CRYSTALLINE HEXOKINASE (HETEROPHOSPHATASE)**‡§

METHOD OF ISOLATION AND PROPERTIES

BY M. KUNITZ AND MARGARET R. MCDONALD

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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INTRODUCTION

Meyerhof (1) in 1927 obtained an "activator" from yeast which restored to aged muscle juice its lost ability to ferment glucose. In 1935 von Euler and Adler (2) found an enzyme in yeast which catalyzed the transfer of phosphorus from adenosine triphosphate to hexoses. They named the enzyme "heterophosphatase." Meyerhof (3) then established that his "activator" of 1927, which he renamed "hexokinase" had the same enzymatic property as von Euler's "heterophosphatase."

Colowick and Kalckar (4) in 1941 showed that hexokinase catalyzes the transfer of one phosphate group from adenosine triphosphate to glucose with the liberation of one hydrogen equivalent of acid. The reaction is represented as follows:

\[
\begin{align*}
R-OH + \text{Glucose} & \rightarrow R'--O--P--O--P--O--P--O' + \\
& \text{Adenosine triphosphate} \quad \text{Glucose-6-phosphate} \\
& \text{Adenosine diphosphate}
\end{align*}
\]

* A preliminary note on the isolation of crystalline hexokinase and three other crystalline proteins from yeast has been published recently (Kunitz, M., and McDonald, M. R., J. Gen. Physiol., 1946, 29, 143).

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§ The writers acknowledge with thanks the kind cooperation of Dr. Sidney P. Colowick.

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This paper describes the isolation from baker's yeast of pure hexokinase in crystalline form. Crystalline hexokinase is a protein of the albumin type. It is crystallized at 5°C. in the presence of ammonium sulfate and dilute phosphate buffer pH 7.0. The crystals become relatively pure after two or three crystallizations as tested by solubility, electrophoresis, and ultracentrifuge measurements. The hexokinase activity of the crystals is associated with the protein nature of the material.

Solutions of crystalline hexokinase in dilute buffers of pH 4.5-7.5 are stable for several days when kept at a temperature of 5°C. or lower. At higher temperatures the hexokinase activity is rapidly lost. The loss in activity is accompanied by denaturation of the protein. The point of maximum stability is around pH 5.0 which is near the isoelectric point of the material. The elementary composition of crystalline hexokinase is that of a typical protein. It contains 0.11 per cent phosphorus which would indicate a minimum molecular weight of about 30,000. Sedimentation and diffusion measurements in acetate buffer pH 5.5 at 1°C. gave a molecular weight of 96,600.

Method of Preparation of Crystalline Hexokinase from Baker's Yeast

Meyerhof's (1) original method for preparing hexokinase from yeast consisted essentially in plasmolyzing baker's yeast by means of toluene and extracting the plasmolyzed yeast with water at 35°C. The active material was then precipitated in 50 per cent alcohol at 0°C. Berger, Slein, Colowick, and Cori (5) reported that purification of crude hexokinase by means of alcohol is more effective if carried out in a solution containing 1 per cent dextrose and 0.05 M acetate buffer pH 5.2-5.4

Meyerhof's method of plasmolysis and of extraction with water was used as a starting step in the present work on the preparation of hexokinase. Advantage was also taken of Meyerhof's method of purification by means of alcohol as modified by Colowick and associates. The basis of the present method, however, is the technique of purification and crystallization of proteins by means of ammonium sulfate from concentrated protein solutions as developed by Northrop, Kunitz, and others for the isolation of crystalline enzymes.

The method consists essentially of the following steps:

1. Plasmolysis with toluene and extraction of the plasmolyzed yeast with water.
2. Concentration and fractionation by means of ammonium sulfate.
3. Removal of "inert" crystalline proteins.
Several crystalline proteins, including a crystalline yellow protein, appear during the process of fractionation with ammonium sulfate. These proteins do not possess hexokinase activity. The separation of these crystalline proteins, however, leads to a considerable improvement in the hexokinase activity of the remaining fractions.
4. Dialysis.
5. Purification by fractional precipitation with alcohol.
6. Crystallization in the presence of ammonium sulfate.
7. Recrystallization.

All operations, except when mentioned otherwise, are done at temperatures of 8-10°C. The pH of the preparations is tested by the drop method, by mixing 1 drop of solution with 1 drop of 0.01 per cent Clark indicator on a test plate and comparing the colors with those of drops of 0.1 m standard buffers mixed on the plate with the same indicators. The saturated ammonium sulfate is prepared at about 20°C.

The details of the method of preparation of crystalline hexokinase are as follows:

1. Plasmolysis and Extraction.—25 pounds of fresh Fleischmann’s baker’s yeast is broken by hand into small fragments and then macerated by means of a wooden paddle in a large aluminum or enameled vessel with 6 liters of warm toluene of about 40°C. The vessel is placed in a water bath of about 45°C. The maceration is continued until the yeast is heated to 37°C. at which temperature the yeast rapidly liquefies and begins to “work.” A rapid liberation of CO₂ takes place and the volume of the mixture increases considerably. It is left in the room for 2 to 3 hours and then cooled to 10°C. in an ice water bath. The thick mixture of plasmolyzed yeast and toluene is distributed in four 10 liter jars, 3 liters of distilled water of about 5°C. is added to each jar and mixed. The jars are left for 18 hours at 5°C. A layer of an emulsion of the toluene with yeast stromata gradually forms above the yeast-water suspension.

The yeast suspension is siphoned off from under the toluene-stromata emulsion and then filtered in the cold room at 8-10°C. with suction on four 32 cm. Büchner funnels with the aid of 100 gm. hyflo super-cel¹ per liter of fluid using Eaton-Dikeman No. 303 paper. The residue on each funnel is washed once with 1 liter of cold water. (The toluene is partly recovered by filtering the toluene-stromata emulsion with the aid of 100 gm. hyflo super-cel per liter.)

2. Fractionation with Ammonium Sulfate.—The clear filtrate and washings are brought to 0.5 saturation with solid ammonium sulfate (314 gm. per liter of filtrate) and the precipitate formed is filtered with suction with the aid of 10 gm. of standard super-cel, plus 10 gm. filter-cell per liter of solution. The residue is discarded. The clear filtrate is brought to 0.65 saturation by further addition of 99.3 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.6 fraction) is filtered with suction on No. 612 E. & D. paper on large funnels. The filtrate is brought to 0.7 saturation by the addition of 33.8 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.7 fraction) is filtered with suction after standing for 16 to 18 hours at a temperature not higher than 10°C. Both 0.6 and 0.7 fractions are used for isolation of hexokinase. The purification of the 0.7 fraction will be described first since it involves fewer steps and the yield of crystalline hexokinase obtainable is often greater than in the 0.6 fraction.

3. Removal of “Inert” Crystalline Proteins.—(a) Isolation of crystals of “yeast

¹ Supplied by Johns-Manville Corporation, New York.
protein, No. 2." Each gram$^2$ of the 0.7 fraction is dissolved in 2 ml. cold water, the pH adjusted to 7.4 with 1 M sodium hydroxide, then enough saturated ammonium

$^2$This expression is used to denote the relative volume of solvent in which the precipitate is dissolved. It does not mean that each gram is dissolved separately.
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sulfate added with stirring to trace of turbidity. It generally requires about 2.5 ml. ammonium sulfate per gm. filter cake. The solution is kept in an ice bath during this operation and then stored at about 5°C for 6 to 8 days. A gel of very fine crystals of yeast protein, No. 2, is gradually formed. This is filtered off with the aid of 5 gm. standard super-cel per 100 ml. of solution. The filtrate is brought to 0.85 saturation with solid ammonium sulfate, added slowly with stirring (21.5 gm. per 100 ml. of filtrate). The precipitate formed is filtered with suction. Filter cake = fraction 0.71.

(b) Isolation of crystals of “yeast protein, No. 2.” Each gram of filter cake of fraction 0.71 is dissolved in 0.5 ml. cold water at 2-5°C. Saturated ammonium sulfate is added to trace of turbidity, then 1 M sodium hydroxide to pH 7.5. The solution is left at 5°C, for 5 to 6 days. Prismatic crystals of yeast protein, No. 3 (Fig. 2), are gradually formed. The solution is centrifuged at 5-10°C. The supernatant solution is dilute with 2 volumes of cold 0.65 saturated ammonium sulfate pH 7.2 (containing 2 ml. 5 M sodium hydroxide per liter), 10 gm. of standard super-cel and 43 ml. saturated ammonium sulfate are then added for each 100 ml. of original supernatant solution. The ammonium sulfate solution is added slowly with stirring. The precipitate formed is filtered with suction, resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction. Filter cake = fraction 0.72.

(c) Isolation of crystals of “yeast yellow protein.” Each gram of 0.6 fraction is dissolved in 1 ml. of cold water and 1 ml. saturated ammonium sulfate is added; a slight precipitate generally forms. The turbid solution is stored for 20 to 24 hours at 5°C. It is then centrifuged and the residue is discarded. The filtrate is titrated with 0.5 N NaOH to pH 7.2 and stored at 5°C for 5 to 7 days. The gelatinous precipitate formed is removed by filtration with the aid of 10 gm. hyflo super-cel per 100 ml. of solution. (The residue may yield No. 2 crystals when treated as described in footnote 3.) The clear filtrate is brought to 0.85 saturation by the slow addition of 233 ml. saturated ammonium sulfate per 100 ml. filtrate, and filtered with suction. Yield 200 to 300 gm. filter cake. This is dissolved in one half volume of H2O, saturated ammonium sulfate is added to trace of turbidity. The solution is then titrated with 1

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3 Recrystallization of yeast protein, No. 2: The protein is extracted from the super-cel with cold water of about 5°C and is reprecipitated with solid ammonium sulfate at 0.85 saturation. The precipitate is dissolved in about 3 volumes of cold water, 8.0 ml. saturated ammonium sulfate is added for every 10 ml. of water, and the solution is titrated with 0.5 N NaOH to pH 7.4 and stored for 7 days or longer at about 5°C. Gradual crystallization of long needles takes place (Fig. 1).

Yeast protein, No. 2, can also be recrystallized at pH 4.3 in 0.5 saturated ammonium sulfate at 20-25°C. Under these conditions the crystals appear in the form of hexagonal and rhomboid plates.

4 Recrystallization of yeast protein, No. 3: The centrifuged residue of crystals is dissolved in about 2 volumes of cold water and 2.5 volumes of saturated ammonium sulfate is added; the clear solution is titrated with 0.5 N NaOH to pH 7.4 and stored for several days at 5°C. Gradual crystallization of well formed prisms takes place.
n NaOH to pH 7.5, and stored at 6°C. Crystals of a yellow protein (Fig. 3) gradually form. These are centrifuged after 1 to 2 weeks.  

Recrystallization of the yeast yellow protein. The centrifuged residue of crystals is dissolved in an equal volume of cold water and one half volume of saturated ammo-
The supernatant solution is diluted with 2 volumes of cold 0.65 saturated ammonium sulfate of pH 7.2. 10 gm. of standard super-cel and 43 ml. saturated ammonium sulfate is gradually added. The solution is titrated with 0.5 N NaOH to pH 7.5 and stored at 5°C. for several days. Rhombohedral crystals of the yellow protein gradually form.

Fig. 3. Crystals of yeast yellow protein. × 128.
sulfate are added for each 100 ml. of the original supernatant solution. The suspension is filtered with suction on No. 3 paper. The precipitate is resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction.
Filter cake about 50 gm.—fraction 0.62—is treated exactly as described in section 3b to yield fraction 0.63. A certain amount of No. 3 crystals are generally formed during this step.\footnote{\ If the activity of fraction 0.72 is less than 50 hexokinase units per mg. protein, then step 3b should be repeated. The final precipitate, fraction 0.72, 0.73 or 0.63, as}
4. Dialysis in Dextrose Solution.—Each gram of filter cake of fraction 0.72, 0.73, or 0.63, as the case may be, is dissolved in 1 ml. of 1 per cent dextrose. The solution is transferred into a collodion bag provided with a large glass bead and is dialyzed in a rocking machine for 18 hours at about 5°C. against slowly running 1 per cent dextrose.

5. Fractional Precipitation with Alcohol.—(a) The dialyzed solution is diluted with cold 1 per cent dextrose to a volume equal in milliliters to 4.75 times the original weight in grams of the filter cake in step 4, and 1 ml. 1 M acetate buffer pH 5.4 is added per 19 ml. of solution. The solution is cooled in a freezing mixture bath to about −2°C. and 35.7 ml. cold 95 per cent alcohol added gradually with stirring to each 100 ml. of solution to a concentration of 25 per cent. The precipitate formed is centrifuged off at about 5°C. The supernatant fluid is measured, cooled again to −2°C., and more 95 per cent alcohol added to a final concentration of 50 per cent (35.5 ml. per 100 ml. supernatant). The suspension is centrifuged, and the supernatant discarded. The residue is resuspended in a volume of cold 1 per cent dextrose equal to 2 times the weight of the filter cake in step 4 and recentrifuged. The clear supernatant is brought to pH 5.4 by the addition of 5 ml. 1 M acetate buffer of that pH to 100 ml. of solution and the alcohol fractionation is repeated. (This repetition is unnecessary if the activity of the material before dialysis is above 100 units per mg. protein. In that case the residue left after centrifugation of the 50 per cent alcohol is suspended not in dextrose but in a volume of cold water equal to 5 times the weight of the filter cake in step 4. The suspension is then centrifuged and the supernatant solution is treated with ammonium sulfate as described in step 5b.)

(b) The final residue left after centrifugation of the 50 per cent alcohol mixture is resuspended in a volume of cold water equal to twice the weight of filter cake before dialysis in step 4. The suspension is recentrifuged. The clear supernatant fluid is brought with solid ammonium sulfate to 0.90 saturation (66 gm. per 100 ml.). The precipitate formed by the addition of ammonium sulfate is filtered with suction. The filtration generally takes 1 or 2 days.

6. Crystallization.—Each gram of filter cake obtained from step 5b is dissolved in 1 ml. 0.1 M phosphate buffer pH 7.0 at about 3°C. and 1 ml. saturated ammonium sulfate is added slowly. If a heavy precipitate is formed then a few drops of phosphate buffer are added to incipient clearing. The solution is centrifuged. The clear supernatant solution is left at about 5°C. Crystals in the form of long prisms or fine needles (Figs. 4a and 4b) gradually appear. Seeding hastens the crystallization, as usual.

7. Recrystallization.—The suspension of crystals is centrifuged after 7 to 10 days. The residue is dissolved in a minimum amount of cold 0.1 M phosphate buffer pH 7.0 and a volume of saturated ammonium sulfate is added equal to 1.4 volumes of the buffer used. The solution is left at 5°C. for crystallization which is usually completed in 2 to 3 days. The crystals are centrifuged or filtered with suction.

The yield of crystalline hexokinase varies considerably with the individual lots of yeast delivered to the laboratory, perhaps because of differences of age of the case may be, is used for dialysis and alcohol fractionation as described in steps 4 and 5.
the yeast. Some lots even fail to yield any hexokinase crystals. It is advantageous to carry through the purification of lots of 25 pounds of yeast to step 4 and to store the filter cakes (fractions 0.63, 0.72, and 0.73) at 5°C. until an accumulated stock of about 100 gm. is obtained for further treatment. In case hexokinase crystals fail to appear in the crystallization mixture within 7 to 10 days the solution is then brought to 0.85 saturation with saturated ammonium sulfate and filtered with suction. The filter cake is reworked through steps 3b to 7.

The total yield of hexokinase can be increased by reworking the various inert protein crystals and super-cell residues of step 3, each according to its place in the general scheme.

The extent of purification of an average lot of 25 pounds of yeast is given in Table I. The specific activity of the various protein fractions is gradually raised on purification. The greatest rise, however, takes place on crystalliza-

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Filter cake</th>
<th>Total hexokinase activity units</th>
<th>Specific activity. Hexokinase units per mg. protein</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original aqueous extract + washing from 25 lb. yeast</td>
<td>400-600</td>
<td>4-8 million</td>
<td>About 20</td>
<td>6,000,000 = 100</td>
</tr>
<tr>
<td>Fraction 0.6</td>
<td>150-250</td>
<td>2-3 million</td>
<td>25-30</td>
<td>40</td>
</tr>
<tr>
<td>Fraction 0.7</td>
<td>5-20</td>
<td>1-2 million</td>
<td>100-170</td>
<td>4</td>
</tr>
<tr>
<td>Fraction 0.63</td>
<td>30-70</td>
<td>200,000-300,000</td>
<td>30-120</td>
<td>8</td>
</tr>
<tr>
<td>Fraction 0.72</td>
<td></td>
<td>400,000-500,000</td>
<td>120,000</td>
<td>2.0</td>
</tr>
<tr>
<td>Fraction 0.63 after alcohol fractionation</td>
<td></td>
<td>100,000</td>
<td>250</td>
<td>1.7</td>
</tr>
<tr>
<td>Fraction 0.72 after alcohol fractionation</td>
<td></td>
<td>120,000</td>
<td>300</td>
<td>2.0</td>
</tr>
<tr>
<td>First crystals (combined 0.63 and 0.72)</td>
<td>167</td>
<td>150,000</td>
<td>900</td>
<td>2.5</td>
</tr>
<tr>
<td>First mother liquor</td>
<td>80</td>
<td>33,000</td>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>Second crystals</td>
<td>38</td>
<td>90,000</td>
<td>1,400</td>
<td>0.9</td>
</tr>
<tr>
<td>Second mother liquor</td>
<td></td>
<td>22,000</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Third crystals</td>
<td></td>
<td>54,000</td>
<td>1,350</td>
<td></td>
</tr>
<tr>
<td>Third mother liquor</td>
<td></td>
<td>27,000</td>
<td>1,200</td>
<td></td>
</tr>
<tr>
<td>Fourth crystals</td>
<td></td>
<td></td>
<td>1,440</td>
<td></td>
</tr>
<tr>
<td>Fourth mother liquor</td>
<td></td>
<td></td>
<td>1,320</td>
<td></td>
</tr>
</tbody>
</table>

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The specific activity of the first crystals is more than double that of the protein solution from which they were crystallized, while the protein left in the first mother liquor has a specific activity of only 11 per cent of that of the crystals. On further recrystallization the specific activity of the crystalline protein is gradually raised. It reaches a constant value after 2 or 3 recrystallizations. The specific activity of the protein in the mother liquor is lower than that of the crystals, the difference, however, becoming less and less as crystallization is repeated.

Tests of Purity of Crystalline Hexokinase

1. Repeated Crystallization.—The new protein has been recrystallized 5 times. The hexokinase activity per milligram of protein reached a constant value after two recrystallizations, while the activity of the mother liquors approached the same constant value after the third crystallization (see Table I).

2. Solubility Curve.—The purity of a crystalline protein is conveniently tested by measuring the solubility of the crystals in a suitable solvent in the presence of increasing amounts of crystals in suspension. A curve is plotted of the amount of protein dissolved vs. the total amount of protein in suspension. The plotted curve is compared with the theoretical phase rule curve for a pure substance (10).

Fig. 5 shows the result of a solubility test on the crystals of the hexokinase protein which has been recrystallized five times.

The crystals were first washed several times by centrifugation at 5°C. with a solution of ammonium sulfate of about the same composition as the solution used for crystallization, namely 2.5 M ammonium sulfate in 0.05 M phosphate buffer pH 7.0. Increasing amounts of the washed crystals were pipetted into 2 ml. test tubes. A glass bead was placed in each tube. The tubes were filled with 2.5 M ammonium sulfate in 0.05 M phosphate buffer pH 7.0 and stoppered. The test tubes were then attached by means of rubber bands on the face of a 5 inch wheel mounted on the shaft of a reducing gear which was slowly driven by a small motor. The rotation of the wheel at the
speed of 5 to 10 r.p.m. brought about continuous rolling of the glass bead along the side of each tube and thus kept the suspension stirred continuously. The suspensions were rotated for 18 hours at 5°C. Samples of each suspension were analyzed for protein, the rest of the material was centrifuged at 5°C., and the clear supernatant fluids were analyzed both for protein and hexokinase activity.

The solid lines in Fig. 5 represent the theoretical solubility curve of a pure substance. The experimental points fall close to the theoretical lines except near the point of their intersection. The irregularity in that region may be due to the presence of a small amount of impurities or denatured protein formed during the stirring as evidenced by the presence of fine strings and broken films of protein.

3. Electrophoresis.—The mobility of five times recrystallized hexokinase protein in the Tiselius apparatus was measured by Dr. A. Rothen. Measurements were made at pH 5.6 and 6.8. In both cases the protein was negatively charged and moved as a single electrophoretic component as shown by the uniformity and sharpness of the moving boundary.

4. Sedimentation Studies by Means of the Ultracentrifuge.—Tests by Dr. A. Rothen show that when centrifuged at pH 5.6 the protein is homogeneous to a high degree. At pH 6.0, however, a double boundary appears. This abnormality may be due to an effect of ultracentrifugation on the homogeneity of the hexokinase protein.

The Protein Nature of Crystalline Hexokinase

That the hexokinase activity of the crystals is associated with the protein nature of the material follows directly from the fact that the hexokinase activity per milligram of protein remains constant on repeated crystallization. Also, the solubility experiment showed that the dissolved material consisted of protein of the same specific activity as the bulk of the material. Additional evidence on the protein nature of the hexokinase is shown by studies on the stability of crystalline hexokinase. Inactivation is accompanied by denaturation of the protein. Hexokinase is inactivated in the presence of a small amount of trypsin. The inactivation is a gradual process and is accompanied by a loss of protein as tested by precipitation in 2.5 per cent trichloracetic acid. The inactivation, however, proceeds at a rate faster than the rate of digestion of the protein. Crystalline chymotrypsin does not appear to affect crystalline hexokinase.

Stability of Crystalline Hexokinase

1. Effect of Temperature and pH.—Crystalline hexokinase dissolved in dilute buffers of pH 4.5–7.5 is stable for 2 to 3 days when kept at a temperature of 5°C. or lower. At temperatures above 5°C. the enzymatic activity is gradually lost. The rate of inactivation increases rapidly with increase in temperature.
The rate of inactivation varies also with the pH of the solution. Hexokinase is most stable at about pH 5.0 which is near its isoelectric point. The inactivation in the region of pH 4.5–7.5 is not accompanied by any significant hydrolysis of the protein. The protein is denatured, however, and precipitates out when the solution containing the inactivated material is brought to pH 5.0.

2. Effect of Various Substances on the Stability of Hexokinase at pH 7.0 and 27°C.—The stability of a 0.0025 per cent solution of hexokinase in 0.02 M phosphate buffer pH 7.0 when stored at 26–27°C. is increased in the presence of certain sugars and also in the presence of glycine. The order of effectiveness of the various sugars tested is shown in Table II.

<table>
<thead>
<tr>
<th>Concentration of sugar in hexokinase solution, per cent</th>
<th>1.0</th>
<th>0.02</th>
<th>0.001</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent activity left after 20 hrs. at 27°C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>74</td>
<td>70</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Mannose</td>
<td>68</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>29</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>17</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>57</td>
<td>22 in 0.1 per cent glycine</td>
<td>0-5</td>
<td></td>
</tr>
</tbody>
</table>

The order of the effectiveness of sugars as stabilizers of hexokinase does not correspond to the order of their effectiveness as acceptors of phosphorus from adenosine triphosphate. The order in that case is:

- Dextrose ............................................... 100 per cent
- Fructose .............................................. 100 " "
- Mannose .............................................. 54 " "
- Galactose ............................................. 0 " "
- Maltose ............................................... 0 " "
- Sucrose ................................... Trace...

Physicochemical Properties of Crystalline Hexokinase

1. Elementary Analysis.—An aqueous solution of five times recrystallized hexokinase protein was dialyzed in a collodion bag with stirring against slowly running distilled water for 24 hours at about 5°C. The dialyzed protein solution was then frozen in dry ice-methyl cellosolve mixture and evaporated to dryness under vacuum while frozen.

The chemical analysis was carried out by Dr. A. Elek of The Rockefeller Institute. The results of analysis are as follows:—
2. Isoelectric Point of Crystalline Hexokinase by Cataphoresis.—Measurements were made of the rate of cataphoretic migration (6) of collodion and of quartz particles which had been soaked for a few minutes in 0.5 per cent hexokinase solution and then suspended in 0.02 M buffer solutions. The region of minimum mobility was found to be at pH 4.5-4.8.

3. Ultracentrifuge Data by Dr. Rothen.—Diffusion constant ($D_{20}$) at 1°C. measured in acetate buffer pH 5.5 = $2.9 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$.
Sedimentation constant ($S_{20}$) at same pH and temperature = $3.1 \times 10^{-13}$ cm. sec.$^{-1}$ dyne$^{-1}$ gm.
Molecular weight at pH 5.5 calculated from the diffusion and sedimentation constants = 96,600.
The specific volume of the material was assumed to be 0.740 at 1°C. which is the usual value for a protein.

The Enzymatic Properties of Crystalline Hexokinase

1. Hexokinase Activity Measurement.—The hexokinase activity is measured by the rate of formation of free acid (4) during the process of the catalytic transfer of phosphorus from adenosine triphosphate to dextrose under standardized conditions. The amount of acid formed is determined by direct titration. Fig. 6 shows that the rate of formation of acid is nearly proportional to
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the concentration of hexokinase over a range extending to the first 75 per cent of the ultimate amount of acid formed at the end of the reaction.

2. The Hexokinase Unit.—1 unit of hexokinase is defined as the amount of enzyme which catalyzes the formation of $1 \times 10^{-9}$ hydrogen equivalents of acid per minute at $5^\circ C$ and pH 7.5, in a standard reaction mixture. This is equivalent to the liberation of about 1 mm$^3$ of CO$_2$ from NaHCO$_3$ per minute at $25^\circ C$, when the acid formed is determined by the usual manometric method.

3. Technique of Activity Measurement.—

Solutions.—Stock solution of ATP (kindly suggested by Dr. Sidney P. Colowick). 0.35 gm. of dibarium adenosine triphosphate, $C_{10}H_{18}O_{23}N_{5}P_{3}Ba_2$, prepared from fresh calf muscle by the method of Kerr (7), is dissolved in 5 ml. 0.2 M hydrochloric acid, then 0.25 gm. of sodium sulfate (anhydrous) is added. The precipitate of barium sulfate is centrifuged off and washed once by centrifugation with 2 ml. of water. The combined supernatant solutions are neutralized to pH 7.5 with 1.0 M sodium hydroxide and the volume is made up to 10 ml. The solution is stable for several weeks when stored at $5^\circ C$.

Stock solution of 5 per cent dextrose (Pfanstiehl) stored with a crystal of thymol at $5^\circ C$.

Stock of 0.1 per cent phenol red in water containing 5.7 ml. 0.05 M sodium hydroxide per 100 ml.

Solution $A$.—20 ml. of stock solution of ATP + 3 ml. of 0.05 M magnesium chloride. Stored with a crystal of thymol at $5^\circ C$.

Solution $B$.—8 ml. 5 per cent dextrose + 3 ml. 0.5 M Sörensen's phosphate buffer pH 7.5 + water to 25 ml. Stored with thymol at $5^\circ C$.

Solution $C$.—2.5 ml. of stock of 0.1 per cent phenol red made up with water to 100 ml.

Standard 0.01 M Sodium Hydroxide Solution.—It contains 6.25 ml. of stock of 0.1 per cent phenol red per liter of solution.

Standard 0.01 M Hydrochloric Acid.—

Reaction Mixture Used for Activity Measurements.—

0.5 ml. solution $A$ +
0.5 ml. solution $B$ +
0.5 ml. solution $C$.

Mixed in test tubes 1.5 x 12 cm.

Measurement.—The test tube containing the reaction mixture is cooled to $5^\circ C$, then 0.5 ml. sample of hexokinase dissolved in ice-cold water is added. The mixture is adjusted immediately with 0.01 M sodium hydroxide or hydrochloric acid from micro-burettes to the color of a standard of pH 7.5, and then titrated with 0.01 M sodium hydroxide to the color of the standard after standing for 30 minutes at $5^\circ C$. The color standard consists of 2.5 ml. 0.1 M phosphate buffer pH 7.5 + 0.5 ml. of solution $C$ (phenol red). The hexokinase units in the 0.5 ml. sample added are then read off the curve (Fig. 6).

4. Studies of the Enzymatic Action of Crystalline Hexokinase.—

(a) The effect of temperature. Fig. 7 shows the curves for the rate of the
reaction between adenosine triphosphate and dextrose at 5, 15, and 25°C. in the presence of a constant amount of crystalline hexokinase (2.5 hexokinase units). The rate of reaction was measured by the rate of formation of free acid in the reaction mixture. The temperature coefficient was estimated from the initial slopes of the curves and is about 2 per 10°C.

(b) Effect of crystalline hexokinase protein on the reaction between adenosine triphosphate and various sugars. The dextrose in the reaction mixture was replaced by the following sugars:

<table>
<thead>
<tr>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoses: -arabinose, d-xylene, l-rhamnose</td>
</tr>
<tr>
<td>Hexoses: d-dextrose, d-fructose, d-mannose, d-galactose</td>
</tr>
<tr>
<td>Disaccharides: sucrose, d-lactose, maltose, trehalose.</td>
</tr>
<tr>
<td>Trisaccharides: raffinose.</td>
</tr>
</tbody>
</table>

It was found that only dextrose, fructose, and mannose react with adenosine triphosphate. The rate of reaction in the presence of 15 hexokinase units is the same for dextrose and fructose and only about half as much for mannose. The samples of sugars used were mostly Pfanstiehl, c. p.

(c) Effect of magnesium ions on hexokinase activity. Crystalline hexokinase requires for its catalytic action the presence of magnesium ions. This is shown in Table III. The usual reaction mixture was used except for varying the concentration of magnesium chloride in solution A.

(d) Effect of inorganic phosphate ions. Crystalline hexokinase does not require the presence of inorganic phosphate ions as shown in Table IV. The
usual reaction mixture was used except for varying the concentration of phosphate buffer in solution B.

(e) Effect of varying the concentration of the substrates. Within certain limits of concentrations of adenosine triphosphate and of dextrose the rate of reaction is practically independent of their concentrations, as shown in Tables V and VI.

**TABLE IV**

<table>
<thead>
<tr>
<th>Molar concentration of phosphate buffer in solution B</th>
<th>0.20</th>
<th>0.10</th>
<th>0.05</th>
<th>0.025</th>
<th>0.0125</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 N acid formed in 30 min. at 5°C., ml.</td>
<td>0.38</td>
<td>0.42</td>
<td>0.47</td>
<td>0.44</td>
<td>0.43</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**TABLE V**

**Effect of Varying the Concentration of ATP**

<table>
<thead>
<tr>
<th>Ml. solution A in 2.5 ml. final reaction mixture. Concentration of magnesium chloride 0.004 M</th>
<th>1.0</th>
<th>0.75</th>
<th>0.50</th>
<th>0.35</th>
<th>0.20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 N acid formed in 30 min. at 5°C., ml.</td>
<td>0.34</td>
<td>0.38</td>
<td>0.39</td>
<td>0.38</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**TABLE VI**

**Effect of Varying the Concentration of Dextrose**

<table>
<thead>
<tr>
<th>Dextrose in 2 ml. final reaction mixture, per cent</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 N acid formed in 30 min. at 5°C., ml.</td>
<td>0.51</td>
<td>0.51</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

**The Extent of Reaction**

The extent of the reaction, as determined by the final amount of acid formed, varied slightly with the samples of adenosine triphosphate used, depending on their percentage content of pure ATP. The rate of the reaction, however, was found not to vary with the sample used, in agreement with the results given in Table V. The final amount of acid formed is equivalent approximately to one-third of the phosphorus content of the adenosine triphosphate in the reaction mixture, thus confirming the findings of Colowick and Kalckar (4). Crude hexokinase preparations gave rise to the formation of acid equivalent to about two-thirds of the phosphorus content of the ATP.

**Protein Determination**

_Protein by Turbidity._—5 ml. sample of protein is mixed with 5 ml. 5 per cent trichloracetic acid. The mixture is left at about 20°C. for 1 hour. The tur-
bidity formed is compared in a photoelectric colorimeter with that of a suspension of the same protein material of a known concentration, as determined by Kjeldahl analysis. A more convenient way is to draw a calibration curve for the turbidity of several concentrations of the standard protein solution as read against a suitable glass disk. This avoids the necessity of preparing fresh standards. This method covers the range of 0.02 to 0.8 mg. protein per ml.

Colorimetric Method (according to Herriot (8)).—1 ml. sample containing 0.2 to 1.2 mg. protein + 1 ml. 0.0025 M cupric sulfate + 8 ml. 0.5 M sodium hydroxide + 3 ml. of 1:3 dilution of Folin's phenol reagent (9) in water. The color developed is measured after 3 to 7 minutes and compared with that of a standard as described for the turbidity method.

SUMMARY

1. Crystalline hexokinase has been isolated from baker's yeast.
2. Crystalline hexokinase is a protein of albumin type of a molecular weight of 96,000. Its isoelectric point is at about pH 4.8.
3. The method of isolation consists in separating the proteins of an aqueous extract of toluene-treated yeast by means of fractional precipitation with ammonium sulfate and with alcohol.
4. The procedure involves also the separation of several crystalline proteins, including one yellow crystalline protein, which do not possess hexokinase activity. The biological and the physicochemical properties of these proteins are still under investigation.
5. The crystallization of hexokinase proceeds at about 5°C. in the presence of ammonium sulfate and dilute phosphate buffer pH 7.0.
6. Crystalline hexokinase becomes relatively pure after 2 or 3 recrystallizations as tested by solubility, sedimentation in the ultracentrifuge, and electrophoresis. The enzymatic activity remains constant on repeated crystallization.
7. The enzymatic activity is associated with the protein nature of the material. Inactivation is accompanied by denaturation of the protein.
8. Crystalline hexokinase is relatively stable when stored in the form of crystalline filter cake. Solutions of hexokinase in dilute buffers are most stable at pH 5.0.
9. Crystalline hexokinase requires the presence of magnesium ions for its catalytic activity.

REFERENCES