Purification and Properties of D-Fructose 1,6-Diphosphatase from Swine Kidney*

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SUMMARY

D-Fructose 1,6-diphosphatase was extensively purified from swine kidney by a simplified procedure which involved chromatography on cellulose phosphate, ammonium sulfate fractionation, and separation on Sephadex G-200. The final specific activity of the enzyme was 25.2 pmoles per min per mg and 34% of the activity present in the crude extract was recovered. The stability of the purified enzyme was greatly increased by the addition of 0.01 M β-mercaptoethanol and 0.0005 M 8-hydroxyquinoline. The purified enzyme was sensitive to inhibition by adenosine monophosphate and high concentrations of D-fructose 1,6-diphosphate. The preparation was essentially homogeneous after electrophoresis, ultracentrifugation, and elution from cellulose phosphate and Sephadex G-200 columns.

Ultracentrifugal sedimentation analysis revealed the presence of a major symmetrical peak with $s_{20,W} = 7.5$ and a minor symmetrical peak with $s_{20,W} = 3.6$. The proportion of the minor peak was increased by dialysis of the enzyme preparation. The molecular weight of swine kidney D-fructose 1,6-diphosphatase calculated from data obtained by ultracentrifugation, chromatography on Sephadex G-200, and sucrose density gradient centrifugation was 129,500 and that of the minor component was about 65,000. The turnover number of the enzyme was 3250 moles of D-fructose 1,6-diphosphate converted to fructose 6-phosphate and inorganic phosphate per min per mole of enzyme. Analysis of the amino acid composition indicated the presence of a comparatively large amount of basic amino acids, particularly lysine.

Numerous studies have now indicated that D-fructose 1,6-diphosphatase (EC 3.1.3.11) may have an important role in the regulation of the rate and direction of glucose and glycogen metabolism in liver and kidney (1, 2). Specific D-fructose 1,6-

diphosphatases have been purified from rabbit liver (3, 4), rat liver (5–7), pig kidney (8), C. utilis (9), spinach leaves (10), Engleria gracilis (11), Pseudomonas saccharophila (12), pea roots (13), Escherichia coli (14), and frog muscle (15). Investigations of the kinetic properties of many of these enzymes have shown that D-fructose 1,6-diphosphate, AMP, and sulfhydryl reagents profoundly influence their activity.

In more recent experiments it was observed that when the enzyme from swine kidney was incubated with ATP and magnesium ion in the presence of crude extracts, a striking decrease in activity occurred (16). To study this effect on a molecular level large amounts of purified enzyme were required. In the present investigation the isolation of D-fructose 1,6-diphosphatase as a homogeneous protein and the stabilization of the enzymatic activity were undertaken in order to use the purified enzyme as a substrate to study the mechanisms involved in the regulation of its activity in renal tissue. The previous purification procedure which yielded a partially purified but unstable preparation (8) was simplified, and the activity was stabilized by the addition of 8-hydroxyquinoline and β-mercaptoethanol. An essentially homogeneous preparation was obtained and the yield of purified enzyme was almost doubled over the previous procedure. The molecular weight, amino acid composition, and physical properties of D-fructose 1,6-diphosphatase from swine kidney are reported.

EXPERIMENTAL PROCEDURE

Determination of Enzyme Activity—D-Fructose 1,6-diphosphatase was assayed as described previously (8). The standard reaction mixture was incubated at 38° and contained, in 9 ml, 100 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 5 mM cysteine, 0.08 mM D-fructose 1,6-di-P, and an appropriate amount of enzyme. After 5 min the reaction was stopped by the introduction of 1 ml of 5 N H₂SO₄-2.5% ammonium molybdate for phosphate analysis. One unit of activity represents the amount of enzyme catalyzing the formation of 1 μmole of P₁ from fructose 1,6-di-P per min and specific activity is expressed as units per mg of protein.

The activities of aldolase (17), lactate dehydrogenase (18), alcohol dehydrogenase (19), and hexokinase were measured by standard spectrophotometric methods. The assays were linear in the concentration ranges used in these studies. One unit of activity is defined as the amount of enzyme catalyzing the con-
Table I

Purification of d-fructose 1,6-diphosphatase from swine kidney

The enzyme was assayed by the standard procedure described in the text. The activity of the crude extract was estimated by isolating the enzyme from an aliquot (1 ml) of the extract on cellulose-P before the determination.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>175</td>
<td>48</td>
<td>1220</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>2. Cellulose-P chromatography</td>
<td>712</td>
<td>0.14</td>
<td>921</td>
<td>9.2</td>
<td>74</td>
</tr>
<tr>
<td>3. Ammonium sulfate precipitation</td>
<td>9.7</td>
<td>3.50</td>
<td>548</td>
<td>16.1</td>
<td>44</td>
</tr>
<tr>
<td>4. Chromatography on Sephadex G-200</td>
<td>40</td>
<td>0.48</td>
<td>485</td>
<td>25.2</td>
<td>38</td>
</tr>
<tr>
<td>5. Second ammonium sulfate precipitation</td>
<td>5.5</td>
<td>3.20</td>
<td>430</td>
<td>24.4</td>
<td>34</td>
</tr>
</tbody>
</table>

version of 1 μ mole of substrate to product per min and specific activity as units per mg of protein in each case.

Analytical Methods—Purification of the enzyme and molecular weight determinations by chromatography on Sephadex G-200 columns were carried out according to the method of Andrews (20). The gel obtained from Pharmacia was washed with 0.1 N NaOH and then with water and 0.05 M Tris-HCl, pH 8.0. The Sephadex G-200 columns were equilibrated at 3°C, with 0.05 M Tris-HCl, pH 8.0, for 5 days before use.

Sucrose density ultracentrifugation was carried out according to Martin and Ames (21). Sucrose gradients of 5 to 20% in 0.01 M Tris-HCl (pH 8.0)-0.005 M β-mercaptoethanol-5 × 10⁻⁴ M 8-hydroxyquinoline were equilibrated at 3°C for several hours before the enzyme sample in 0.1 ml was layered on top. Sedimentation was carried out in a SW-50 rotor in a Spinco model L centrifuge for 14 hours at 37,000 rpm. After centrifugation, the tubes were punctured and 0.15-ml fractions were collected. Aldolase and alcohol dehydrogenase were used as standards.

The properties of the enzyme were also examined with the Spinco model E analytical ultracentrifuge in sedimentation velocity experiments. The procedures and calculations for the ultracentrifuge runs were carried out according to Schachman (22). The mobility of the purified enzyme at 3°C as a function of pH was examined by free boundary electrophoresis in a Perkin-Elmer model 38A apparatus.

The amino acid composition of protein hydrolysates was determined by chromatographic procedures (23) on dialyzed samples of the purified enzyme. The samples, 6 mg of protein, were hydrolyzed with constant boiling HCl at 110°C in evacuated sealed tubes for 22 and 48 hours. Inorganic phosphate was estimated colorimetrically (24, 25). In concentrated solutions protein was determined by the method of Lowry et al. (26), with crystalline bovine albumin as standard, and in dilute solutions it was assayed by the spectrophotometric method (27).

Materials—Cellulose phosphate obtained from Schleicher and Schuell was washed with 0.1 N NaOH and then with water to remove fines and alkali. The columns were equilibrated with 0.01 M Tris-HCl, pH 8.0, before use.

Enzyme grade tris(hydroxymethyl)aminomethane (General Biochemicals) and ammonium sulfate and sucrose (Mann) were used without purification. Aldolase, yeast alcohol dehydrogenase, lactate dehydrogenase, and hexokinase were purchased from Boehringer.

RESULTS

Purification of d-fructose 1,6-diphosphatase—The previous method used for the isolation of the enzyme was modified to exclude the steps involving heat treatment in the presence of 0.04 M MgCl₂, lyophilization, and extensive dialysis, since these procedures resulted in a decrease in the activity of the enzyme. Rapid inactivation of d-fructose 1,6-diphosphatase occurred when magnesium ion was added to the crude extract. This effect is probably due to the presence of an inactivating system in these extracts which is dependent on divalent ion (16), and it may be responsible for the very low and variable specific activities of enzyme preparations obtained by the previous procedure (8).

Fresh or frozen swine kidney, 75 g, was homogenized in 75 ml of ice-cold 0.05 M Tris-HCl, pH 8.0, for 5 min in a Waring Blender. The homogenate was then centrifuged at 34,000 × g for 10 min and the precipitate was resuspended in 75 ml of 0.05 M Tris-HCl, pH 8.0, and centrifuged. The combined extracts, 175 ml (Fraction 1, Table I), were diluted to 4 liters with distilled water and passed into a cellulose-P column (12 × 18 cm). The cellulose-P column was prepared by washing it with 0.1 N KOH and then with distilled water. Fines were removed by decantation and the washed resin was suspended in 0.01 M Tris-HCl, pH 8.0, before use. After the solution containing the enzyme had passed into the column the resin was washed with 3 liters of 0.05 M Tris-HCl, pH 8.0, and d-fructose 1,6-diphosphatase was eluted with 3 liters of 0.05 M Tris-HCl (pH 8.0)-0.5 M NaCl.

The enzyme was concentrated by a simplified procedure which involved dilution of the combined active fractions from the first cellulose-P column to 0.1 N NaCl with cold distilled water. Then 8-hydroxyquinoline (5 × 10⁻⁴ M) and β-mercaptoethanol (0.005 M) were added. The enzyme in the diluted salt solution was absorbed onto a much smaller cellulose-P column (5 × 10 cm) and it was eluted from this column with 300 ml of 0.05 M Tris-HCl (pH 8.0)-0.5 M NaCl (Fraction 2). This solution was dialyzed twice against 4 liters of 0.005 M β-mercaptoethanol-0.001 M 8-hydroxyquinoline-0.001 M Tris HCl (pH 8.0) for 1-hour periods and it was passed into two small cellulose-P columns (3 × 3 cm). The enzyme was finally eluted with 50 ml of 0.05 M Tris-HCl (pH 8.0)-0.5 M NaCl and this solution was dialyzed against 10 volumes of a solution 0.5 saturated with respect to ammonium sulfate at 4°C and adjusted to pH 8.0 with NH₄OH. The dialyzing solution also contained 0.01 M β-mercaptoethanol and 0.001 M 8-hydroxyquinoline. The resulting suspension was removed from the dialysis tubing and centrifuged, and the precipitate, which contained very little activity, was discarded. The supernatant was dialyzed against a solution of saturated ammonium sulfate at 4°C and pH 8.0. Solid ammonium sulfate was added to the dialyzing solution until the suspension inside the dialysis tubing was saturated with respect to ammonium sulfate. The suspension was centrifuged at 34,000 × g for 15 min and the precipitate was dissolved in 0.05 M Tris-HCl (pH 8.0)-0.005 M β-mercaptoethanol-0.005 M 8-hydroxyquinoline (Fraction 3). The procedure involving dialysis against ammonium sulfate was necessary in order to reduce further the volume of the solution and increase the protein concentration, thereby ensuring complete precipitation of d-fructose 1,6-diphosphatase in this step.
The enzyme was dialyzed for 1 hour against 4 liters of the same buffer and 2-ml aliquots were placed on Sephadex G-200 columns (2.5 × 35 cm), which were equilibrated with 0.05 M Tris-HCl, pH 8.0. The column was then eluted at a flow rate of 15 ml per hour and fractions of 2 to 4 ml were collected. Tubes containing n-fructose 1,6-diphosphatase activity were pooled (Fraction 4). This preparation was concentrated by ammonium sulfate precipitation as described for Fraction 3. The precipitate was dissolved in a small volume of 0.05 M Tris-HCl (pH 8.0)-0.01 M β-mercaptoethanol-0.0005 M 8-hydroxyquinoline (Fraction 5). All operations were carried out at 3°C.

The enzyme could be left at 3°C for at least 12 hours after adsorption on cellulose-P or precipitation with ammonium sulfate with little loss of activity. However, over 90% of the activity was lost when the crude extract was left overnight at 3°C or when dialysis against ammonium sulfate was carried out for more than 12 hours in the same solution. Purified enzyme preparations began to lose activity soon after isolation, and retained only about 60% of their activity when stored at −20°C for a period of 3 weeks. Repeated freezing and thawing or decreasing the salt concentration by dilution or dialysis resulted in a large variable loss of activity.

The addition of small amounts of 8-hydroxyquinoline to preparations at all stages of purification led to a significant increase in the stability of the enzyme. Renal n-fructose 1,6-diphosphatase requires thiol reducing agents for maximal activity (8) and, since the autoxidation of thiol groups is known to be accelerated by traces of metal ions (28), these findings suggest that small amounts of metal ion or other compounds which can react with 8-hydroxyquinoline may be associated with or bound to the enzyme protein. An interesting observation in this regard was that preparations which had been stored for long periods at −20°C could be completely reactivated by precipitating the enzyme with ammonium sulfate and redissolving it in a fresh solution containing β-mercaptoethanolamine and 8-hydroxyquinoline. For example, the activity of one preparation which was stored at −20°C declined from 610 units to 404 units in 3 weeks. When the enzyme was precipitated and redissolved in a solution containing fresh sulfhydryl reagents the activity of the preparation increased to 640 units. This treatment was effective with all of the partially inactive preparations, but it had no influence on the activity of fully active freshly prepared enzyme.

The purification obtained from 75 g of renal tissue is summarized in Table 1. The enzyme was purified approximately 160-fold and the final specific activity was about 25 units per mg. The purification summarized in Table 1 was obtained with at least five preparations. However, the yield of enzyme was usually higher than that reported in the table, and the recovery with some preparations was as high as 75%.

Elution Profile of n-Fructose 1,6-Diphosphatase on Sephadex G-200—The enzyme solution from Fraction 5 in 0.8 ml was applied to a Sephadex G-200 column (2.5 × 27 cm). The column was drained until the solution entered the gel and it was then eluted with 0.05 M Tris-HCl, pH 8.0. Fractions of 2 ml were collected at flow rates from 0.2 to 0.5 ml per min. The elution pattern of a typical gel filtration run at this stage of purification is illustrated in Fig. 1. The enzyme activity was eluted in a nearly symmetrical peak, and the active fractions had an almost constant specific activity. These fractions were combined and the enzyme was concentrated by precipitation with ammonium sulfate. Most of the work described in this communication was performed with enzyme purified to this stage.

Sucrose Density Gradient Centrifugation—The comparative distribution of n-fructose 1,6-diphosphatase, aldolase, and purified n-fructose 1,6-diphosphatase in 0.1 ml of 0.01 M Tris-HCl, pH 8.0, were layered on a 5 to 20% linear sucrose gradient (4.6 ml) containing 10 mM Tris-HCl (pH 8.0)-5 mM β-mercaptoethanolamine-0.5 mM 8-hydroxyquinoline. After centrifugation at 37,000 rpm for 14 hours at 3°C the solution was fractionated as described by Martin and Ames (21) and the activity of each enzyme was assayed as indicated in the text. The tubes containing 0.15-ml fractions were numbered starting from the bottom of the sucrose gradient.

Sedimentation Properties of Enzyme—Sedimentation velocity...
FIG. 3. Influence of dialysis on the ultracentrifuge patterns of purified swine kidney D-fructose 1,6-diphosphatase which was precipitated with ammonium sulfate and dissolved in a solution containing 0.05 M Tris-HCl (pH 8.0)-0.01 M β-mercaptoethanol-0.0005 M 8-hydroxyquinoline. The protein concentration in A analysis was carried out in a Spinco model E ultracentrifuge at 20°. The purified enzyme obtained after chromatography on Sephadex G-200 (25 units per mg) was concentrated by precipitation with ammonium sulfate and dissolved in 0.05 M Tris-HCl (pH 8.0)-0.01 M β-mercaptoethanol-0.0005 M 8-hydroxyquinoline. When this preparation was dialyzed for 50 min against 4 liters of the same buffer at 2°, the sedimentation pattern of the enzyme (10 mg per ml, 21 units per mg) showed a major symmetrical peak (s20,w = 7.5) and a minor slower moving symmetrical component (s20,w = 3.8) (Fig. 3, Series A). The major peak contained at least 80% of the total protein and contained all of the enzyme activity. About 20% of the total protein was present in a fraction, devoid of catalytic activity, which had a sedimentation constant which was about half of the major component. The presence of this second peak may be related to the fact that the preparation had lost 20% of its activity on dialysis.

Further dialysis against the same buffer for 4 hours caused denaturation and precipitation of some protein and decreased the specific activity of the enzyme preparation. The resulting turbid solution was centrifuged at 34,000 × g for 20 min and the precipitate was washed once with buffer. This material was insoluble in solutions containing up to 3.0 M salt at pH 8.0 and it was devoid of D-fructose 1,6-diphosphatase activity. When the enzyme (7 mg per ml) in the supernatant solution was examined in the ultracentrifuge the same major component (s20,w = 7.5) and minor peak (s20,w = 3.6) were observed (Fig. 3, Series B). However, the amount of the major component in the sample had decreased.

The preparation was then dialyzed for 16 hours against the same buffer and more protein precipitated. The specific activity of D-fructose 1,6-diphosphatase in the supernatant solution after centrifugation was 7.5 units per mg and the protein concentration was 3.7 mg per ml. Ultracentrifugal analysis of the sample (Fig. 3, Series C) showed that the amount of protein in the slower moving peak (s20,w = 4.6) had increased by 30%. The quantity present comprised 70% of the protein in the sample and was noticeably greater than the amount of the faster component (s20,w = 7.6).

The lower molecular weight material was readily isolated by chromatography on Sephadex G-200. The two components separated by chromatography on a Sephadex G-200 column (2.2 × 35 cm) were assayed for enzyme activity. Only the higher molecular weight component (s20,w = 7.5) contained activity and the specific activity of this fraction was once again 25 units per mg. Examination of samples after various periods of dialysis indicated that the loss of catalytic activity could be related to the appearance of the low molecular weight peak and the precipitation of protein from the solution. It is likely that the minor component represents a less soluble monomeric unit of D-fructose 1,6-diphosphatase which is formed during denaturation and precipitation of the enzyme by dialysis.
These results indicate that before dialysis the purified preparation was probably a single protein and that the protein was D-fructose 1,6-diphosphatase. The active enzyme in the preparation sedimented as a single boundary in the ultracentrifuge with an $s_{20, w}$ of 7.5 and there was no significant effect of concentration in the range from 10 to 3.7 mg per ml of protein. The sedimentation constant of renal D-fructose 1,6-diphosphatase reported here, 7.5, is of the same order of magnitude as that found by Pontremoli et al. (4) for the crystalline enzyme from liver, 7.2. The molecular weight of the enzyme was determined on three different preparations by combining information from sedimentation analysis and chromatography on Sephadex G-200 (22, 29). The molecular weight was calculated from the formula

$$\text{Mol wt} = \frac{RT_{s_{20, w}}}{D_{p,s}(1 - \beta)}$$

The partial specific volume, $\beta = 0.735 \text{ cm}^3 \text{ g}^{-1}$, was calculated from the amino acid composition and a diffusion constant of $5.44 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ was calculated from data obtained by chromatography on Sephadex G-200 (29, 30). The other terms were values from the literature. From the sedimentation constants obtained by ultracentrifugation and sucrose density centrifugation an average value of 129,500 ± 700 g per mole was obtained. The molecular weight of the slower moving component was approximately 65,000 ± 900 g per mole.

**Determination of Molecular Weight by Chromatography on Sephadex G-200—**A comparison of the elution volume of n-fructose 1,6-diphosphatase with the elution volume of several

![Graph](image)

**Fig. 4**. Estimation of the molecular weight of D-fructose 1,6-diphosphatase by chromatography on Sephadex G-200 columns equilibrated with 0.05 M Tris-HCl, pH 8.0, for 5 days. The enzymes were eluted with the same buffer and 0.5-ml fractions were collected. Each fraction was assayed for enzymatic activity as described in the text. In the figure, the elution volume of n-fructose 1,6-diphosphatase with the elution volume of several other enzymes was obtained.

In the inset, approximately 100 units each of renal n-fructose 1,6-diphosphatase, aldolase, lactate dehydrogenase, and hexokinase in 0.7 ml were applied to a Sephadex G-200 column (2.2 X 50 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.0, for 5 days. The enzymes were eluted with the same buffer and 0.5-ml fractions were collected. Each fraction was assayed for enzymatic activity as described in the text. In the figure, the elution volume of n-fructose 1,6-diphosphatase with the elution volume of several other enzymes was obtained.

The partial specific volume, $\beta = 0.735 \text{ cm}^3 \text{ g}^{-1}$, was calculated from the amino acid composition and a diffusion constant of $5.44 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ was calculated from data obtained by chromatography on Sephadex G-200 (29, 30). The other terms were values from the literature. From the sedimentation constants obtained by ultracentrifugation and sucrose density centrifugation an average value of 129,500 ± 700 g per mole was obtained. The molecular weight of the slower moving component was approximately 65,000 ± 900 g per mole.

### Table II

**Amino acid composition of swine kidney D-fructose 1,6-diphosphatase**

Approximately 6 mg of purified D-fructose 1,6-diphosphatase were hydrolyzed for the indicated time at 110° at a concentration of 1 mg per ml in constant boiling HCl.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount of amino acid after hydrolysis for</th>
<th>Amount of residue</th>
<th>No. of residues per 100 g of protein</th>
<th>Minimum molecular weight</th>
<th>Residues per 130,000 g of protein</th>
<th>Nearest integer per 130,000 g of protein</th>
<th>Integral no. X mol wt of residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 hrs</td>
<td>48 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>6.04</td>
<td>6.10</td>
<td>6.04</td>
<td>86.9</td>
<td>1.176</td>
<td>110.5</td>
<td>111</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.10</td>
<td>6.11</td>
<td>6.10</td>
<td>39.5</td>
<td>5.629</td>
<td>51.4</td>
<td>51</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.83</td>
<td>11.05</td>
<td>10.83</td>
<td>94.1</td>
<td>1.062</td>
<td>122.4</td>
<td>122</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.16</td>
<td>1.10</td>
<td>1.10</td>
<td>11.3</td>
<td>8.888</td>
<td>14.6</td>
<td>15</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.76</td>
<td>10.65</td>
<td>10.76</td>
<td>83.4</td>
<td>1.199</td>
<td>108.4</td>
<td>108</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.72</td>
<td>4.75</td>
<td>4.72</td>
<td>82.6</td>
<td>1.210</td>
<td>107.4</td>
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<tr>
<td>Histidine</td>
<td>2.29</td>
<td>2.31</td>
<td>2.29</td>
<td>16.7</td>
<td>5.901</td>
<td>21.7</td>
<td>22</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.63</td>
<td>5.94</td>
<td>5.63</td>
<td>49.8</td>
<td>2.009</td>
<td>64.7</td>
<td>65</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.64</td>
<td>8.91</td>
<td>8.64</td>
<td>76.1</td>
<td>1.914</td>
<td>99.0</td>
<td>99</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.49</td>
<td>9.53</td>
<td>9.49</td>
<td>74.0</td>
<td>1.352</td>
<td>96.2</td>
<td>96</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.13</td>
<td>3.07</td>
<td>3.13</td>
<td>23.9</td>
<td>4.103</td>
<td>31.0</td>
<td>31</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.29</td>
<td>4.40</td>
<td>4.29</td>
<td>28.1</td>
<td>3.433</td>
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<tr>
<td>Proline</td>
<td>4.20</td>
<td>4.34</td>
<td>4.20</td>
<td>43.8</td>
<td>2.282</td>
<td>57.0</td>
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<tr>
<td>Serine</td>
<td>4.50</td>
<td>4.76</td>
<td>4.50</td>
<td>51.7</td>
<td>1.935</td>
<td>97.3</td>
<td>97</td>
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<tr>
<td>Threonine</td>
<td>5.38</td>
<td>5.31</td>
<td>5.38</td>
<td>53.3</td>
<td>1.877</td>
<td>69.2</td>
<td>69</td>
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<tr>
<td>Tyrosine</td>
<td>5.17</td>
<td>5.34</td>
<td>5.17</td>
<td>31.7</td>
<td>3.159</td>
<td>41.1</td>
<td>41</td>
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<tr>
<td>Tryptophan</td>
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<td>0.97</td>
<td>0.97</td>
<td>5.2</td>
<td>19.230</td>
<td>6.8</td>
<td>7</td>
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<tr>
<td>Valine</td>
<td>6.16</td>
<td>6.19</td>
<td>6.16</td>
<td>62.1</td>
<td>1.609</td>
<td>80.8</td>
<td>81</td>
</tr>
<tr>
<td>Ammonia</td>
<td>1.27</td>
<td>1.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>99.88a</td>
<td>100,790</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Values were estimated by taking the nearest whole number.

*b The 22-hour hydrolysis values were used for these amino acids.

*c The total amino acid residues do not include ammonia.
standard enzymes of known molecular weight and Stokes radius is presented in Fig. 4. The empirical correlation of the log of the molecular weight with elution volume indicated a molecular weight of about 130,000 g per mole for n-fructose 1,6-diphosphatase.

The Stokes radius calculated by the method of Acker (30) was 39.6 A. The effective pore radius of the gel used in these calculations was 187 A. The diffusion constant of the enzyme was estimated according to the procedure of Siegel and Monty (29) from the relationship $D_r = kT/6\pi\eta r$, where $r$ is the Stokes radius, 39.6 A, calculated from the elution volume; $k$ is the Boltzmann constant; and $\eta$ is the viscosity of water at 20°C, 0.01005 poise. The diffusion constant of n-fructose 1,6-diphosphatase measured by this method was $5.44 \times 10^{-4}$ cm$^2$ sec$^{-1}$. The position of the lighter component ($r_{90,000} = 3.6$ to 3.8) on elution from Sephadex G-200 indicated a molecular weight of 65,000 g per mole.

Amino Acid Composition—The analysis was performed on samples which had been hydrolyzed with constant boiling HCl at 110°C for 22 and 48 hours (Table II). The data were obtained according to the chromatographic method of Spackman, Stein, and Moore (23). Tryptophan was determined by alkaline hydrolysis and was assayed by the procedure of Goodwin and Morton (31). The value of amide residues of 1.27 g/100 g of protein was obtained by measuring the release of ammonia during acid hydrolysis. The data presented in the table were not corrected for the destruction of serine and threonine or for the incomplete hydrolysis of valine and isoleucine.

Renal n-fructose 1,6-diphosphatase contained relatively large amounts of alanine and arginine, which may in part explain the great affinity of this enzyme for cellulose-P resin. The minimum molecular weight, calculated from the data presented in Table II, was 129,308 g per mole, which compares well with the values obtained by sedimentation analysis and Sephadex G-200 chromatography. As indicated in the table, the calculated number of amino acid residues approaches a whole number in most cases. The enzyme contained relatively small amounts of half-cystine and tryptophan. The percentage weight of nitrogen of the enzyme determined from the amino acid composition was 18%.

After the removal of $\beta$-mercaptoethylamine and 8-hydroxyquinoline the A$_{280}$:A$_{650}$ ratio of the preparation was 1.72. From the average amino acid content and values of $\beta$ of amino acid residues obtained as described by Cohn and Edsall (32) a partial specific volume of 0.735 cm$^3$ per g was calculated for n-fructose 1,6-diphosphatase.

With an average molecular weight value of 130,000 g per mole and a maximum specific activity of 25.2 mmoles per min per mg, the turnover number of the enzyme was calculated to be about 3250 moles of n-fructose 6,16-diphosphate converted to fructose-6P and P$_1$ per min per mole of enzyme under the standard assay conditions described in the text.

Electrophoretic Properties of Renal n-Fructose 1,6-Diphosphatase—Electrophoretic analyses were performed at pH 5.5, 7.0, 8.0, and 8.8 in a Perkin-Elmer model 38A apparatus (Fig. 5). At pH 5.5 some protein was denatured and precipitated, although there was only a 10% decline in the specific activity of the protein which remained in solution. The homogeneity of purified n-fructose 1,6-diphosphatase by electrophoresis was examined with several different buffers. The enzyme migrated essentially as a single component in each buffer. It was apparent that the peaks were not symmetrical and only at pH 7.0 in 0.1 M phosphate buffer was the resolution adequate enough to permit the observation of a minor component. The preparation contained from 80 to 90% active enzyme and the minor component could represent denatured monomeric enzyme. An approximate isoelectric point of 5.9 was obtained by interpolation of the data derived from the mobilities which are listed in the legend of Fig. 5.

DISCUSSION

In previous studies (8) the purification of n-fructose 1,6-diphosphatase from extracts of renal tissue was impeded by the instability of the enzyme. Addition of $\beta$-mercaptoethylamine and 8-hydroxyquinoline to the preparation in the presence of 0.1 M NaCl or other salts stabilized the activity of the enzyme considerably, permitting a 34% minimum recovery of the purified enzyme from kidney extracts. In some preparations yields of 50 to 75% were obtained. The highly purified enzyme preparations showed constant specific activities in the elution patterns from cellulose-P and Sephadex G-200 columns.

The purified preparations appeared nearly homogeneous on
sedimentation velocity analysis and electrophoresis. The major component, with $s_{20,w} = 7.5$ was converted to the minor component, with $s_{20,w} = 3.8$, during dialysis, which suggested that the minor component might be a disaggregated form of the enzyme. The sedimentation coefficient and molecular weight of swine kidney D-fructose 1,6-diphosphatase (129, 500), as determined by several independent measurements, are similar to those reported for the crystalline enzymes from rabbit liver (137,000) (4) and C. utilis (about 100,000) (33). These enzymes can also be dissociated into subunits, having about half of the molecular weight of the native enzyme under certain conditions (33, 34). The subunits prepared from rabbit liver enzyme were inactive, whereas those obtained from the C. utilis enzyme still had some activity. When the liver enzyme subunits were reassOCIatEd the catalytic activity was restored. The lower molecular weight component formed from the swine kidney enzyme was inactive, whereas those obtained from the C. utilis enzyme still had some activity. When the liver enzyme subunits were reassOCIated the catalytic activity was restored. The lower molecular weight component formed from the swine kidney enzyme was inactive, whereas those obtained from the C. utilis enzyme still had some activity. When solutions of this component were concentrated the protein precipitated and the subunits did not reassociate. The data thus far suggest that all three enzymes are composed of at least two subunits.

The principal step for the purification of swine kidney D-fructose 1,6-diphosphatase by the procedure reported in this paper depends upon its affinity for cellulose phosphate resin. The reason of this tight binding may be explained, in part, by the high content of lysine in this protein.

The purified enzyme was relatively stable for at least 1 month at $-20^\circ$ and could be reactivated when the specific activity fell to a very low level. The remarkable stabilizing effect of 8-hydroxyquinoline may be due to the prevention of autoxidation of the sulphydryl groups of the enzyme or to a retardation of the oxidation of $\beta$-mercaptoethylamine which in turn protects the enzyme. These results may also explain the stabilizing effect of ethylenediaminetetraacetate on this enzyme in crude extracts, since this compound would also be expected to chelate metal ions. The yield of D-fructose 1,6-diphosphatase from swine kidney was greatly increased; with a 34% yield about 17.6 mg of purified enzyme was obtained from 75 g of kidney cortex. However, a more important objective was achieved, in that the final specific activity of the purified enzyme prepared by the procedure described in this report was constant. Furthermore, the inherent instability of this enzyme to storage at $-20^\circ$ which previously interfered with its use as a substrate in other reactions was circumvented by reactivation of stored preparations to their original specific activity.

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