilled in ice. Further steps were conducted in the cold room at 4°. The muscle tissue was passed through a chilled meat grinder, weighed, and homogenized twice with 2 or 3 volumes of extractant for 30 seconds in a Waring Blendor. The homogenates were centrifuged for 30 minutes at 10,000 X g at 0°, and the clear supernatant solutions were assayed immediately for P-fructokinase activity as described under "Methods.

<table>
<thead>
<tr>
<th>Extractant</th>
<th>pH of the final extract</th>
<th>Protein (mg/ml)</th>
<th>P-Fructokinase activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOH*</td>
<td>6.8 ± 0.1</td>
<td>20.8</td>
<td>0.93</td>
</tr>
<tr>
<td>2 volumes of 0.03 M KOH</td>
<td>6.8 ± 0.2</td>
<td>19</td>
<td>1.02</td>
</tr>
<tr>
<td>K₃HPO₄ 10⁻³ M EDTA, 3 volumes of 0.03 M KOH</td>
<td>6.5 ± 0.1</td>
<td>19.7</td>
<td>2.03</td>
</tr>
<tr>
<td>KF⁻¹ M EDTA, 3 volumes of 0.03 M KOH</td>
<td>6.5 ± 0.1</td>
<td>19.7</td>
<td>2.03</td>
</tr>
</tbody>
</table>

* Enhancing the volume of KOH solution did not increase the yield of enzyme nor the specific activity.
† The figures in parentheses indicate the number of experiments in each case.
‡ Values are expressed in milligrams or units per g of fresh muscle tissue.

Protein concentrations were estimated colorimetrically by the biuret method of Gornall, Bardawill, and David (28). The estimation was usually performed with the protein solutions as obtained, except when Tris or ammonium sulfate was present. In these cases the protein was precipitated with 10% trichloroacetic acid and then resuspended in water. Crystalline bovine serum albumin was used as the standard.

A unit of P-fructokinase activity is defined as that amount of enzyme which catalyzes the formation of 1 mmole of fructose-1,6-diphosphate per minute at 28° under the conditions described. Activity in units is obtained directly by multiplying the change of absorbance at 340 µm, per minute, by 0.24 (derived from the molar absorbance index of 6.22 X 10³). Protein concentrations were estimated calorimetrically by the biuret method of Gornall, Bardawill, and David (28). The estimation was usually performed with the protein solutions as obtained, except when Tris or ammonium sulfate was present. In these cases the protein was precipitated with 10% trichloroacetic acid and then resuspended in water. Crystalline bovine serum albumin was used as the standard.

**RESULTS**

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The protective effect of fluoride was also shown by adding 10⁻³ M KF to a freshly prepared phosphate extract, in which case the high initial level of P-fructokinase activity again remained unchanged for at least 1 week.

The activity of the KOH extracts is stable during storage. The loss of activity from phosphate extracts cannot be ascribed to changes of pH for the pH of all three types of extracts does not change during a period of 50 hours. However, the P-fructokinase activity of the phosphate extract could be stabilized by adjusting the pH to 8.0 immediately after centrifugation.

The nature of the activity loss occurring in fresh phosphate extracts in the absence of KF has been further examined with the finding that it is reversible by ATP in the presence of magnesium (Fig. 1). The reactivation is particularly striking in fresh extracts prepared with 0.03 M KCl or water, for these have extremely low initial activity and may be restored to nearly the level of activity found in KF extracts (Table II).

The high yields and stability of P-fructokinase in KF extracts led us to adopt this method of extraction in the purification procedure described in the following section.

**Purification of P-Fructokinase from Rabbit Skeletal Muscle**

Among the various fractionation procedures developed, the one described below is most effective and convenient. It has been repeated successfully 15 times and the quantitative data presented are for a typical preparation.

**Step 1: Extraction**—A rabbit was decapitated after deep anesthesia with Nembutal and the muscles from the hind legs were taken as the first estimation of P-fructokinase activity done immediately after the animal was killed.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Loss of P-fructokinase activity during storage and restoration by MgATP. A K₃HPO₄ extract of rabbit skeletal muscle was prepared as described in Table I. The extract was kept at 4° and samples were taken at time intervals and assayed for P-fructokinase activity before (O——O) and after (●——●) incubation with MgATP as described in Table II. Zero time was taken as the first estimation of P-fructokinase activity done with the freshly prepared extract (90 minutes after the animal was killed).
and back rapidly removed and chilled in ice. Unless otherwise stated, the manipulations were carried out in a cold room at 4° and a Lourdes model IRA refrigerated centrifuge at 0° was used for all centrifugations. The muscles were passed through a chilled meat grinder, weighed (652 g), and extracted for 5 minutes with occasional stirring with 3 volumes of cold, freshly chilled meat grinder, weighed (652 g), and extracted for 5 minutes with occasional stirring with 3 volumes of cold, freshly prepared, 0.03 M KF containing 0.001 M disodium EDTA. The mixture was then homogenized twice for 30 seconds in a Waring Blender, and centrifuged for 60 minutes at 1,300 × g at 0° in an International model SR-3 refrigerated centrifuge. The residue was discarded and the supernatant fraction centrifuged for 30 minutes at 10,000 × g, in order to remove yellowish gelatinous material. The pH of the supernatant solution (Fraction 1) was 6.6 (glass electrode), and its volume was 1,580 ml. Fraction 1 can be kept at 4°, for at least 1 week, without loss of P-fructokinase activity.

Step 2: Heating and Precipitation with Isopropyl Alcohol—This step was carried out with two 790-ml portions of Fraction 1. The solution, in a 1-liter flask, was immersed in a water bath at 45° and stirred efficiently until the temperature had risen to 40°; 0.10 volume (79 ml) of 2-propanol was then added at a rate of 10 ml per minute. The solution, still immersed in the 45° water bath, was stirred for an additional 10 minutes. It was cooled to 20° in an ice bath, and the precipitate was removed by centrifuging for 20 minutes at 10,000 × g.

The supernatant fraction (830 ml) was transferred to a 1-liter flask, chilled to about 0° in a →4° bath, and 0.10 volume of isopropyl alcohol at −4° was added at a rate of 10 ml per minute with mechanical stirring. The mixture was stirred for an additional 20 minutes in the →4° bath and the precipitate collected by centrifuging for 20 minutes at 10,000 × g at →4°. The white precipitate was kept at 4° until the next batch was obtained, and then both were redissolved with 45 ml (3.0 of the volume of Fraction 1) of 0.1 M Tris-phosphate, pH 8, containing 2 × 10⁻⁴ M EDTA; 51 ml of an opalescent solution were obtained (Fraction 2).

Step 3: Fractionation on DEAE-cellulose Column—Fraction 2 was dialyzed against 0.1 M Tris-phosphate, pH 8, containing 2 × 10⁻⁴ M EDTA and 2 × 10⁻⁴ M fructose-1,6-di-P. Two changes of 500 ml of the solution were made during 16 hours. The dialyzed Fraction 2, was applied to a DEAE-cellulose column, 2.5 × 15 cm, which had been equilibrated with 0.1 M Tris-phosphate, pH 8, containing 2 × 10⁻⁴ M EDTA. The effluent from the column was connected to an ultraviolet absorption meter (Gilson Medical Electronics model UV-2801), and per cent transmittance at 280 nm was recorded (Esterline Angus model A.W.). Once the enzyme solution had passed into the resin, the inside of the column was washed twice with 50-ml portions of 0.1 M Tris-phosphate, pH 8, containing 2 × 10⁻⁴ M EDTA, and then 400 ml of the same buffer were passed through the column to remove a large peak of protein, without P-fructokinase activity. Elution was carried out with 0.3 M Tris-phosphate, pH 8, containing 2 × 10⁻⁴ M EDTA. A large peak, with P-fructokinase activity, appeared with the change of eluant. The main portion of the peak (fractions showing less than 80% transmittance at 280 nm) was collected (78 ml) and precipitated by addition of solid ammonium sulfate to 0.66 saturation. After 1-hour equilibration the precipitate was collected by centrifuging for 1 hour at 10,000 × g, and redissolved with 14 ml (1/3 of Fraction 1) of 0.1 M potassium phosphate, pH 8, containing 10⁻³ M EDTA (Fraction 3).

Effect of incubation with ATP on phosphofructokinase activity

Extracts were obtained from the muscle of the same rabbit, as described in Table I. Three volumes of extractant were used in each case, and P-fructokinase activity was assayed (see "Methods") before and after holding for 10 minutes at 28° in a medium containing 0.004 M ATP, 0.004 M MgSO₄, 0.04 M Tris-HCl, pH 8, and 0.10 volume of extract.

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Amount</th>
<th>Protein</th>
<th>P-Fructokinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>units/mg</td>
<td>Not treated with ATP</td>
</tr>
<tr>
<td>KF</td>
<td>0.03</td>
<td>22</td>
<td>2.40</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.03</td>
<td>24</td>
<td>1.93</td>
</tr>
<tr>
<td>KCl</td>
<td>0.03</td>
<td>20</td>
<td>0.32</td>
</tr>
<tr>
<td>H₂O</td>
<td>0.03</td>
<td>20</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Activity did not increase in controls held for 10 minutes at 28° without ATP and MgSO₄.

A summary of the data obtained for a typical preparation is given in Table III. Specific activities of different preparations vary from 130 to 150. Most of the more recent preparations have the higher specific activity.

Hereafter, Fraction 3 that has been dialyzed for 16 hours at 4° against 0.1 M potassium phosphate, pH 8, and 10⁻³ M EDTA, is referred to as purified P-fructokinase.

Attempts at Further Purification of Enzyme

Various techniques were used to fractionate further the proteins of the purified P-fructokinase. Those that led to lowest specific activities are not mentioned. The following were not destructive but failed to increase the specific activity of the purified enzyme: (a) ammonium sulfate fractionation; (b) ammonium sulfate precipitation and back extraction with ammonium sulfate of gradually decreasing concentration, as described by Zalun and Stahl (32); (c) 2-propanol fractionation at −4°; and (d) DEAE-cellulose chromatography. When linear gradient elution from 0.1 to 0.48 M Tris-phosphate, pH 8, was used instead of the stepwise elution developed for Step 3 of the purification procedure, the sharpness and symmetry of the peak was greatly affected, but the specific activity of the wide trailing peak was constant. (e) Molecular sieve chromatography on Sephadex G-100, Sephadex G-200, 3.3% agar, and 7% agar gel; (f) heat treatment of purified P-fructokinase (8 mg per ml) for 5 minutes at 70° in the presence of 0.02 M ATP and 0.02 M MgSO₄, or for 15 minutes at 60° (2 mg per ml) in the presence or absence of 5 × 10⁻² M fructose-1,6-di-P; and (g) ammonium sulfate fractionation after 3 hours at 0° in the presence of 2 M urea and 10⁻³ M fructose-1,6-di-P. Protein concentration was 14 mg ml⁻¹.

Stability of Purified P-Fructokinase

The purified enzyme at 20 mg per ml Solution a, 0.1 M potassium phosphate, pH 8, containing 10⁻³ M EDTA, lost 30% of its activity after 1 month at 4°. At 0.1 to 1 mg per ml, concentration likely to be found in column chromatography effluents, the purified P-fructokinase was stable for at least 45 hours at 4° in Solution a, in Solution b, 0.2 M Tris-phosphate, pH 8, and 2 × 10⁻⁴ M EDTA, or in Solution c, 0.2 M Tris-sulfate, pH 8, and 2 × 10⁻⁴ M EDTA.
1. Initial extract (from 652 g of muscle) .................................
2. After heating and 2-propanol precipitation ..........................
3. After DEAE-cellulose ..............................................

TABLE III
Purification of rabbit skeletal muscle phosphofructokinase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Concentration</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial extract (from 652 g of muscle)</td>
<td>1,580</td>
<td>45.6</td>
<td>72,050</td>
<td>18.1</td>
<td>2.52</td>
<td>(100)</td>
<td>(1)</td>
</tr>
<tr>
<td>2. After heating and 2-propanol precipitation</td>
<td>51</td>
<td>978.4</td>
<td>49,900</td>
<td>12.35</td>
<td>79.2</td>
<td>09.2</td>
<td>31.4</td>
</tr>
<tr>
<td>3. After DEAE-cellulose</td>
<td>14</td>
<td>3,240</td>
<td>45,300</td>
<td>25</td>
<td>129.6</td>
<td>62.9</td>
<td>51.4</td>
</tr>
</tbody>
</table>

Tris-HCl, pH 8, containing 10^{-4} M EDTA, and one of the following substrates at 2 \times 10^{-4} M: ATP, ADP, fructose 6-phosphate, or fructose-1,6-di-P.

At all protein concentrations tested the enzyme proved to be very unstable in pH 7 buffers, including 0.1 M potassium phosphate. It was also unstable in Tris-HCl buffer at pH 8. Purified P-fructokinase was also unstable when dialyzed for 16 hours at 4°, but could be stabilized by dialysis against Solution a or Solution e, even if any of the four substrates are as low as 2 \times 10^{-4} M. Recovery of activity after dialysis, in the above mentioned buffers, was about 90%.

FIG. 2. Sedimentation patterns of purified P-fructokinase
The photographs were taken at about 24 minutes after top speed (59,780 r.p.m.) was reached. The direction of sedimentation is from left to right. A, P-fructokinase (10 mg per ml) in 0.1 M potassium phosphate, pH 8, containing 10^{-3} M EDTA and 2 \times 10^{-4} M fructose-1,6-di-P. Corrected sedimentation coefficients expressed as s_{20,w} are 13.8, 20.9, and 31 S. B, P-fructokinase (10 mg per ml) in 0.1 M potassium phosphate, pH 8, containing 10^{-3} M EDTA, 10^{-2} M fructose-1,6-di-P, and 2 M urea. Run started 40 minutes after urea was added. s_{20,w} = 13.7 S. C, urea-treated P-fructokinase after dialysis against 0.1 M potassium phosphate, pH 8, containing 10^{-3} M EDTA and 2 \times 10^{-4} M fructose-1,6-di-P. Protein concentration was 6.8 mg per ml. s_{20,w} are 14.1, 19.9, and 31.7 S. D, urea-treated P-fructokinase after ammonium sulfate precipitation to 0.4 saturation and redissolution in 0.1 M potassium phosphate, pH 8, containing 10^{-3} M EDTA. Protein concentration was 10.1 mg per ml. s_{20,w} was not calculated since ammonium sulfate was present. There was no appreciable difference in the specific activity (100 to 125 units per mg) in the four samples shown.

At 4 mg per ml, concentrations required for activity assays and kinetic studies, the purified P-fructokinase was stable for at least 2 hours at 4° in Solution a, or in Solution d, 0.1 M Tris-phosphate, pH 8, and 2 \times 10^{-4} M EDTA, or in Solution e, 0.01 to 0.1 M Tris-HCl, pH 8, containing 10^{-4} M EDTA, and one of the following substrates at 2 \times 10^{-4} M: ATP, ADP, fructose 6-phosphate, or fructose-1,6-di-P.

Protein concentration was 6.8 mg per ml. s_{20,w} are 14.1, 19.9, and 31.7 S. D, urea-treated P-fructokinase after ammonium sulfate precipitation to 0.4 saturation and redissolution in 0.1 M potassium phosphate, pH 8, containing 10^{-3} M EDTA. Protein concentration was 10.1 mg per ml. s_{20,w} was not calculated since ammonium sulfate was present. There was no appreciable difference in the specific activity (100 to 125 units per mg) in the four samples shown.

Sedimentation Behavior
Sedimentation studies were performed in a Spinco model E ultracentrifuge at 20° at 59,780 r.p.m. The usual procedure was employed for the determination of sedimentation coefficients and for the conversion of the experimental values into those for standard conditions, s_{20,w} (33).

A typical sedimentation pattern of purified P-fructokinase showing three major components with s_{20,w} values of 13.8, 20.9, and 31 S is shown in Fig. 2a. The same pattern was observed at various protein concentrations, from 5 to 15 mg per ml, and was not altered when the purified P-fructokinase was dialyzed for 16 hours against 0.1 M Tris-HCl, pH 8, containing either 0.01 M ATP, 0.01 M AMP, or 0.01 M fructose-1,6-di-P.

This heterogeneous pattern was rather surprising in view of our inability to achieve further purification of the enzyme by several methods as described earlier. The possibility of heterogeneity due to polymerization of the protein as the result of the use of organic solvent in the purification method (34–36) was excluded since P-fructokinase prepared by a modified method in which an ammonium sulfate fractionation replaces the isopropyl alcohol step (Step 2) of the purification procedure, led to a preparation of the same final specific activity and the same sedimentation pattern.

When purified P-fructokinase (10 mg per ml) in 0.1 M potassium phosphate, pH 8, containing 0.001 M EDTA and 0.01 M fructose-1,6-di-P was incubated for 40 minutes in 2 M urea at 4°, and then analyzed in the ultracentrifuge, the sedimentation pattern (Fig. 2b) showed a single symmetrical peak with s_{20,w} of 13.7 S, in good agreement with the S value of the slower moving component of Fig. 2a. As no new peaks, or light material appeared, the result must be taken as indicating the conversion of the 20.9 and 31 S peaks to the 13.7 S one. This suggests that disaggregation of polymeric forms of P-fructokinase to a monomeric one occurred in the presence of urea.

Sucrose gradient experiments, described in the following section, also tend to support the existence of more than one form of the enzyme in purified preparations of P-fructokinase.
The time course of the inactivation of purified P-fructokinase, when treated with 2 M urea in the presence of 0.01 M fructose-1,6-di-P, is shown in Table IV. Under the same experimental conditions, but in the absence of fructose-1,6-di-P, the rate of inactivation of P-fructokinase is markedly increased.

When P-fructokinase was treated with 2 M urea as described above and urea was then removed by dialysis for 16 hours against 0.1 M potassium phosphate, pH 8, containing 10-3 M EDTA and 2 x 10-4 M fructose-1,6-di-P, a return to the original pattern was observed (Fig. 2c), and the \( s_{0.01} \) values obtained in this case were 14.1, 19.9, and 31.7 S.

Fig. 2d shows that the same pattern was obtained when a sample of the urea-treated P-fructokinase was precipitated at 0.40 saturation with solid ammonium sulfate, redissolved in 0.1 M potassium phosphate, pH 8, containing 10-3 M EDTA, and then analyzed in the ultracentrifuge.

Sucrose density gradient studies were performed by the method of Martin and Ames (37). The 5 to 20% sucrose gradient contained 0.1 M potassium phosphate, pH 8, and 0.001 M EDTA. Centrifugation was at 38,000 r.p.m. for 6 hours at 4°C in a Spinco model L SW-39 rotor. Pyruvate kinase, \( s_{0.01} = 10.0 \) S (38), was used as reference standard in all centrifugations. After removal from the rotor, the tubes were punctured with a 26-gauge syringe needle and 2-drop fractions collected in tubes containing 0.5 ml of 0.1 M potassium phosphate, pH 8, and 0.001 M EDTA. Usually 40 fractions were obtained, and were assayed for P-fructokinase and pyruvate kinase activity as described under "Methods."

With 0.1 to 1 mg of purified P-fructokinase in 0.1 ml, sedimentation patterns indicating heterogeneity of P-fructokinase activity were obtained. A typical centrifugation pattern is shown in Fig. 3A (continuous line). However, when only 0.020 mg of purified P-fructokinase was used a more homogeneous sedimentation pattern was obtained (Fig. 3A, broken line) that could be taken as an indication of disaggregation at lower protein concentration. By using pyruvate kinase as the reference standard (not shown in the figure), a \( s_{0.01} \) of 12.3 was calculated for P-fructokinase. This is somewhat lower than the value of 13.8 S obtained for the slower moving component in the ultracentrifuge studies. In these experiments the recovery of P-fructokinase activity was always greater than 70% and of pyruvate kinase, greater than 90%.

These changes in pattern with protein concentration were not observed with crystalline pyruvate kinase preparations.

When 0.1 ml (2 mg) of a KF extract of rabbit muscle was placed on a sucrose gradient and centrifuged, only one peak of P-fructokinase was observed with crystalline pyruvate kinase preparations. As demonstrated by Passonneau and Lowry (39) for less highly purified preparations of skeletal muscle P-fructokinase, and by several workers (4, 8, 40, 41) for P-fructokinase preparations from various sources, the purified preparation reported in the present work, when studied at pH 7 with ATP fixed at a high inhibitory concentration, yielded sigmoid curves when velocity was plotted against fructose 6-phosphate. At pH 8, the enzyme yielded normal hyperbolic plots of activity with changes of concentration of either of the two substrates. Detailed kinetic studies of the highly purified enzyme are under investigation and will be the subject of a future publication.

**TABLE IV**

Phosphofructokinase activity after treatment with urea and fructose diphosphate

<table>
<thead>
<tr>
<th>After urea addition</th>
<th>P-Fructokinase</th>
<th>P-Fructokinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hours</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>122</td>
<td>100</td>
</tr>
<tr>
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**FIG. 3.** Sedimentation pattern of P-fructokinase in a 5 to 20% sucrose gradient. A, ---, 0.200 mg of purified P-fructokinase in 0.10 ml were placed on top of a gradient, and after 6 hours of centrifugation at 38,000 r.p.m. at 4°C the gradient was fractionated and analyzed. Activity is expressed in units per 0.01 ml of the 2-drop fractions collected in 0.5 ml of 0.1 M potassium phosphate, pH 8, and 10-3 M EDTA. ---, as above, but 0.020 mg of purified P-fructokinase in 0.10 ml were used and activity is expressed in units per 0.10 ml of the collected fractions. B, in this gradient experiment, 0.10 ml of a KF extract of rabbit skeletal muscle (2 mg of protein) were used. P-Fructokinase activity is expressed in units per 0.05 ml of the collected fractions.

**Preliminary Kinetic Studies**

As demonstrated by Passonneau and Lowry (39) for less highly purified preparations of skeletal muscle P-fructokinase, and by several workers (4, 8, 40, 41) for P-fructokinase preparations from various sources, the purified preparation reported in the present work, when studied at pH 7 with ATP fixed at a high inhibitory concentration, yielded sigmoid curves when velocity was plotted against fructose 6-phosphate. At pH 8, the enzyme yielded normal hyperbolic plots of activity with changes of concentration of either of the two substrates. Detailed kinetic studies of the highly purified enzyme are under investigation and will be the subject of a future publication.

**DISCUSSION**

Phosphofructokinase activity is affected by many substances (2, 5, 8-10, 23, 42) and it is therefore not surprising that there
may be considerable variation in the amount of enzyme extracted from tissues by different procedures. It has been stated that the total P-fructokinase activity extracted from rabbit skeletal muscle is less than one-fourth that of aldolase activity in a similar extract (23). The present study shows that in extracts prepared by other techniques, P-fructokinase activity is 5 times as high as that of the KOH extracts reported earlier, and that loss of activity occurs unless fluoride is present in the extract. Therefore, the potential activity of P-fructokinase is fully as great as that of aldolase in rabbit skeletal muscle.

The decreasing activity of P-fructokinase in phosphate extracts, as well as the low activity in H_2O or KC1 extracts, is not caused by the low pH, since KF extracts have the same pH butunchanging activity for at least 1 week of storage at 4°C. It indicates instead an alteration of Tuesday, May 2, 1956. The molecular weight of the active form of guinea pig heart P-fructokinase has been reported by Mansour (45) as 300,000 to 450,000.

It is suggested that the two other components (20.4 S and 31.4 S) are formed by a concentration-dependent aggregation of this monomeric form of P-fructokinase. Many examples of association-dissociation phenomena for other proteins have been reported (46-53). When the molecular weight of these two peaks was calculated in a similar fashion, the values of 600,000 and 1,300,000, respectively, were obtained. The ratio of the molecular weight of the three peaks was 1:1.94:3.70, relatively close to a possible monomer-dimer-tetramer system.

Aggregation of purified P-fructokinase has also been observed by Morrison (data discussed in Reference 54) and Parmeggiani, Love, and Krebs (18). The latter report suggests aggregation occurring during storage of partially purified P-fructokinase. They also gave the sedimentation value of 35 S as the major component of their final mixture, in close agreement with the heaviest component of our studies. Unfortunately no data were given for the minor peaks to permit a comparison with our data.

SUMMARY

1. Rabbit skeletal muscle phosphofructokinase was purified by means of KF extraction, heating and 2-propanol fractionation, and DEAE-cellulose chromatography.

2. The high initial activity in KF extracts made possible the purification in only three steps, with 63% recovery and a final specific activity of 130 to 150 units per mg of protein.

3. No further purification was obtained by several methods of protein fractionation.

4. Ultracentrifuge studies showed the presence of three major components, but one single symmetrical peak was obtained with purified phosphofructokinase in 2 m urea.

5. Sucrose density gradient patterns were found to be dependent on protein concentration. Heterogeneity of phosphofructokinase activity was observed when 0.1 mg was used, but not when 0.020 mg of the purified enzyme was applied.

6. The above results appear to indicate disaggregation to a
single form of phosphofructokinase (molecular weight about 360,000). Only this form was shown to be present in sucrose gradient centrifugation of KF extracts of muscle.

Acknowledgments.—We wish to acknowledge fruitful discussions with Dr. John Morrison and to thank Dr. Pauline Yang and Gerardo Velasquez for their professional assistance in the analytical ultracontributions.

REFERENCES
