Preparation and Properties of Yeast Aldolase*

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The enzyme aldolase discovered by Meyerhof and Lohmann (1) catalyzes the following reaction:

\[
\text{dihydroxyacetone phosphate} \rightarrow \text{Fructose diphosphate} + \text{d-glyceraldehyde-3-phosphate}
\]

Aldolases derived from various sources may be differentiated into two types according to whether or not they are inhibited by metal-chelating agents (2). Warburg and Christian (3) first recognized a distinctive difference in the properties of yeast and muscle aldolase. The yeast enzyme was strongly inhibited by cyanide, pyrophosphate, cysteine, and \(\alpha,\alpha'-\text{dipyrridyl}\), and the inhibition was reversed by divalent metal ions (\(\text{Zn}^{2+}, \text{Fe}^{2+}, \text{Co}^{2+}, \text{Cu}^{2+}\)). A dissociable metal ion activator (probably \(\text{Fe}^{2+}\)) for the yeast enzyme was therefore proposed. It was later reported that the highly purified preparations of Warburg and Gawehn (4) contained considerable quantities of zinc and only traces of iron (5). The inhibition of the yeast enzyme by metal-chelating agents thus was correlated with the presence of zinc in the protein. In contrast to the yeast enzyme, muscle aldolase was not affected by the presence of metal-chelating agents (3), and did not contain significant quantities of any divalent ion (3, 5).

In line with the above, muscle aldolase is considered the prototype of the metal ion-dependent (type I), and similarly, yeast aldolase is the prototype of the metal ion-independent (type II) aldolases. In addition to the muscle enzyme (studied in the rat and rabbit), the bovine liver enzyme is appropriately classified as a type I aldolase since it is not inhibited by chelating agents, and does not contain divalent ions (6). The aldolase from peas (7) also may be placed tentatively in this category. In contrast, most aldolases which have been studied in microbiological systems are apparently type II aldolases. For example, the activity of the aldolase from several \(\text{Aspergillus}\) species is inhibited by chelating agents, and the inhibition is reversed by \(\text{Zn}^{2+}, \text{Fe}^{2+}, \text{Co}^{2+}\) and \(\text{Mn}^{2+}\); moreover, a highly purified preparation of the enzyme from \(\text{Aspergillus niger}\) contains 1 mole of \(\text{Zn}\) per 50,000 g of protein (8). The aldolase activity in aged preparations of Warburg and Gawehn (4) contained considerable quantities of zinc and only traces of iron (9). The inhibition of the yeast enzyme by metal-chelating agents thus was correlated with the presence of zinc in the protein. In contrast to the yeast enzyme, muscle aldolase was not affected by the presence of metal-chelating agents (3), and did not contain significant quantities of any divalent ion (3, 5).

A definitive comparison of the molecular and catalytic properties of the aldolases is not possible at present since the rabbit muscle enzyme is the only member which has been studied extensively.

The present paper presents a method for the isolation of yeast aldolase and reports some of its properties.

**EXPERIMENTAL PROCEDURE**

Materials—Fleishmann’s type 20–40 dry yeast (distributed by Standard Brands, Inc.) was the source for yeast aldolase. Fructose-di-P was obtained from Schwarz Laboratories. The commercial barium salt was converted to the sodium salt for use in routine assays. In other instances, the crystalline cyclohexylammonium salt was used (14). DPN was procured from Pabst Laboratories or Sigma Chemical Company. DPNH was prepared by chemical reduction of DPN according to the procedure of Beisenherz et al. (15). Crystalline \(\alpha\)-glycerophosphate dehydrogenase was obtained from the Sigma Chemical Company as an ammonium sulfate suspension. Protamine sulfate was a product of Eli Lilly Company. DEAE-cellulose powder was obtained from Brown and Company. Alumina \(\gamma\) gel was prepared according to the procedure of Willstätter and Kraut, outlined by Colowick (16). The stock gel suspension was centrifuged at 10,000 \(\times\) g for 10 minutes, and the pellet was resuspended in an equal volume of water to obtain the 50 volumes % gel used in the aldolase purification.

**Aldolase Assays**—A unit of aldolase activity is defined as the quantity of enzyme catalyzing the synthesis or cleavage of 1 \(\mu\) mole of fructose-di-P per minute, under the conditions of the assays described below. Specific activity is defined as units per mg of protein. Protein was determined by the spectrophotometric procedure of Warburg and Christian (17).

All assays were performed at pH 7.5 and 25°C. Aldolase preparations were diluted in 0.1 M glycylglycine, pH 7.5, 0%, immediately before assay. When EDTA (always less than 10⁻⁴ M) was present with yeast aldolase, a 1 to 2 times molar excess of zinc was added for optimal activity.

Two assays, based on the spectrophotometric detection of triosephosphates formed on aldol cleavage of fructose-di-P, were employed. In the first instance, a suitably diluted aliquot containing 0.01 to 0.1 unit of aldolase was added to a mixture containing 150 \(\mu\) moles of glycylglycine, 5 \(\mu\) moles of fructose-di-P, 0.5 \(\mu\) mole of DPNH, 5 \(\mu\) moles of cysteine, 300 \(\mu\) moles of potassium acetate, and 20 \(\mu\) g of crystalline \(\alpha\)-glycerophosphate dehydrogenase, in a total volume of 3.0 ml. The decrease in absorbancy at 340 nm was recorded immediately. In the absence of isomerase, it was assumed that the oxidation of 1 \(\mu\) mole of DPNH (O.D. = 2.07 with a cuvette of 1-cm light path) reflected the cleavage of 1 \(\mu\) mole of fructose-di-P.
For routine measurements, a modification of the hydrazine assay described by Jagannathan et al. (8) was employed. A suitably diluted aliquot containing 0.03 to 0.5 unit of aldolase was added to a solution containing 150 µmoles of glycglycine, 7 µmoles of hydrazine, 5 µmoles of fructose-di-P, and 300 µmoles of potassium acetate, in a final volume of 3.0 ml. The activities obtained in assay were standardized by the α-glycero-P dehydrogenase assay. With equivalent quantities of triosephosphate isomerase-free yeast and muscle aldolase, the hydrazine assay served with the α-glycerophosphate dehydrogenase assay.

In the third assay, the fructose-di-P formed from dihydroxyacetone-P and β-glyceraldehyde 3-P in the presence of the enzyme was detected by the Roe colorimetric test for fructose (18). The assay system contained 25 µmoles of glycglycine, 0.5 mg of bovine serum albumin, 2 µmoles of dihydroxyacetone-P, 0.7 µmoles of β-glyceraldehyde-3-P, 50 µmoles of potassium acetate, and 0.02 to 0.2 unit of yeast aldolase, in a total volume of 0.5 ml. The reaction was initiated by the addition of aldolase; after 1 minute of incubation at 25°, it was stopped by the addition of 1.75 ml of 30% HCl. Subsequently, 0.25 ml of resorcinol thiourea reagent (0.1% resorcinol plus 0.25% thiourea in glacial acetic acid) was added to give a total volume of 2.5 ml. The solutions were then heated at 80° for 10 minutes and air-cooled to room temperature. The optical density at 520 m was measured with a non-incubated control as a blank, and related to fructose-di-P standards (0.015 to 0.15 µ mole).

The response in the three assays described is linear with enzyme concentration under the conditions stipulated. In the two aldol cleavage assays, the concentration of fructose-di-P employed was more than 20 times greater than the K_m in this system (19). The concentrations of triosephosphates in the fructose-di-P synthetic assay only approximates the K_m values, because higher concentrations cause marked inhibition (19). In the assay of muscle or liver aldolase, potassium acetate may be deleted from any of the assay mixtures without affecting the response.

Zinc Analysis—Zinc analyses were performed by the dithizone procedure of Vallee et al. (20, 21). Solutions were stored in polyethylene containers that had been washed with 2 N nitric acid and previously extracted with a dithizone solution. All suitable quantities of a dialyzed sample of aldolase were analyzed for zinc content, and an equal sample of the dialysate was used as a blank in the analysis. Three trichloroacetic acid precipitations were carried out to ensure quantitative removal of zinc from the protein.

Physical Methods—Electrophoretic measurements were made at 0.1° in a Pearson electrophoresis apparatus with use of Tiselius-type cells with shear boundaries. The Longsworth scanning technique was used. Conductivity measurements were made on the appropriate buffer at 0.1°.

Molecular weights were determined by the Archibald technique as modified by Ehrenberg (22) with the Spinco model E analytical ultracentrifuge. The same 12-mm synthetic boundary cell (rubber valve) was employed throughout the work. Experiments were conducted at temperatures between 23 and 26°. Measurements on photographic plates were made by means of a Gaertner microcomparator.

RESULTS

Purification of Yeast Aldolase—A procedure for the purification of yeast aldolase was developed from the method reported by Warburg and Gawehn (4). In addition to minor modifications of several fractionation steps, column chromatography on DEAE-cellulose was successfully employed. A summary of the purification of yeast aldolase is shown in Table I. A more detailed description of the purification procedure follows.

Dry yeast, 1.5 kg, NaHCO_3, 45 g, and cysteine hydrochloride, 93 mg, were mixed with 4.5 liters of deionized water at 25°. Autolysis was allowed to continue at this temperature for 9 hours with occasional stirring. The mixture was then cooled to 5°, and the cell debris was removed by centrifugation. The crude extract (2345 ml) was used for further fractionation; it could be stored for at least 2 to 3 months at −15° without altering the specific activity or behavior in the fractionation procedure.

The crude extract was adjusted to pH 0.8 at 0° with cold 0.1 N HCl or NaOH and cooled to about 2°. Acetone fractionation was then performed in a room maintained at −15°. Three equal portions of 271 ml of acetone at −60° were added to the solution to bring the concentration to 35% acetone. Each acetone addition was made during a period of approximately 2 minutes, with rapid stirring (total acetone added, 812 ml). A 20-minute period with occasional stirring was allowed between each acetone addition, and after the final acetone addition, 1 hour of equilibration was permitted. The solution was centrifuged at 10,000 × g, −10°, for 30 minutes. The 35% acetone precipitate was discarded. An additional 587 ml of acetone at −20° were added to the supernatant solution with rapid stirring to bring the solution to 60% acetone. Each acetone addition was made during a period of approximately 2 minutes, with rapid stirring (total acetone added, 812 ml). A 20-minute period with occasional stirring was allowed between each acetone addition, and after the final acetone addition, 1 hour of equilibration was permitted. The solution was centrifuged at 10,000 × g, −10°, for 30 minutes. The 35% acetone precipitate was discarded. An additional 587 ml of acetone at −20° were added to the supernatant solution with rapid stirring to bring the solution to 60% acetone. After 1 hour of equilibration at 15° with occasional stirring, the solution was centrifuged as before and the supernatant solution was discarded. The 60% acetone precipitate was quickly dissolved in 500 to 600 ml of distilled water at 0°, and the turbid protein solution was dried from the frozen state (87 g). The powder could be stored at 0° without loss of activity.

The dry crude enzyme (87 g) was dissolved in 1500 ml of ice-cold water. The pH of the solution was adjusted to 7.1 by the addition of cold 0.1 N HCl. A 2% protamine sulfate solution, 70 ml (no pH adjustment), was added carefully with stirring during a period of 15 minutes. After equilibration for 30 minutes

<table>
<thead>
<tr>
<th>Fractionation step</th>
<th>Total</th>
<th>Recovery</th>
<th>Total mg</th>
<th>Recovery</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td>1. Crude extract</td>
<td>529,000</td>
<td>100</td>
<td>166,000</td>
<td>100</td>
<td>3.15</td>
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<tr>
<td>2. Acetone precipitation, 35-60% saturation</td>
<td>425,000</td>
<td>81</td>
<td>97,000</td>
<td>58</td>
<td>4.38</td>
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<tr>
<td>3. Acetone-dry powder</td>
<td>498,000</td>
<td>89</td>
<td>76,000</td>
<td>48</td>
<td>6.16</td>
</tr>
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<td>4. Prolamine supernatant</td>
<td>457,000</td>
<td>87</td>
<td>58,000</td>
<td>35</td>
<td>7.88</td>
</tr>
<tr>
<td>5. Heat supernatant</td>
<td>435,000</td>
<td>83</td>
<td>17,000</td>
<td>10</td>
<td>25.6</td>
</tr>
<tr>
<td>6. Alumina gel</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1st eluate</td>
<td>151,000</td>
<td>29</td>
<td>3,260</td>
<td>2</td>
<td>46.3</td>
</tr>
<tr>
<td>2nd eluate</td>
<td>80,200</td>
<td>15</td>
<td>1,450</td>
<td>0.9</td>
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<tr>
<td>3rd eluate</td>
<td>49,700</td>
<td>9.5</td>
<td>810</td>
<td>0.5</td>
<td>61.2</td>
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<tr>
<td>Total alumina gel eluates</td>
<td>280,900</td>
<td>54</td>
<td>5,500</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>7. Combined DEAE-cellulose-lose fractions</td>
<td>116,500</td>
<td>22</td>
<td>1,100</td>
<td>0.7</td>
<td>105</td>
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</table>
at 0°C with continuous stirring, the solution was centrifuged at 10,000 x g for 15 minutes at 0°C. The precipitate was discarded.

The supernatant was adjusted to 0.05 M cysteine and 0.01 M ZnSO₄ by the addition of appropriate quantities of 0.5 M cysteine, pH 7.1, and 0.1 M ZnSO₄ solutions, with rapid stirring at 0°C. The total volume was 1440 ml. Solid ammonium sulfate, 470 g, was added to bring the solution to 0.4 saturation. The solution, in a 3-liter stainless steel beaker, was stirred for 20 minutes and then heated over an interval of 6 minutes to 66°C in a water bath maintained at 75-80°C. The solution was maintained between 66 and 67°C for 11 minutes and then cooled in a water-ice bath to 20°C within 3 minutes. The mixture was centrifuged at 10,000 x g for 30 minutes at 0°C, and the precipitate was discarded. Solid ammonium sulfate, 485 g, was added to the supernatant solution (1850 ml) to increase the ammonium sulfate saturation to 0.8; the solution was equilibrated with stirring for 1 hour. The enzyme activity of this suspension was stable when stored at -15°C for at least several weeks.

The ammonium sulfate suspension was thawed, and the precipitate was collected by centrifugation at 22,000 x g for 30 minutes at 0°C; and dissolved in 110 ml of cold 0.01 M NH₄OH. This solution was re-centrifuged at 30,000 x g for 30 minutes and the precipitate was discarded. The supernatant solution was diluted with stirring to 1700 ml with water at 0°C. The pH of the solution was 6.45. 355 ml of 50 volumes % alumina gel suspension was added rapidly to the protein solution with stirring. After stirring for 30 minutes at 0°C, the suspension was centrifuged for 15 minutes at 10,000 x g at that temperature. The supernatant solution exhibited little activity and was discarded. The gel was washed with 1500 ml of water at 0°C and collected by centrifugation at 10,000 x g for 15 minutes. The enzyme was eluted by three successive treatments of the gel with 250 ml portions of a solution consisting of 0.05 M cysteine, 0.01 M ZnSO₄, and 0.01 M NH₄OH in 0.3 saturation ammonium sulfate at 0°C. The gel was mixed with the eluting solution in a Potter-Elvehjem homogenizer attached to a cone-driven motor. After each resuspension, the mixture was centrifuged as before, and the supernatant solutions were saved as the first, second, and third alumina eluates. These eluates could be stored successfully in 0.8 saturation ammonium sulfate at -15°C.

Further purification of yeast aldolase was accomplished by chromatography on a DEAE-cellulose column at 5°C. From 400 to 600 mg of protein were collected from the ammonium sulfate suspension by centrifugation and dissolved in about 9 ml of cold 0.01 M histidine, pH 6.5. This solution was then dialyzed twice for 1½ hours; each time against about 1 liter of cold 0.01 M histidine, pH 6.5, at 1°C. The solution was then immediately placed on a properly prepared DEAE-cellulose column (15 g of DEAE-cellulose powder in a 2.8- × 31-cm column was washed with 1 liter each of 2 M KCl, 0.2 M histidine, pH 6.5, and 0.01 M histidine, pH 6.5, in succession). The enzyme solution was carefully placed on the column; afterwards, about 50 ml of 0.01 M histidine, pH 6.5, were added, and then gradient elution was begun. The system consisted of a 300-ml mixing flask filled initially with 0.01 M histidine, pH 6.5, and connected with a reservoir which contained 0.75 M KCl. Thirteen milliliter fractions were collected. Yeast aldolase was eluted from the column within tubes 21 to 28. The appropriate tubes of high aldolase activity were combined and freeze-dried. Fig. 1 illustrates a DEAE-cellulose elution pattern which was obtained by chromatography of an alumina eluate.

The final yeast aldolase preparations contained no measurable hexokinase, glyceraldehyde-3-P dehydrogenase, α-glycero-P dehydrogenase, or lactic dehydrogenase activities; they were also devoid of triosephosphate isomerase activity, an enzyme which is difficult to separate from muscle aldolase.

Instability of Yeast Aldolase Preparations—Although crude preparations of yeast aldolase could be stored at 30°C at pH values from 6 to 9 for extended periods without appreciable loss of activity, the enzyme activity became progressively mor-
labile with purification. Fractions of highest specific activity from DEAE-cellulose columns, for example, frequently lost 50% of the aldolase activity within a few hours, and dialysis for extended periods has never been performed without extensive loss of activity. The addition of crude extracts, protein fractions, reducing agents (cysteine, sulfite, dithionite, ascorbate, thiocyanate, etc.), EDTA, or metal ions (Zn$^{2+}$, Fe$^{3+}$, K$^+$) either by themselves or in various combinations did not result in recovery of activity. It seemed possible that the apparent instability could be caused by proteolytic contaminants; however, no protection was achieved by the addition of diisopropyl fluorophosphate; moreover, addition of trypsin, chymotrypsin, or papain did not alter the rate of activity loss. Stabilization of the activity of highly purified yeast aldolase preparations was achieved in high salt (0.8 saturation ammonium sulfate) containing 0.01 M EDTA. The addition of crude extracts, protein fractions, reducing agents (cysteine, sulfite, dithionite, ascorbate, thiocyanate, etc.), EDTA, or metal ions (Zn$^{2+}$, Fe$^{3+}$, K$^+$) either by themselves or in various combinations did not result in recovery of activity.

Zinc in Muscle Aldolase—Yeast aldolase was assayed semi-quantitatively for metal ions by emission spectrographic procedures. Zinc was shown to be present in significant quantities (about 1 μg of Zn per mg of protein), whereas other divalent metal ions (Fe, Mg, Mn, Cu) were detected in traces if at all. Samples of the enzyme were therefore assayed for zinc by the quantitative chemical procedure of Vallee et al. (20, 21). In 5.1 and 17 mg, respectively, of two separate aldolase preparations, 5.2 and 22.8 μg of zinc were found. The minimal molecular weight for the yeast aldolase (assuming 1 mole of zinc per mole of enzyme) is therefore in the range of 50,000 to 65,000.

Electrophoretic Analysis—Fractions with specific activity of approximately 100 were pooled and solid ammonium sulfate was added to 0.8 saturation. After 1/2 hours of equilibration with stirring, the suspension was centrifuged at 54,000 × g for 15 minutes, and the precipitate was dissolved in 1/20.1 buffer (either glycylglycine at pH 7.5 or histidine at pH 5.5). The solution was dialyzed against two changes of 100 volumes of the same buffer for 20 hours at 0°C. Afterward, the specific activity of the protein was less than 10% of the original. The dialyzed protein solution was adjusted to the appropriate protein concentration by the final dialyzing medium and submitted to electrophoretic analysis. Fig. 2 presents the electroforetic patterns observed in the experiments at pH 7.5 and 5.5. A rather prominent boundary anomaly is evident in the electrophoretic experiments in glycylglycine. This boundary migrated little, if any, during the duration of the run, and direct analysis of the fluid in the descending arm of the electrophoretic cell indicated the boundary did not reflect the presence of protein. The fairly symmetrical gaussian curve at pH 7.5 is suggestive of an "essentially homogeneous" protein with a mobility of $-8.5 \times 10^{-9}$ cm$^2$ volt$^{-1}$ sec$^{-1}$. At pH 5.5, on the other hand, the enzyme exhibited a heterogeneous behavior; at least four components were evident. Since the enzyme activity is lost very rapidly and irreversibly at this pH, the electrophoretic behavior may reflect denaturation and dissociation of the protein.

Other zinc-containing proteins have been shown to dissociate at acidic pH. Carboxypeptidase, for example, readily loses zinc at pH values below 5.5 (23). Unlike yeast aldolase, however, the enzymatic activity of carboxypeptidase is restored by the readdition of zinc ions.

Sedimentation Behavior—A control sedimentation analysis of muscle aldolase (Fig. 3a) showed a homogeneous peak, $s_{20,w}$ 7.13 at 0.8% protein concentration, which is within the range of values reported by Taylor, Lowry, and Keller (24). A yeast aldolase preparation with a specific activity of 115 exhibited a single sedimenting boundary (Fig. 3b) corresponding to a sedimentation coefficient $s_{20,w}$ of 5.40 at 0.8% protein. Other sedimentation experiments were carried out in a medium containing EDTA with alumina eluate fractions of specific activity 70 to 90, since the enzyme activity was retained during the course of the experiments. In these experiments, two peaks appeared (Fig. 3c), the major peak corresponding to that observed with the more pure preparations (Fig. 3b). A plot of the sedimentation coefficients as a function of protein concentration for the major peak is presented in Fig. 4; an $s_{20,w}$ of 5.4 was obtained. The minor peak has an $s_{20,w}$ of 11.5 and 9.9, for 0.55 and 1.75% protein concentrations, respectively. If the enzyme solutions were allowed to incubate in the sedimentation medium (0.1 M KC1-0.01 M EDTA-0.02 M phosphate, pH 6.9, 1/2 0.18) for 2 to 3 hours at 25°C, the enzymatic activity was lost, and the sedimentation coefficient of the major peak was lowered considerably (witness the closed points on Fig. 4). Neither the relative size nor the sedimentation coefficient of the minor peak was influenced by this treatment.

**Fig. 2.** Electrophoresis of yeast aldolase. The experiments were performed at 0.1°C in 1/20.1 buffers; histidine buffer, pH 5.50, 10 mg of protein per ml; glycylglycine buffer, pH 7.50, 4 mg of protein per ml. Other pertinent information concerning the electrophoretic analysis may be found in "Experimental Procedure."
Molecular Weight of Yeast Aldolase—The weight average molecular weights of several separate yeast aldolase preparations were determined as described in the experimental procedure. The technique employed was checked experimentally by a determination of the molecular weight of a crystalline rabbit muscle aldolase preparation. In a single experiment at 0.8% protein concentration, a molecular weight of 161,000 to 164,000 was obtained. This value may be compared to 149,000 g, which was derived by Taylor et al. from sedimentation and diffusion data (24).

A yeast aldolase preparation with a specific activity of 115 yielded molecular weights between 66,500 and 73,500 (average 70,000) in 10 separate determinations at two protein concentrations (1.05 and 1.74%). In these experiments, the variation in calculated values of molecular weights seemed random, i.e. there was no apparent decrease in apparent molecular weight with increasing time of the run.

In other experiments, in which the enzyme had been allowed to stand several hours at 25°C, so that enzyme activity was lost, the apparent molecular weight was markedly lower. In eight separate determinations at protein concentration from 0.6 to 1.35% and with two separate preparations, the apparent molecular weight varied from 41,500 to 51,500. The latter observations together with the lowered sedimentation coefficients obtained after standing at a moderate temperature suggests that a modification of molecular structure may occur in dilute salt solutions even at moderate temperatures. It is possible that these changes are associated with a loss of zinc from the protein under these conditions.

Effect of EDTA and Divalent Metal Ions on Enzyme Activity—The activity of yeast aldolase is inhibited by EDTA and this inhibition is reversed by Zn²⁺ ions (Fig. 5), whereas the activity of muscle aldolase is indifferent to the presence of EDTA. At the high level of EDTA required to achieve essentially complete inhibition of activity, only a partial reactivation by ZnSO₄ could be obtained. Excess zinc ions caused an inhibition of activity. However, when the enzyme was preincubated in high concentrations of EDTA (10⁻¹ M) at pH 7.0 and diluted so that lower concentrations (10⁻⁴ M) of EDTA were present in the assay, full reactivation could be readily obtained by the addition of ZnSO₄ (2 × 10⁻⁴ M). At optimal concentrations, both Fe²⁺ and Co²⁺ were only partially effective in reversing inhibition by EDTA.

In all stages of the isolation procedure, maximal activity was observed in the absence of metal ions. Zn²⁺, Co²⁺ and Fe²⁺ salts, all inhibited when added in concentrations greater than 10⁻⁴ M, and even at lower concentrations on prolonged incubation with the enzyme. No activation of enzyme activity by metal ions in the absence of chelating agents has been observed. There
is, thus, no evidence that the zinc in yeast aldolase dissociates, even in the presence of chelating agents.

Activation by Potassium Ions—Greater than 100% recovery of yeast aldolase activity was routinely achieved upon elution of DEAE-cellulose columns with KCl according to the last step in the purification procedure. Subsequent investigation showed this effect was a result of a stimulation of the enzyme activity by potassium ions (Table II). Stimulations of 7, 10, and 12-fold were achieved in the α-glycero-P, hydrazine, and fructose-di-P synthetic assays, respectively. These stimulations were evident with enzyme obtained at all stages of purification. Since the first two assays are performed at concentrations more than two orders of magnitude greater than the $K_m$ of fructose-di-P, the stimulatory effects of potassium cannot be ascribed to an influence of this ion on the $K_m$, but must reflect a more direct role in the catalytic activity ($V_{max}$) of the yeast enzyme.

A summary of the effects of various salts as a function of concentration is shown in Fig. 6. Maximal activity is observed with potassium fluoride or acetate at a final concentration of 0.1 M. The lower activity obtained with other potassium salts indicates a modifying effect of the anionic species. Sodium and lithium salts at various concentrations were completely inactive in this system. Furthermore, it was shown that the action of potassium ions is independent of the ionic strength of the medium, and not affected by the presence of sodium ions. Appropriate experiments have shown no activation of crystalline muscle or liver aldolases by potassium ions.

Enzyme activity in all the assay systems appears maximal at

![Fig. 4. Sedimentation coefficients of yeast aldolase. ○, fresh preparations; ●, aged preparations (24-26°C for more than 2 hours). Sedimentation coefficients have been corrected to standard conditions (20°C, and pure water as the solvent).](#)

![Fig. 5. Activity of effect of EDTA on yeast and muscle aldolases. Enzyme activity (hydrazine assay) was detected kinetically with a Cary spectrophotometer. Activity is expressed in terms of the increment in optical density at 240 nm per mg of protein. The EDTA levels were 2 × 10^{-3} M and 6.7 × 10^{-6} M for muscle (MA) and yeast (YA) aldolases, respectively. The zinc sulfate level was 4 × 10^{-3} M.](#)

### Table II

<table>
<thead>
<tr>
<th>Assay system</th>
<th>Aldolase activity</th>
<th>Stimulation</th>
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<tr>
<td></td>
<td>Potassium acetate, 0.1 M</td>
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</tr>
<tr>
<td>1. Fructose-di-P synthesis</td>
<td>44.2</td>
<td>3.55</td>
</tr>
<tr>
<td>2. Fructose-di-P cleavage:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Hydrazine</td>
<td>42.7</td>
<td>4.12</td>
</tr>
<tr>
<td>b. Coupled α-glycero-P dehydrogenase</td>
<td>52.9</td>
<td>7.52</td>
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</table>

Assay systems are described in "Experimental Procedure." The enzyme employed was an alumina eluate fraction.
The catalytic activity of yeast aldolase is, however, unusually sensitive to the buffer species present in the assay system. Most buffers are inhibitory; as shown in Fig. 7, aldolase activity in the presence of imidazole, phosphate, and Tris buffers is always less than that observed in the absence of buffer. Glycylglycine on the other hand appears to stimulate activity with increasing concentration. Enzyme assays are therefore routinely carried out in 0.05 M glycylglycine buffer at pH 7.3 and with 0.1 M potassium acetate.

**DISCUSSION**

The catalytic activity of the present preparation approximates that of the crystalline preparation of Krebs and Vanderheiden (25). It is difficult to compare quantitatively the present preparation with the "crystalline" yeast aldolase of Warburg and Gawehn (4) because of the difference in assay conditions, but it is pertinent that the assay employed by these investigators contained approximately 0.04 M potassium ions and a favorable buffer ion (glycine). The best reported preparations of Warburg and Gawehn have a specific activity of 17 at 20° (in the presently defined units), and may be compared with preparations obtained in this work having specific activities of 70 to 75 at this temperature. The specific activity of highly purified *Aspergillus niger* aldolase (8) is similar to the yeast aldolase preparations of Warburg and Christian, but this may also be misleading, since it is possible that the *Aspergillus* enzyme is potassium-activated. The specific activity of yeast aldolase is about three times greater than the muscle aldolase, and the turnover number is still somewhat greater (assuming one catalytic site per molecule, and molecular weights of 70,000 and 150,000 for yeast and muscle aldolase, respectively).

Yeast aldolase is a highly negatively charged molecule at pH 7.5 \( (\mu = -8.5 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{sec}^{-1}) \). The *Aspergillus* aldolase, which also contains zinc, is reported to have a mobility, \( \mu \), of \( -5.4 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{sec}^{-1} \) at pH 7.6 in 1/2 0.2 phosphate buffer (8). On the other hand, liver and muscle aldolase have only slightly negative mobilities (\(-0.57\) and \(-1.6 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{sec}^{-1}\)).
volt$^{-1}$ sec$^{-1}$, respectively, at $\Gamma/2 0.1$ phosphate, pH 7.5). Veltick (20) has shown that the strong dependence of the net electrical charge of muscle aldolase is associated with a binding of buffer anions, especially phosphate. It is not known whether the yeast enzyme binds phosphate ions strongly, but it is pointed out that the large negative mobilities were observed in glycyglycine buffer. It seems likely that the large difference in electrophoretic mobility between the type I (liver, muscle) and the type II (yeast, A. niger) aldolases may be attributed to basic differences in the molecules themselves.

It is presumed the molecular weight of yeast aldolase is approximately 70,000 though a definitive assignment cannot be made until stabilization of the activity of the best preparations has been achieved and the effects of chelating agents have been clarified. The finding of zinc in this highly purified yeast preparation substantiates the earlier findings on this point (5). A molecular weight of 70,000 would be consistent with a stoichiometry of 1 atom of zinc per molecule.

The above results, considered with the known inhibition of activity by chelating agents, and the effect of EDTA on the sedimentation properties of the enzyme, suggest a role of zinc in yeast aldolase. However, definitive evidence on the role of the metal in the structure or catalytic action of the enzyme is lacking. It has not yet been possible to achieve reversible inactivation.

The specific effect of potassium ions on the yeast aldolase activity are analogous to those reported for other potassium-activated enzymes: pyruvate kinase (27), fructokinase (28, 29), acetyl coenzyme A kinase (30), tryptophanase (31), pantothenate activated enzymes: pyruvate kinase (27), fructokinase (28, 29), malic enzyme of Lactobacillus arabinosus (34, 35) and Moraxella (36)) phosphotransacetylase from Clostridium kluyveri (37). The finding of zinc in this highly purified yeast preparation appears consistent with this generalization.

4. The catalytic activity is inhibited by chelating agents and reversed by the addition of certain divalent metal ions.

5. The highly purified aldolase preparations contain 1 g atom of zinc per 50,000 to 65,000 g of protein.

6. Potassium ion specifically stimulates the catalytic activity 7- to 12-fold depending on the assay system used. The potassium activation is independent of the ionic strength, and of added sodium ions.

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SUMMARY

1. A procedure is described for the isolation of yeast aldolase. Under optimal conditions, the enzyme has a higher specific activity than other reported aldolases.

2. The yeast aldolase preparation behaved as an "essentially homogeneous" protein electrophoretically:

$$\mu = -8.5 \times 10^{-4} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \left(\frac{\Gamma}{2} 0.1 \text{ glycyglycine at pH 7.5} \right)$$

and during sedimentation in the ultracentrifuge

$$s_{20, w} = 5.4 \times 10^{-12} \text{ sec}$$

3. A molecular weight of approximately 70,000 is estimated for yeast aldolase from ultracentrifugal studies.
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